Therapeutic Effects of Ca$^{2+}$ on Peritoneal Dissemination of Gastric Carcinoma in Vivo

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It is well known that oral calcium supplementation has inhibited the gastric carcinogenesis in animals treated with chemical carcinogen and sodium chloride. However, the therapeutic effects of calcium ion (Ca$^{2+}$) for peritoneal dissemination of gastric carcinoma in vivo remain unknown. Here, we showed that Ca$^{2+}$ could be effective for inhibiting the peritoneal metastasis of gastric carcinoma MKN-45 cells along with apoptosis in vivo. Significantly prolonged survival was obtained for the peritoneal dissemination mouse models of MKN-45 cells after the intraperitoneal injection (i.p.) of Ca$^{2+}$. These results suggest that Ca$^{2+}$ could be a new candidate i.p. drug against peritoneal dissemination of gastric cancer in human.

Key words —— calcium, antitumor effect, gastric carcinoma, peritoneal dissemination, apoptosis

INTRODUCTION

Gastric carcinoma is the fourth most frequent cancer in the world. It is known that the cancer metastasizes to lymph nodes, liver and peritoneum after invasion through stratum submucsum, muscularis propria, stratum subserosum and serosa. Especially, peritoneal dissemination of gastric carcinoma is a refractory disease, which often causes multiple bowel obstructions and severe ascites.$^{1,2}$ The optimal treatment for peritoneal dissemination of gastric carcinoma has not yet been defined,$^{2}$ and novel effective agents are needed.

On the other hand, calcium ion (Ca$^{2+}$) is one of the most important inorganic metal cations in the living body and mediates various biofunctions such as activation of enzymes, muscular contraction, neurotransmitter release, differentiation and apoptosis. Epidemiological studies suggested that the daily oral intake of food and drink including calcium supplements decreased the risk of carcinogenesis.$^{3–5}$ In addition, oral calcium supplementation inhibited the gastric carcinogenesis in animals treated with chemical carcinogen and sodium chloride.$^{6}$ However, the therapeutic effects of Ca$^{2+}$ for peritoneal dissemination of gastric carcinoma in vivo remain unknown.

In previous studies, we have investigated the membrane targeted chemotherapy with hybrid liposomes$^{7,8}$ composed of phospholipids and nonionic surfactants for tumor cells in vitro,$^{9–14}$ in vivo,$^{14,15}$ and in clinical applications.$^{13,16}$ More recently, we have found the markedly inhibitory effects of extracellular Ca$^{2+}$ on the growth of human gastric carcinoma (MKN-45) cells through the induction of apoptosis in vitro.$^{17}$ On the basis of these studies, we investigated the therapeutic effects of Ca$^{2+}$ on mouse models with human gastric MKN-45 cancer cells in vivo.

MATERIALS AND METHODS

Materials —— Calcium chloride CaCl$_2$ and magnesium chloride MgCl$_2$ were of reagent grade from Nacalai Tesque (Kyoto, Japan) and dissolved in 5% glucose solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Human stomach tumor MKN-45 cell line was purchased from Riken Cell Bank (Tsukuba, Japan).

Assessment of Antitumor Activity in Vivo —— The animals were handled in accordance with the guidelines for animal experimentation under Japanese law during the study. Female mice with...
severe combined immunodeficiency (C.B-17/scid/scidIcl) were obtained from CLEA Japan Inc. (Tokyo, Japan). The mice (5-week-old) were randomly grouped on the basis of body weight on the day of tumor cell inoculation using the stratified randomization method. MKN-45 cells (5.0×10^6 cells) were intraperitoneally inoculated into the mice, as a model of peritoneal dissemination. The mice were divided randomly into three groups (control, Ca^{2+}, and Mg^{2+} groups). Control group was received 5% glucose solution. Either Ca^{2+} or Mg^{2+} ([Ca^{2+} or Mg^{2+}] = 10 mM; dose of Ca^{2+} was 11.1 mg/kg per d, dose of Mg^{2+} was 9.5 mg/kg per d) were intraperitoneally administrated once each day for 19 d after one hour of the peritoneal inoculation of MKN-45 cells. The mice were sacrificed 22 d after the inoculation of tumor cells. The tumor nodules were counted macroscopically, and the volume of ascites was weighted.

Survival Rate in Vivo —— Female mice (C.B-17/scid/scidIcl) were randomly grouped (n = 5) on the basis of body weight on the day of MKN-45 cells inoculation using the stratified randomization method and were treated with Ca^{2+} or Mg^{2+} as described above. The median lifespan was calculated using the following equation, median lifespan = (median survival days after treatment)/(median survival days of control group) × 100.

TUNEL Method —— Detection of apoptotic cells was performed on the basis of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using an in situ oxynucleotidyl transferase dUTP nick end labeling (TUNEL) method (ApopTag Plus Peroxidase, Millipore, Billerica, MA, U.S.A.). Tumors were removed and were fixed in 10% formalin solution. Paraffin-embedded sections were cut with a microtome, de-waxed in xylene and rehydrated through a series of ethanol to water. The sections were incubated with proteinase K for 15 min at room temperature and endogenous peroxidase was blocked with a solution of phosphate buffered saline (PBS) and 2% H_{2}O_{2} for 5 min. The sections were then incubated with a solution of digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 30°C for 60 min. Subsequently, anti-digoxigenin antibody was applied and the sections were incubated for 30 min at room temperature. Detection of the antigen-antibody link was made through immunoperoxidase followed by 3,3′-diaminobenzidine chromogen. The sections were counterstained with 5% methyl green, rinsed in distilled water, and observed using optical microscopy (Nikon TS-100, Nikon, Tokyo, Japan).

Statistical Analysis —— Results are presented as the mean ± S.D. Data were statically analyzed using Student’s t-test. A p-value less than 0.05 was considered to represent a statistically significant difference.

RESULTS AND DISCUSSION

First, the therapeutic effects of Ca^{2+} on the peritoneal dissemination mouse models of MKN-45 cells were examined in vivo. We used Mg^{2+} as negative control. The results are shown in Fig. 1. Fewer nodules were observed on the peritoneal cavity of the mice treated with Ca^{2+} as compared with the control and Mg^{2+} groups (Fig. 1A). The number of nodules of the control and Mg^{2+} groups were 159 ± 25 and 120 ± 23, respectively, whereas that of the Ca^{2+} group was 49 ± 29 (p < 0.01, Fig. 1B). The tumor weight of the Ca^{2+} treated mice (162 ± 33 mg) was fairly decreased compared with those of the control (412 ± 129 mg) and Mg^{2+} (492 ± 133 mg) groups (Fig. 1C). Moreover, the Ca^{2+} treated mice had lower ascites weights (38 ± 19 mg) compared with the control (172 ± 138 mg) and Mg^{2+} groups (184 ± 130 mg), although these values did not reach statistical significance. These results indicate that Ca^{2+} could be effective for inhibiting the peritoneal dissemination of MKN-45 cells in vivo.

Next, we observed the survival span of peritoneal dissemination mouse models after the treatment with Ca^{2+}. The results are shown in Fig. 2. The median lifespan was 108% for the Mg^{2+} group. On the other hand, significantly prolonged survival (123%) was obtained in mouse models treated with Ca^{2+} (p < 0.05).

Furthermore, we examined the mechanism of the therapeutic effects of Ca^{2+} on the peritoneal dissemination mouse models of MKN-45 cells on the basis of histological analysis using TUNEL method. The results are shown in Fig. 3. Significant number of apoptotic cells was stained in brown in the tumor tissues of the group treated with Ca^{2+}, although the apoptotic cells were not observed in that of the group treated with Mg^{2+}. These results indicate that Ca^{2+} could induce apoptosis of MKN-45 gastric carcinoma cells in vivo.

In addition, no abnormal finding was observed
How does Ca\textsuperscript{2+} induce apoptosis in tumor cells? It is well known that Ca\textsuperscript{2+} can chelate with anionic lipids such as phosphatidylinerines (PS) and induce the phase separation in the artificial cell membranes (phosphatidylcholine liposomes), though Mg\textsuperscript{2+} less interacts with PS.\textsuperscript{19–22} On the other hand, it was reported that the expression of PS in outer plasma membranes was more increased in the cancer cells as compared with in the normal cells.\textsuperscript{23, 24} Most recently, we have found the markedly inhibitory effects of extracellular Ca\textsuperscript{2+}, but not Mg\textsuperscript{2+}, on the growth of human stomach carcinoma (MKN-45) cells through the induction of apoptosis \textit{in vitro}.\textsuperscript{17} Furthermore, the localization of lipid rafts and the decrease of fluidity in MKN-45 cell membranes were observed in the presence of Ca\textsuperscript{2+}. On the basis of these results, we propose a hypothesis that Ca\textsuperscript{2+} would induce apoptosis toward tumor cells through the localization of lipid rafts by the electrostatic interaction between extracellular Ca\textsuperscript{2+} and PS in tumor cells. These findings could contribute to the
elucidation of antitumor effects of Ca\textsuperscript{2+} in vivo.

In conclusion, we clearly demonstrated for the first time that remarkable therapeutic effects and survival benefits of Ca\textsuperscript{2+} were obtained on the peritoneal dissemination mouse models of gastric carcinoma along with apoptosis in vivo.

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