Effects of casein glycomacropeptide on the early development of primary colorectal cancer in rats

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Abstract

In this paper, we utilized dimethylhydrazine (DMH)-induced colorectal cancer (CC) model rats to explore the effects of casein glycomacropeptide (CGMP) on colorectal cancer. Rats with CC were orally administrated with 10, 50, or 100 mg/kg bw d CGMP, or the same volume of phosphate-buffered saline for 15 weeks. The total numbers of aberrant crypt foci (ACF) and crypts per focus in colon were scored using a light microscope at low magnification after the colon was stained with methylene blue solutions. The methylation level of DNA extracted from colon was detected using methylation-specific PCR. The expression of p16 and mucin 2 (MUC2) proteins were measured by immunohistochemistry. The results showed that although ACF were found in rats treated with CGMP, their number was significantly decreased compared to that of model rats. In addition, methylation and expression levels of p16 and MUC2 were also inhibited by CGMP, which were more obvious in rats treated with 50 mg/kg bw d CGMP. In conclusion, CGMP has potential application as nutritional therapy for preventing colorectal cancer.

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Keywords: Casein glycomacropeptide; Colorectal cancer; DNA methylation; p16; Mucin 2

1. Introduction

Delfour et al. [1] in 1965 found a sialic acid containing polypeptide from milk-derived κ casein. This polypeptide contained two components: one was insoluble sub-κ-casein produced by chymosin digestion of κ-casein at Phe (105) and Met (106) residues during cheese production, and the other was the TCA-soluble caseino-macropeptide (CMP). About 30%–50% CMP was present in glycosylated form and named as casein glycomacropeptide (CGMP). Studies have found that CGMP has multiple biological activities such as promoting probiotics growth [2], regulating immune activity [3] and inhibiting influenza virus [4]. However, whether it plays a role in colorectal cancer has not been reported at home and abroad.

In recent years, the incidence of colorectal cancer has risen to the third place and its mortality rate ranked the second among malignant tumors. Thus, in-depth studies on the apparent molecular and genetic mechanisms of colorectal tumorigenesis and development of effective early molecular marker would have important implications for the prevention, diagnosis and treatment of colorectal cancer.

Studies on bioactive peptides are extremely active at home and aboard. Jolles [5] isolated a functional peptide from the degradation products of casein by chymosin and chymotrypsin and found the peptide could stimulate macrophages and erythrocytes. Parker et al. [6] isolated two active peptides from casein and found they could inhibit the growth of tumor cells. Belshaw et al. [7] laid the foundation of colorectal cancer screening by detecting methylation of 6 genes using feces as sample and first confirmed the feasibility of detecting fecal DNA methylation for colorectal cancer screening. Huang et al. [8] found that expression of mucin 2 (MUC2) might be involved in the tumorigenesis and development of colorectal cancer, and closely related with its invasion, lymph node metastasis and Dukes staging, implicating that MUC2 expression level may be correlated with the invasiveness of colorectal cancer. Pang et al. [23] reviewed the effects of nutrients, functional food ingredients and dietary on DNA methylation and believed that individual nutrients and functional food ingredients could alter DNA methylation and expression, and these epigenetic changes may affect peoples’
health by influencing bodies’ physiological and pathological processes.

In this study, we preliminarily studied the therapeutic effects of CGMP treatment on DMH induced colorectal cancer in dimethylhydrazine (DMH) induced colorectal cancer model rats by measuring body weight, aberrant crypt foci formation, p16 methylation, as well as expression of p16 and MUC2 proteins, and discussed molecular markers for CGMP intervention in rats with early colorectal cancer. The study revealed that CGMP might have certain prevention and therapeutic effects on the occurrence and development of early colorectal cancer.

2. Materials and methods

2.1. Animal experiment

50 female SPF grade Wistar rats weighting 80–90 g from the Experimental Animal Center of the Chinese People’s Liberation Army Academy of Military Medical Sciences were used in the experiments. The animal experimental protocols were approved with permit number of SYXX (Jin) 2012–2014 by Institutional Ethic Committee. The rats were fed conventionally. After accommodated to the environment for one week, they were randomly assigned into normal, model, as well as three CGMP (Tatata New Zealand) treatment groups with 10 rats in each group. Rats in all groups except normal group were intraperitoneally injected with dimethylhydrazine (DMH) (Sigma, USA) at 25 mg/kg w once a week [9,10]. Meanwhile, rats in CGMP groups were intraperitoneally injected with 10, 50 or 100 mg/kg bw d of CGMP, respectively. Their diet, drinking and behavior were observed daily and their body weights were recorded weekly.

2.2. Detection of colon aberrant crypt foci (ACF)

Rat colon was taken from the incisions at the junction of cecum and colon as well as pelvis, and cut open longitudinally. After removal of its content and rinsed with phosphate buffered saline, the colon was spread between two layers of filter papers and placed in a glass container with 10% formalin for 24 h. The fixed sample was stained with 0.2% methylene blue solution (Fermentas) for 10–20 min and transferred onto a glass slide. The number of ACF was counted with a microscope at 40× magnification [11]. The pathological morphology includes (1) mild or focal cell congestion, normal nuclear shape, no stratified cell layers, no neoplastic cells, and normal mucus secretion; (2) hyperplasia, crypt opening expansion, volume increase, but no nuclear atypia; (3) diffused or focal atypical hyperplasia, nuclear enlargement, congestion, increased nuclear layer, and reduced mucus secretion.

2.3. Detection of p16 gene methylation in rat colon

DNA purity was examined using a spectrophotometer (Beijing Purkinje General Instrument Co., Ltd.). 20 μL of extracted DNA was used to detect methylation using an EZ DNA Methylation-Gold Kit (ZYMO Research Country) according to the protocol provided by the manufacturer.

2.4. Methylation specific PCR (MSP)

The methylation of p16 promoter were detected using methylation primers 5′-AATTCGAGGAGGCGATTCG-3′ and 5′-ACCTATATCGAATACGACCGA-3′ to amplify a 155 bp fragment and non-methylation primers 5′-ACCTATATCGAAATACGACCGA-3′ and 5′-GTGAAATTGAGGAGGTGATTTG-3′ to amplify a 156 bp fragment [12]. MSP was performed in a 25 μL system containing 0.2 mM PCR buffer, 0.2 mM dNTP, 10 pmol each primer and 30–50 ng DNA template. After the reaction mixture was heated at 97 °C for 5 min and cooled down on ice for 3 min, 2.5 units of Taq polymerase was added to initiate the reaction under the following conditions: 40 cycles of 30 s at 94 °C, 30 s at 51 °C (U) and 30 s at 72 °C. The products were detected by 2.5% agarose electrophoresis. Appearance of methylation band was considered as methylation while appearance of only non-methylation band was considered as non-methylation.

2.5. Detection of p16 and MUC2 expression in rat colon mucosa

p16 and MUC2 expressions in rat colon mucosa were detected using immunohistochemical methods and quantified using Image Pro-plus software by calculating its accumulated image optical density (IOD) value [13].

2.6. Statistical analysis

All data was expressed as mean ± standard deviation (x ± s) and analyzed using SPSS11.5 statistical software with variance test. P value of less than 0.05 was considered as statistical significance.

3. Results

3.1. The effects of CGMP on rat general status and body weight

During the test, rats in all groups ate normally, showing no significant differences. Rats in the model group showed obvious physical and mental abnormalities: their hair was scattered, and their eyes were dull and off-white. It can be seen from Table 1 that body weights of rats in all groups showed an increasing trend. Statistical analysis of variance indicated that weekly changes in body weights of rats in different groups were not significant (P > 0.05), i.e. CGMP has no significant effect on the body weights of rats with colon cancer.

3.2. Effect of CGMP on ACF formation in rat colon

Abnormal enlargement of the midgut gland and the area around it, increased staining intensity and crack-like openings were observed under light microscope at 40× magnification.
Table 1
Weight changes of rats in different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1st week</th>
<th>3rd week</th>
<th>5th week</th>
<th>7th week</th>
<th>9th week</th>
<th>11th week</th>
<th>13th week</th>
<th>15th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>80.5 ± 1.3</td>
<td>116.8 ± 1.9</td>
<td>150.3 ± 6.8</td>
<td>198.2 ± 6.2</td>
<td>220.0 ± 5.7</td>
<td>243.8 ± 3.3</td>
<td>270.8 ± 7.5</td>
<td>300.1 ± 2.3</td>
</tr>
<tr>
<td>Model control</td>
<td>85.3 ± 2.5</td>
<td>120.1 ± 9.2</td>
<td>158.4 ± 1.3</td>
<td>189.4 ± 9.5</td>
<td>210.7 ± 3.9</td>
<td>235.8 ± 1.9</td>
<td>260.1 ± 5.9</td>
<td>290.5 ± 3.1</td>
</tr>
<tr>
<td>Low CGMP dosage</td>
<td>82.1 ± 4.3</td>
<td>125.5 ± 7.9</td>
<td>160.7 ± 4.6</td>
<td>190.3 ± 4.3</td>
<td>213.4 ± 2.6</td>
<td>239.8 ± 5.4</td>
<td>265.7 ± 4.9</td>
<td>295.3 ± 8.7</td>
</tr>
<tr>
<td>Medium CGMP dosage</td>
<td>84.5 ± 5.7</td>
<td>127.2 ± 4.3</td>
<td>165.2 ± 5.3</td>
<td>188.9 ± 3.1</td>
<td>208.5 ± 9.1</td>
<td>236.7 ± 3.5</td>
<td>264.3 ± 5.7</td>
<td>295.9 ± 3.0</td>
</tr>
<tr>
<td>High CGMP dosage</td>
<td>89.1 ± 3.1</td>
<td>129.2 ± 5.7</td>
<td>175.6 ± 2.1</td>
<td>189.5 ± 5.2</td>
<td>215.3 ± 4.7</td>
<td>242.3 ± 5.8</td>
<td>270.3 ± 4.1</td>
<td>305.3 ± 4.8</td>
</tr>
</tbody>
</table>

Table 2 shows the ACF number in colon of rats in all groups. From Table 2 and Fig. 1 it can be seen that there was no ACF in rats of the normal group. However, there were on average 33.1 ± 3.1 ACF and 17.7 ± 5.7 large ACF in rats of the model group, indicating the successful establishment of DMH-induced colon cancer model. In addition, there were 27.8 ± 4.5, 22.8 ± 5.4 and 20.6 ± 1.9 ACF and 13.2 ± 4.8, 14.1 ± 2.7 and 8.7 ± 4.3 large ACF in rats of 10, 50 and 100 mg/kg bw d CGMP groups, respectively. Although the number of ACF and large ACF in CGMP groups were significantly higher than that in the normal group (P < 0.01), they were much lower than that in the model group. Rats in the 100 mg/kg bw d CGMP group

![Image of pathological slices](image_url)
showed statistical significance ($P<0.05$). The results suggested that CGMP could inhibit ACF formation. It showed the best effect in high dose group from the standpoint of the number of ACF formation. But CGMP also showed significant effect in the middle dose group if other characters, including epithelial thickening, methylation and formation of crack-like openings/irregular shape/projections were taken into account. It also suggests that the effects of milk-derived CGMP on ACF controlling is dose-dependent.

3.3. The effect of CGMP on p16 gene methylation in rat with colorectal cancer

The purity test showed that the DNA extracts from colon tissues, containing intact colon genomic DNA, was not degraded and contaminated with protein and RNA. The ratio of absorption at 260 and 280 nm was between 1.8 and 2.0, suggesting the high purity of the obtained DNA.

The methylation specific primer pair could only amplify the methylated fragment of p16 gene promoter, while the non-methylation primer pair could only amplify the non-methylated p16 gene promoter. The changes in the methylation of p16 gene promoter in rats from different groups are shown in Fig. 2. Compared with normal control group, the methylation of p16 gene promoter in model group was significantly enhanced ($P<0.05$).

4. Discussion

The development of colorectal cancer is a complex pathological process which involves multiple steps and stages with changes from normal crypt foci to aberrant crypt foci, adenomas formation, expansion and eventual development to colorectal cancer [14,15]. In recent years, the role of dietary factors in the development of colorectal cancer is highly concerned. Intake of food with high animal protein and fat content has been found to positively correlate with the occurrence and development of colorectal cancer, while intake of large amounts of vegetables and fruits would significantly inhibit this process.
Moreover, studies on the bioactive peptides have become more and more active, with increasing numbers of biologically active peptides and their corresponding nutrients being separated. These peptides, such as anti-thrombosis peptides, anti-tumor peptides, immunomodulation peptides, etc., can not only function as amino acid donors, but also have a wide range of physiological functions roles.[16,17,18,19]

In this study, the effects of CGMP on rats with DMH induced colorectal cancer were analyzed. The results showed that CGMP could inhibit DMH-induced formation of ACF, which was positively related with the dosage of CGMP.

Existing evidences have shown that DNA methylation is due to the methylation of cytidine residues in CpG dinucleotide by intracellular methyl transferase (Dnmt). This modification can change chromosome structures. Some functional food ingredients in human diet could alter protein expression by modifying the methylation of their gene promoters, enhancers, silencers and insulators (non-coding region). p16 gene is one of the most widely studied tumor suppressor gene in recent years. It is extensively involved in the occurrence and development of varieties of tumors, such as colorectal cancer, breast cancer and others. Herman et al. [20] first used methylation-specific PCR (MSP) to detect changes of abnormal DNA methylation. Because of its specificity and sensitivity, this method has been widely accepted. In addition, it was reported that, in colorectal cancer, the positive rate of p16 gene promoter methylation detected by MSP was significantly higher than that using Southern blot method and restriction endonuclease method [21]. In this study, abnormal methylation of the p16 gene promoter was detected in rats with DMH-induced colorectal cancer using MSP and this abnormal methylation was reduced by administration of 10, 50 or 100 mg/kg bw d CGMP. Moreover, methylation of the p16 gene promoter showed significant differences between untreated and 50 mg/kg bw d treated rats with DMH-induced colorectal cancer. The results suggested that CGMP may change the activities of enzymes involved in abnormal methylation, such as Dnmts.

The p16 gene encodes an inhibitory factor of cell cycle-dependent kinase 4 (CDK4), one of the key regulators for G1 phase of the cell cycle. Thus, it could prevent cell division and growth, and regulate cell cycles. When there are deletion, mutation or methylation on p16 gene, its encoded p16 protein is unable to bind to CDK leading to increased binding between Cyclin D and CDK4 and enhanced cell division and differentiation. This will eventually result in the development of malignant cancer. In contrast, enhanced p16 expression could inhibit the growth of cancer cells. Spillare et al. [22] found that p16 protein could inhibit the proliferation of colorectal cancer cells both in vivo and in vitro.

Hanski et al. [23] found that expression of MUC2 protein was low in colorectal adenocarcinoma and it was further decreased with the reduction of metastatic potential of tumor cells. Cho et al. [24] also showed that MUC2 expression was related with local invasion and distant metastasis of colorectal cancer. In this study, rats with DMH-induced colorectal cancer were intragastrically administrated with different doses of CGMP and the expression of p16 and MUC2 were examined using immunohistochemistry. We found that the expression of p16 and MUC2 was enhanced in the model rats, which was significantly attenuated by intragastrical administration of different doses of CGMP. There was a significant difference between untreated model rats and 50 mg/kg bw d CGMP treated model rats, indicating that CGMP could enhance p16 and MUC2 protein expression in colorectal cancer.

5. Conclusion

This study demonstrated that intragastrical administration of 100 mg/kg bw d CGMP could significantly reduce the number of ACF in rats with DMH-induced colorectal cancer, indicating that CGMP plays certain preventive roles in colorectal carcinogenesis. From the epigenetics point of view, intragastrical administration of 50 mg/kg bw d CGMP could effectively inhibit aberrant methylation of p16 gene promoter in rats with DMH-induced colorectal cancer and enhance the expression of p16 and MUC2 in intestinal mucosa in rats with DMH-induced colorectal cancer, prompting that administration of bioactive peptide CGMP is a feasible way for the control and prevention of early colorectal cancer. The study also showed that the dose of CGMP resulting in physiological changes in epithelium observed during the development process using routine pathological sections was different from that resulting in epigenetic alteration. Thus, analysis of the expression of genes related to colorectal cancer is of important values for early warning of colorectal cancer. However, further researches are needed to clarify the molecular mechanisms and pathways by which CGMP could enhance the expression of genes related to colorectal cancer.

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References


