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Effect of Butyric Acid on p53 Expression and Apoptosis in Colon Epithelial Cells in Mice after Treated with 9,10-dimethyl-1,2-benz(a)anthracene

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Abstract

As the most common cancer, colorectal cancer is the fourth leading cause of death among this malignancy disease. Surgery procedure with chemotherapy and radiotherapy for colorectal cancer treatment may cause unpleasant side effects. Therefore, prevention and early detection of the disease is important. Butyrate, a short chain fatty acid, has a protective effect against colon cancer by inhibiting cell proliferation and inducing apoptosis. We conduct a research to investigate the effect of butyrate as a possible agent to decreased mutant p53 gene expression.

Keywords: butyrate; DMBA; p53 mutan inhibition

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1. Introduction

Colorectal cancer is one of the most common cancers worldwide, ranks fourth leading cause of death due to cancer\(^1\). The incidence of colorectal cancer in the United States is still high around more than 145,290 new cases with a mortality rate reached 56,290 in 2005 and is the second largest cause of death of all patients with cancer\(^2\). While in Indonesia, based on data from the Cancer Registration Bureau of the Department of Pathology, Faculty of Medicine, Airlangga University Surabaya, from 2002-2005, about 146 new cases of colorectal cancer were found\(^3\). The incidence of colon cancer in several cities in Indonesia as reported by Soetiarto was 5.5 per 100,000 in Yogyakarta and in Ujung Pandang up to 4.31 per 100,000\(^4\).

Colorectal cancer associated with low fiber eating habits and several epidemiological studies reported that diets high in fat and protein and low in fiber is one of the risk factors for colon cancer\(^6\). Diets high in fiber turns correlated of concentration butyrate in providing a protective effect against rat colon carcinogenesis induced chemical carcinogens material as reported by McIntyre et al\(^7\). This underlies later studies focused on Short Chain Fatty Acid (SCFA) mainly butyrate which has a protective effect against colon cancer by inhibiting cell proliferation and inducing apoptosis\(^8,9\).

Butyrate is a SCFA; the result of fermentation of carbohydrates by an-aerobic bacteria in the human colon\(^10\). Several in vitro studies have reported protective effect of butyrate on colon cancer occur through inhibition of cell proliferation and induced cell apoptosis. Butyrate protective effect against colon cancer through its ability to inhibit the enzyme activity of histone deacetylase (HDAC) in the p21 gene locus (P21) that produce histone hyperacetylation mainly H3 and H4 cause transcriptional activation of P21 to halt the cell cycle\(^9\).

P53 gene (P53), known as the “master guardian of the genome” is one of the tumor suppressor gene which regulates cell proliferation via activation of P21 and the control of cell apoptosis through activation of BAX\(^11,12\). Under normal circumstances, the p53 protein will halt the cycle of cells with DNA damage so there is no cell proliferation but if there is a mutation in the p53 than cell cycle continues and the cells with DNA injury remain replicates producing cells with DNA damage\(^11,12\).

However, the protective effect of butyrate against colon cancer via decreased expression of mutant p53 protein (P53) is not clearly known. If we do not know the mechanism of butyrate to decrease p53 mutant expression then the sustained P53 mutations will cause the cells undergo rapid and continuous proliferation and develop into cancer cells so cancer problem can not solved.

Based on this set out the research is to prove the protective effect of butyrate against expression of mutant P53 protein mice in colonic epithelial cells and apoptosis of mice colon epithelial cells after DMBA induced.

2. Methods

Carcinogenesis test material used is sodium butyrate (WAKO) and cancer induction material used is DMBA (9,10-dimethyl-1,2-benz(a)anthracene) from WAKO. This study uses laboratory mice (*Mus musculus* Swiss Webster strain (Balb/c) 12-week-old male, weighing 25-35 g, housed at Experimental Animal Unit, Laboratory of Biochemistry, Faculty of Medicine, Airlangga University, Surabaya.

In this true experimental study, male Swiss-Webster (Balb/c) *Mus musculus* (12 weeks old, weighing 25-35 g) divided in three groups of nine. No treatment applied to group one, group two received DMBA (10mg/100g body weight), group three received butyrate (2.5 g/kg body weight for 14 days) and DMBA (10mg/100g body weight) on day 15. Mice sacrificed on day 20 to collect colonic tissues. Immunohistochemical staining and Tunel assay performed for expression of mutant p53 and detect apoptotic colon epithelial cells, respectively. Normally and non-normally distributed data analyzed with one-way Anova with least significant difference test (\(\alpha=0.05\)) and Kruskal-Wallis with Mann-Whitney test (\(\alpha=0.05\)), respectively.

3. Results and discussion

3.1 Immunohistochemical examination of epithelial cells expressing mutant p53 in the colon of male Balb/c mice

The results of counting the number of epithelial cells expressing mutant p53 in the colon of male Balb/c mice seen in Table 1. Number of epithelial cells expressing mutant p53 in the colon of male Balb/c mice showed a significant difference (p < 0.0001; p < 0.05). Furthermore, the data analyzed by Mann-Whitney test and showed a
significant difference in the value of the median number of colonic epithelial cells expressing mutant p53 between the treatment group that were not administered butyrate nor DMBA and the treatment group that were not administered butyrate but dosed with DMBA ($p < 0.0001$; $p < 0.05$); as well as in the treatment group without butyrate administration but dosed with DMBA no significant difference with treatment group that administered butyrate and DMBA ($p < 0.0001$; $p < 0.05$). And there is no difference between treatment group that were not administered butyrate nor DMBA and the treatment group that administered butyrate and DMBA ($p = 0.387$; $p > 0.05$).

Table 1. Median number of epithelial cells expressing mutant p53 in the colon male Balb/c mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Median (n=9)</th>
<th>Min</th>
<th>max</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (no treatment)</td>
<td>0$^a$</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>P2 (received DMBA)</td>
<td>3$^b$</td>
<td>2</td>
<td>5</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>P3 (received butyrate)</td>
<td>0$^a$</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

$^a, ^b$ Different superscripts in the same column indicate significant differences ($p < 0.0001$)

3.2 Results of examination on epithelial cells undergoing apoptosis in the colon of male Balb/c mice

The results of counting the mean number of epithelial cells undergoing apoptosis in the colon of male Balb/c mice seen in Table 2. Results on analysis the number of cells undergoing apoptosis using one way ANOVA test showed that there was no difference in the number of colonic epithelial cells undergoing apoptosis in all groups treatment ($p = 0.129$; $p > 0.05$).

Table 2. Mean number of epithelial cells undergoing apoptosis in the colon of male Balb/c mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD (n=9)</th>
<th>min</th>
<th>max</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (no treatment)</td>
<td>25.111 ± 31.362</td>
<td>0</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>P2 (received DMBA)</td>
<td>29.778 ± 33.082</td>
<td>3</td>
<td>97</td>
<td>0.129</td>
</tr>
<tr>
<td>P3 (received butyrate)</td>
<td>77.444 ± 89.727</td>
<td>1</td>
<td>218</td>
<td></td>
</tr>
</tbody>
</table>

3.3 Results of immunohistochemical staining and TUNEL assay

The results of immunohistochemistry to detect mutant p53 expression of colonic epithelial cells of male Balb/c mice, using mutant p53 monoclonal antibodies with 400x magnification seen in Fig. 1. Image of the TUNEL assay staining to detect epithelial cells undergoing apoptosis in the colon of male mice Balb/c with 400x magnification, seen in Fig. 2. The arrows indicate positive results if a brown staining appeared.

Fig. 1. Immunohistochemical staining (a) Colon slice of male Balb/c mice. P1=No treatment (400 X, magnification ); (b) Colon slice of male Balb/c mice. P2= received DMBA (10mg/100g BW) (400 X, magnification ); white arrow marked positive reaction (brown) ; (c) Colon slice of male Balb/c mice. P3= received butyrate (2.5g/kg BW) and DMBA (10mg/100g BW) (400 X, magnification )
Carcinogenesis is a process of development towards malignancy characterized by transform normal cells from the initiation and promotion stages up to the stage of progression that occurs after the cancer is formed\textsuperscript{13}. The main targets of genetic damage are three classes of regulatory gene; protooncogene that promote growth; tumor suppressor gene which inhibits the growth as well as gene that regulates programmed cell death or apoptosis\textsuperscript{11}.

P53 is a tumor suppressor gene that plays a role in cell cycle regulation and apoptosis by controlling a number of gene when DNA injury was occur\textsuperscript{14}. P53 role as a cell cycle regulator is through stop the cell cycle in G1 phase which caused by the increase transcription of p21 that would inhibition formation of cyclin-CDK complexes. Cell cycle arrest will allow time for DNA repair injury so the cell with injured DNA do not do replication and mitosis. Repair of cells with DNA injury occurs through activation of repair Growth Arrest and Damage\textsuperscript{45} (GADD45) gene which induced by P53. When DNA injury repaired by the cell, P53 will increase transcription of MDM2 which then will suppress P53 so the cell cycle cessation eliminated. If the DNA injury is irreparable, P53 will direct the cell to eliminated by apoptosis\textsuperscript{11}. Hence, P53 become target of the most frequent genetic changes in humans. More than 50\% of human tumors contain mutations in the P53\textsuperscript{11}.

DMBA belongs to polycyclic aromatic hydrocarbon (PAH) procarsinogen group\textsuperscript{11,15}. DMBA known as mutagenic\textsuperscript{16}. DMBA metabolism forms 8,9-diol-10,11- epoxide which is highly reactive and can cause DNA injury. If a DNA injury occurs in P53 tumor suppressor gene, it will form mutant p53.

The results of immunohistochemical staining in this study show the number of colonic epithelial cells expressing mutant p53 in the treatment group without butyrate administration but dosed with DMBA, more significant than in the group of mice that were not administered butyrate nor DMBA (p < 0.0001; p < 0.05). This suggests that the DMBA is a mutagenic compound that causes P53 mutations in colonic epithelial cells.

In this study, it turns out that the treatment group receiving DMBA without butyrate, were unable to cope with the DNA injury in P53 causing mutations of P53, DNA repair mechanisms can not repair injured DNA in P53 or through cell apoptosis. When cells with DNA injury stay in the cell cycle, there will be mutations and excessive gene expression continuously, causing uncontrolled cell proliferation. However, by administration of butyrate, the number of colonic epithelial cells expressing mutant p53 in the group that receive butyrate and DMBA are significant fewer than in the group that were not administered butyrate but dosed with DMBA (p < 0.0001; p < 0.05). Ability cells to cope the DNA injury in P53 by butyrate administration gives the same result as in normal epithelium, it seen from the number of cells expressing mutant p53 in the group that administered butyrate and DMBA are not different from the treatment group that were not administered butyrate nor DMBA (p = 0.387; p > 0.05).

Results showed that butyrate is able to inhibit mutant p53 expression in colonic epithelial cells induced DMBA. Butyrate is the end product of carbohydrate fermentation by anaerobic bacteria in the colon\textsuperscript{10}. Butyrate concentration which emerges from the carbohydrate fermentation depends on the type of substrate available and colon microflora composition. Colon microflora that ferment carbohydrates producing butyrate are \textit{Clostridia}, \textit{Fusobacteria}, \textit{Eubacteria} and \textit{Peptococci}\textsuperscript{17}. Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of cancer of the colon, by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes; inhibiting the enzyme histone deacetylase\textsuperscript{18}. Butyrate is a histone deacetylase inhibitors (HDACIs), a new class of anti-cancer drugs\textsuperscript{19,20}. The results of this study support previous studies that butyrate can inhibit mutant p53 expression in cell cultures derived from surgical specimens of patients with colon cancer\textsuperscript{21}. 

Fig 2. Tunnel Assay Staining (a) Colon slice of male Balb/c mice. P1=No treatment (400 X, magnification ), white arrow marked apoptotic cell; (b) Colon slice of male Balb/c mice. P2= received DMBA (10mg/100g BW) (400 X, magnification ), white arrow marked apoptotic cell; (c) Colon slice of male Balb/c mice. P3= received butyrate(2.5g/kg BW) and DMBA (10mg/100g BW) (400 X, magnification ), white arrow marked apoptotic cell
Apoptosis is a cell death through genetic mechanisms (destruction/fragmentation of chromosomes or DNA). Apoptosis can occur physiologically or pathologically. Apoptosis is an important regulatory process to prevent cancer development. Increasing number of cells undergoing apoptosis during the initiation phase of colon carcinogenesis is the result of increasing the elimination cells with DNA injury (DNA defect) that can lead to malignancy.

Butyrate can induce apoptosis in vitro in Caco-2 cells via mitochondrial pathway by activating BAX proapoptotic gene and BCL-2 antiapoptotic gene barriers resulting in translocation cytochrome C from mitochondria to cytosol and then bind to Apoptotic protease activating factor 1 (Apaf1) activating caspase cascade through caspase 9 so that caspase 3 binds to DNAase breaking down DNA into fragments and apoptosis.

In this study, TUNEL assay used for detect DNA fragmentation which is the characteristic of apoptosis. Result showed, no difference was found in the number of colonic epithelial cells undergoing apoptosis in all treatment groups (p = 0.129; p > 0.05), it was indicate that butyrate is able to inhibit mutant p53 the expression in colonic epithelial cells induced by DMBA without increase in cell apoptosis. This is due to ability of cell to stop the cell cycle and allow time for the cell to repair DNA injury in P53 so the cell does not need to sacrificed to go through apoptosis. And apoptosis that occurs in this study is a physiological apoptosis, not the pathological apoptosis in response to DNA injury that can not be repaired by the cell.

These results do not support previous study as reported by Le-Leu et al who conducted research to assess the acute apoptotic response upon carcinogens material induction in mice. Mice were fed a diet of three different kinds of carbohydrates (resistant starch, wheat bran, cellulose) for four weeks and then induced by Azoxymethane to cause DNA injury. The study results found that wheat bran increases faecal and colon butyrate concentration and increases apoptosis as response to carcinogenic materials induction in mice. Increased butyrate concentration positively correlated with the apoptotic index. Mastutik also reported that administration of cellulose for 14 days in mice followed by DMBA would increase the number of colonic epithelial cells undergoing apoptosis.

The difference in this study result due to butyrate dose given to mice at a dose of 2.5 g/kg body weight, can increase GADD45 activity that plays a role in DNA repair mechanisms so that injuries through the mechanism of DNA repair was occur, not through increased cell apoptosis to eliminate cells with DNA injury. Dose of 2.5g/kg body weight administered to the mice was 50% of the maximum dose (5g/kg) that given orally to mice. Butyrate administration at 5g/kg orally to mice will reach butyrate plasma concentration of 9 mM (after 15 minutes of administration). Normal concentrations of butyrate in mice colon approximately 1-4 mM. Ragione et al reported that butyrate is able to increase GADD45 expression based on in vitro HT-29 cells which incubated with 2 mm sodium butyrate would increase GADD45 expression by 3 ± 4 times compared to no incubation with butyrate. Chen et al reported that butyrate has the same ability with TSA to halt the cell cycle and induction of GADD45 in SW 620 human colon cancer cell line. Dronmraju et al also reported that in patients with colorectal cancer who were given resistant starch diet, a main substrate fermented by colon microflora producing butyrate; would increase GADD45 expression as much as two times higher than in those with no resistant starch in the diet.

In this study it was found that administration butyrate at a dose of 2.5g/kg followed by 10mg/100g body weight DMBA induction, there was an increase in colonic epithelial cells undergoing apoptosis in the treatment group that were given butyrate and DMBA although after statistical analysis there was no difference among all treatment groups. Increasing number of cells undergoing apoptosis in the treatment group that were administered butyrate and DMBA, probably cause by some cells undergoing DNA injury fail to be repaired by DNA repair so those cells would be eliminated through apoptosis (pathological apoptosis). This shows that apoptosis occurred in the treatment group that were given butyrate and DMBA is a physiological apoptosis and an increase in cells undergoing apoptosis in response to eliminate cells undergoing DNA injury (pathological apoptosis). This may explain the wide range between minimum and maximum result. So this study concluded that butyrate was able to inhibit the expression of mutant p53 mainly through the mechanism of DNA repair rather than through cell apoptosis mechanism, proved by fewer number of cells expressing mutant p53 in the treatment group that were administered butyrate and DMBA compared to treatment group that were not administered butyrate but dosed with DMBA (p < 0.0001) and there is no difference in the number of cells undergoing apoptosis in all treatment groups (p = 0.129).
4. Conclusion

From the study results, it can be concluded that butyrate has a protective effect against colon carcinogenesis through inhibition against mutant p53 expression in the DMBA-induced colonic epithelial cells by DNA repair mechanism, and not through enhancement in colonic epithelial cell apoptosis due administration of DMBA.

References

1. International Agency for Research on Cancer; 2014.