Cytotoxic Effect of Cholesterol Metabolites on Human Colonic Tumor (Caco-2) and Non-Tumor (CCD-18Co) Cells and Their Potential Implication in Colorectal Carcinogenesis [†]

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Abstract: The unabsorbed cholesterol, along with that of bile secretions and flaked colon cells, can be metabolized by the colonic microbiota. The generated metabolites have been proposed as promoters of colorectal cancer (CRC). In this study, the cytotoxicity (MTT assay) of the main commercially available cholesterol-derived metabolites (coprostanol, cholestanol, coprostanone and cholestenone) on human colon cancer (Caco-2) and non-tumor (CCD-18Co) cells is evaluated at different physiologically relevant concentrations (9.4–300 µM) and incubation times (24–72 h). In general, the metabolites that most reduced cell viability are coprostanone (54–85% in Caco-2 and 20–81% in CCD-18Co) and cholestenone (17–91% in Caco-2 and 14–81% in CCD-18Co). These two metabolites are the most hydrophobic, thus reflecting a possible relationship between hydrophobicity and cytotoxicity. Moreover, cholestenone (IC_{50} at 72 h: 5 ± 1 µg/mL) should be considered cytotoxic on CCD-18Co cells (non-tumor cells) since it shows an IC_{50} close to the one considered toxic (<4 µg/mL). Furthermore, CCD-18Co cells are more vulnerable to the cytotoxic effect of cholesterol metabolites. Possible compensatory responses, attenuating the reduction in cell viability caused by cholesterol metabolites, is observed, however these reactions could favor inflammation, resistance to apoptosis and cellular proliferation, likely contributing to the development of CRC. In conclusion, cholesterol metabolites, mainly the most hydrophobic, could act as promoters of CRC through their cytotoxic activity.

Keywords: colorectal cancer; cytotoxicity; cholesterol metabolites; Caco-2 cells; CCD-18Co cells

1. Introduction

CRC was the second most prevalent cancer in the world in 2018 and the second cause of cancer-related death worldwide [1]. Some authors propose that diets rich in fats and proteins of animal origin are related to an increased risk of CRC through an increase in the production of cholesterol-derived metabolites by the intestinal microbiota [2,3]. Since cytotoxicity may be a mechanism in cancer induction [4], the objective of the present study is to evaluate the cytotoxic activity of the main commercially available cholesterol-derived metabolites (coprostanol, cholestanol, coprostanone and cholestenone) on undifferentiated human colonic epithelial adenocarcinoma cells (Caco-2) and non-tumor human colon fibroblasts (CCD-18Co).

2. Results

Cytotoxic effect of cholesterol metabolites and 5-fluorouracil (5-FU) on CCD-18Co and Caco-2 cells is shown in Table 1.

Table 1. Cytotoxicity assay with metabolites of cholesterol and 5-FU in CCD-18Co and Caco-2 cells.

	Cell Viability (% Control)					
	CCD-18Co			Caco-2		
[μM]	24 h	48 h	72 h	24 h	48 h	72 h
Coprostanol						
9.4	98 ± 6 abA	90 ± 2 aA‡	93 ± 3 aA	106 ± 15 aA	105± 4 aA	108 ± 15 abA
18.75	103 ± 4 bA	97 ± 3 abA‡	109 ± 9 aA	109 ± 22 aA	117 ± 4 *bA	105 ± 8 abA
37.5	109 ± 3 bA	91 ± 5 aB‡	101 ± 2 aAB	93 ± 6 aA	102 ± 2 aAB	121 ± 15 bcB
75	101 ± 7 abA	106 ± 4 bA	99± 19 aA	108 ± 9 aA	102 ± 7 aA	111 ± 17 abA
150	100 ± 8 abA‡	89 ± 7 aAB‡	84 ± 7 aB‡	137 ± 8 aA	132 ± 4 *cA	142 ± 9 *cA
300	89 ± 4 aA‡	72 ± 6 *cB‡	48 ± 5 *aC‡	146 ± 54 aA	103 ± 3 aB	90 ± 10 aBC
Cholestanol						
9.4	86 ± 13 aA	79 ± 8 *aA‡	93 ± 22 aA	86 ± 11 aA	96 ± 1 aA	94 ± 22 aA
18.75	72 ± 7 *abA	74 ± 5 *aA‡	84 ± 12 aA	86 ± 10 aA	102 ± 2 aA	93 ± 16 aA
37.5	70 ± 4 *abA	73 ± 9 *aA‡	80 ± 10 aA	80 ± 8 *aA	90 ± 10 aA	99 ± 27 aA
75	67 ± 8 *abA	75 ± 5 *aA‡	77 ± 4 aA	76 ± 4 *aA	92 ± 9 aA	80 ± 9 aA
150	61 ± 9 *bA	70 ± 5 *aA‡	75 ± 4 aA	77 ± 3 *aA	91 ± 2 aAB	104 ± 17 aB
300	59 ± 10 *bA‡	72 ± 5 *aA‡	98 ± 15 aB	79 ± 1 *aA	97± 2 aAB	108 ± 24 aB
Coprostanone						
9.4	94 ± 10 aA	101 ± 11 aA	80 ± 2 *aB‡	90 ± 18 aA	106 ± 11 abA	105 ± 16 aA
18.75	107 ± 15 aA	91 ± 5 aB‡	86 ± 15 aB	115 ± 11 aA	127 ± 6 *bA	115 ± 4 aA
37.5	87 ± 11 aA	100 ± 4 aA‡	66 ± 9 *aB‡	108 ± 29 aA	120 ± 13 bA	112 ± 9 aA
75	65 ± 2 *bA‡	99 ± 8 aB‡	47 ± 8 *bC‡	132 ± 16 aA	123 ± 10 *bA	102 ± 7 aA
150	62 ± 6 *bcA‡	52 ± 13 *bA‡	25 ± 1 *cB	106 ± 8 aA	117 ± 6 bA	46 ± 3 *bB
300	44 ± 3 *cA‡	25 ± 3 *cB‡	19 ± 4 *cB	103 ± 16 aA	95 ± 9 aA	15 ± 3 *cB
Cholestenone						
9.4	86 ± 4 *aA	90 ± 7 aA	61 ± 31 *aB	83 ± 3 *aA	93 ± 5 aA	83 ± 19 aA
18.75	76 ± 5 *bA	82 ± 5 *aA	51 ± 25 *abB‡	70 ± 5 *bA	77 ± 2 *bA	81 ± 7 abA
37.5	56 ± 4 *cA‡	81 ± 19 *aB	61 ± 4 *aA	70 ± 5 *bA	68 ± 6 *bA	61 ±10 *bA
75	37 ± 3 *dA‡	21 ± 3 *bA‡	21 ± 2 *bA	60 ± 7 *bA	51 ± 5 *cA	27 ± 3 *cB
150	27 ± 2 *eA‡	19 ± 2 *bA	24 ± 5 *abA	37 ± 6 *cA	17 ± 1 *dB	10 ± 3 *cB
300	29 ± 3 *deA	19 ± 1 *bA	31 ± 10 *abA	29 ± 3 *cA	12 ± 2 *dB	9 ± 1 *cB
5- Fluorouracil (25 μM)	90 ± 3 *aA‡	65 ± 5 *bA	64 ± 2 *bA	64 ± 10 *aB	70 ± 10*aA	67 ± 7*aA

Data are shown as mean ± standard deviation (n = 4). The asterisk indicates statistically significant differences ($p < 0.05$) between the treatments and the control. Different lowercase letters (a–e) indicate statistically significant differences ($p < 0.05$) at different concentrations for the same incubation time, cell line and cytotoxic agent. Different capital letters (A-C) indicate statistically significant differences ($p < 0.05$) at different times at the same concentration, cell line and cytotoxic agent. The ‡ sign indicates a statistically significant difference ($p < 0.05$) vs. Caco-2 cells at the same concentration, incubation time cytotoxic agent.

2.1. Coprostanol

After treatment with coprostanol a reduction in the viability of CCD-18Co cells is observed at 300 μM after 48 (28% vs. control) and 72 h (52% vs. control). However, Caco-2 cells do not experience a reduction in viability, whilst an increase in cell viability is observed at 18.75 and 150 μM (117 and 132% vs. control, respectively). This fact could indicate a possible hyperproliferative response to the toxic effect of coprostanol. Therefore, the Caco-2 cells could be less susceptible to the cytotoxicity of coprostanol and could be capable of making a compensatory response earlier.

2.2. Cholestanol

The incubation of cholestanol for 24h, at concentrations of 18.75 μM or above, reduces statistically ($p < 0.05$) CCD-18Co cells viability (28–41% vs. control), whilst at 48 h a cell viability reducion at all concentrations is observed (21–30% vs. control). At 72 h, a reduction in the viability of CCD-18Co cells is not observed, which could indicate a possible compensatory response. This response is verified by observing that the reductions in cell viability at 300 μM decrease with time (41, 28 and 2% vs. control at 24, 48 and 72 h, respectively). In addition, in Caco-2 cells, cholestanol at 24 h reduces the cell viability from 37.5 μM (20–24% vs. control), without effect at 48 and 72 h. Therefore, compensatory response is again observed (earlier than in CCD-18Co cells), which is confirmed with cell viability values after 72 h at 150 (104% vs. control) and 300 μM (108% vs. control) higher than 24 h (77 and 79% vs. control, respectively). On the other hand, the CCD-18Co cells are more susceptible since the reduction in cell viability at 150 and 300 μM after 24 h is greater than that observed in Caco-2 cells (39 vs. 23% control; 41 vs. 21% control, respectively).

2.3. Coprostanone

Coprostanone at 24 h significantly reduces the viability of CCD-18Co cells from 75 μM (35–56% vs. control), at 48 h from 150 μM (48–75% vs. control), and at 72 h at all concentrations except 18.75 μM (20–81% vs. control). A time-response relationship is observed, with an increase in the reduction of viability with time at 150 and 300 μM. In Caco-2 cells, coprostanone does not reduce cell viability at 24–48 h, and only at 72 h a reduction of cell viability from 150 μM (54–85% vs. control) is observed.

2.4. Cholestenone

In general, the incubation of cholestenone at 24 h reduces CCD-18Co (14–73% vs. control) and Caco-2 cells (17–71% vs. control) viability at all concentrations investigated in a dose-dependent manner. The effect at 48 h is slightly lower for both cell lines (18–81% and 23–88% vs. control, respectively), since the minimum dose requiered is 18.75 μM. At 72 h, cholestanone produces a similar cell viability reduction in CCD-18Co cells (39–79% vs. control) compared to 48 h, but in Caco-2 cells a dose ≥37.5 μM is necessary to produce a cell viability reduction (39–91% vs. control). The effect of the cholestenone on cell viability is time-dependent for certain conditions. In this regard, in the CCD-18Co cells there is a higher reduction of cell viability at 9.4 and 18.75 μM at 72 h, and in the Caco-2 cells at 75, 150 and 300 μM at 72 h. In addition, it appears that Caco-2 cells give a compensatory response at 9.4 and 18.75 μM with a trend of lower cell viability decrease at 48 and 72 h.

2.5 5-Fluorouracil

5-FU reduces the cell viability of both cell lines at all incubation times (CCD18-Co: 10–36%; Caco-2: 30–36% vs. control). The reduction in the viability of Caco-2 cells is not time-dependent, while in the non-tumor line the effect increases with the time (24–48 < 72 h). Regarding the sensitivity of the cell lines, only at 24 h the reduction in cell viability is more pronounced in the Caco-2 vs. CCD-18Co cells (36 vs. 10% control), although these differences are not satistically significant ($p < 0.05$) at 48 and 72 h.

3. Discussion

The cholesterol that reaches the colon can be metabolized by the colonic microbiota (mainly bacteria of the genus *Eubacterium* and *Bacteroides*) [5]. There are studies which observe that subjects with CRC present a significantly higher fecal concentration (vs. control) of cholesterol metabolites (21.1 vs. 14.5 mg/g dry feces) [3]. The authors attribute these observations to the abundance of anaerobic bacteria capable of metabolizing cholesterol to potential CRC promoters, and attribute the differences in intestinal flora to the composition of the diet [2,3]. The cytotoxicity may be a key mechanism in cancer induction [4], therefore, in the present study, the cytotoxic activity of the coprostanol, cholestanol, coprostanone and cholestenone on Caco-2 and CCD-18Co cells is evaluated.

A pure compound is cytotoxic when the half maximal inhibitory concentration (IC_{50}) value is less than 4 µg/mL after 48–72 h of incubation [6]. Based on this, cholestenone (IC_{50} at 72 h: 5 ± 1 µg/mL) would be considered cytotoxic on CCD-18Co cells. Studies in rats indicate that cholestenone induces nuclear aberrations [7] and exchange of sister chromatids [8] in the colonic epithelium. These data suggest that cholestenone could be the metabolite with the greatest capacity to promote CRC. Furthermore, coprostanone is the second most cytotoxic metabolite (IC_{50} at 72 h: 15.9–46.8 µg/mL). Therefore, the two most cytotoxic metabolites are, in turn, the most hydrophobic. Probably the greater hydrophobicity favors the ability to alter the structure of the cell membrane and thereby generate cell damage, as occurs with secondary bile acids (promoters of colorectal carcinogenesis, structurally similar to cholesterol metabolites) [9]. In contrast, coprostanol and cholestanol are the metabolites with the lowest cytotoxic activity. In the case of coprostanol, the equatorial position of the C-3 hydroxyl group reduces the ability of coprostanol to bind to the cell membranes of the colon, which would facilitate its elimination through the feces [10].

On other hand, it is observed that CCD-18Co cells are more sensitive to the cytotoxicity of cholesterol metabolites. In other studies, it is also observed that the viability of certain tumor lines (human esophageal, gastric and colorrectal, and mouse colorectal) is not affected by cholestanol [11,12], observing, instead, a slight reduction in the viability of non-tumor human esophageal cells [11]. If cholesterol metabolites exert their cytotoxicity by inducing oxidative stress, such as secondary bile acids [13], one possible reason why CCD-18Co cells are more vulnerable to metabolite cytotoxicity is related to the antioxidant status. The Caco-2 cells are thought to exhibit high intracellular ferritin (antioxidant) levels due to exposure to the heme group through mucosal bleeding and dietary protein during neoplasia [14]. So, the ferritin content in Caco-2 cells is likely to be higher than that of CCD-18Co cells, which would provide protection against possible oxidative damage of cholesterol metabolites. In addition, the composition of the plasma membrane could also be responsible for the difference in the sensitivity of cell lines to metabolites. During colon carcinogenesis, tumor cells undergo changes that allow them to acquire resistance to hydrophobic cytotoxic agents, through an increase in the expression of adenosine triphosphate-binding cassette transporters (ABC), membrane proteins that induce the efflux of substances toxic to cells [15]. Given that these transporters are involved in the efflux of cholesterol and plant sterols from the enterocyte to the intestinal lumen [16], it would be plausible to think that they may also act as transporters for metabolites, making Caco-2 cells less sensitive to their toxicity. In summary, Caco-2 cells are less sensitive to the cytotoxicity of cholesterol metabolites due to their higher antioxidant status and expression in ABC transporters.

It is observed that the reduction in cell viability decreased in some cases with time, which could indicate that the cells adopt a compensatory response. As occurs when cells are exposed to secondary bile acids, the toxic effect of metabolites could lead to the activation of nuclear factor-kappa B (NF-κB) [17]. It is known that NF-κB is a transcriptional regulator that increases the expression of genes that encode antioxidant enzymes and ABC transporter, stimulates cell proliferation, induces resistance to apoptosis, and is related to inflammatory processes [18,19]. Therefore, a hyperproliferative and antioxidant response, together with an increase in the expression of ABC protein and resistance to apoptosis, would explain the lower reduction in cell viability observed. Furthermore, the increase in the cell proliferation, resistance to apoptosis and inflammation could be the mechanisms through which cholesterol metabolites are involved in colorectal tumorigenesis, as occurs with secondary bile acids [13]. Furthermore, it is observed that the compensatory response in Caco-2 cells could occur much earlier than in the non-tumor line. The higher proliferative activity of Caco-2 cells, together with their higher antioxidant status [14] and the higher expression of ABC proteins [15], would explain why the compensatory response of Caco-2 cells occurred earlier than in CCD-18Co cells.

4. Materials and Methods

Caco-2 (passages: 10–17) and CCD-18Co (passages: 3–9) cells come from American Type Culture Collection (HTB-37 and CRL-1459, respectively) (Rockville, MD, USA). Both cells line are seeded at a density of 25,000 cells/well in 96-well plates and incubated at 37 °C, at 95% relative humidity and with 5% (*v/v*) of CO_2. At 24 h post-seeding, the cells are treated with metabolites individually at different concentrations (9.4, 18.75, 37.5, 75, 150 and 300 μM) and incubated for 24, 48 and 72 h.

Untreated cells are the control and 5-FU (25 μM) are a well-known cytotoxic agent on human colon cancer cells [20]. The cytotoxic activity of metabolites on Caco-2 and CCD-18Co cells is evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Briefly, after treatment with metabolites, the culture medium is removed and 90 μL of phosphate-buffered saline (PBS) and 10 μL of MTT (0.5 mg/mL in PBS) are added. After 4 h of incubation, the MTT solution is removed and the formazan salts are solubilized with 10 μL of dimethylsulfoxide. Formazan formation is determined by spectrophotometry at 570 nm with background subtraction at 690 nm.

5. Conclusions

The metabolites produced by intestinal bacteria from cholesterol, mainly those of a hydrophobic nature (cholestenone and coprostanone), could be involved in colorectal carcinogenesis through their cytotoxic activity. More in-depth biochemical and molecular assays are needed to decipher the specific mechanisms involved in their deleterious activity.

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Abbreviations

The following abbreviations are used in this manuscript:

ABC	Adenosine triphosphate-binding cassette
CRC	Colorrectal cancer
5-FU	5-Fluorouracil
IC$_{50}$	Half maximal inhibitory concentration
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NF-κB	Nuclear factor-kappa B
PBS	Phosphate-buffered saline

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