Production of Diacylglycerol, an Activator of Protein Kinase C, by Human Intestinal Microflora

Masami Morotomi, Jose G. Guillem, Paul LoGerfo, and I. Bernard Weinstein

INTRODUCTION

Colon cancer is the second most frequent cancer in the United States. Over 6% of Americans living today are expected to develop colon cancer at some time during their lives, of whom about one-half will die from this disease. Its etiology, like that of most human neoplasms, is probably multifactorial. Hereditary factors certainly play a predominant role in the development of familial polyposis coli and familial nonpolyposis colon cancer. However, in the majority of colon cancer patients, dietary factors probably provide the overriding impetus for tumor development (1). Evidence obtained from epidemiological and experimental studies suggests that high fat diets increase the risk of cancer of the colon (1-3). At the same time, there is evidence that the intestinal microflora also plays a role. Several studies have shown differences between germ-free and conventional animals with regard to incidence, latency, and histology of spontaneous and chemically induced colonic tumors (for review see Ref. 4). However, the mechanisms by which the intestinal microflora exert these effects are not known. Mutagens produced by the intestinal bacteria have been demonstrated in human feces, but their presence does not show an association with colon cancer risks (5, 6) and the action of mutagens would not explain the generalized increase in cell proliferation of the colonic epithelium seen in high risk populations (7, 8).

ABSTRACT

Although dietary lipids have been implicated in colon cancer causation, the underlying mechanisms are not known. This paper indicates that when bacteria obtained from normal human feces are incubated with 14C-labeled phosphatidylcholine there is appreciable production of diacylglycerol (DAG), monoacylglycerol, and free fatty acid. Curiously, the production of DAG and monoacylglycerol, but not fatty acid, is strictly dependent on addition of certain bile acids to the incubation system. Among the bile acids tested deoxycholic acid is the most active. Assays of fecal samples from 10 normal individuals demonstrate a 27-fold interindividual variation in the production of DAG in the in vitro assay system, and also in the absolute levels of DAG present in the same fecal samples. On the other hand, both parameters of DAG are quite constant in repeated fecal samples obtained from the same individual over a period of about 4 months. DAG is a normal physiological activator of protein kinase C, an enzyme that plays a key role in growth control and tumor promotion. We speculate, therefore, that DAG produced by the intestinal microflora might stimulate growth of colonic epithelial cells. Thus an interaction between dietary lipids, bile acids, and specific bacteria in the intestinal lumen could contribute to the risk of colon cancer development in humans.

MATERIALS AND METHODS

Assay of DAG Production. For the standard assay of DAG production from PC, a stock solution of labeled PC was prepared as follows; an aliquot of l-3-phosphatidylcholine, 1,2-dipalmityl (10 mg/ml of chloroform solution) was mixed with labeled PC (l-3-phosphatidylcholine, 1,2-di[l-14C]palmitoyl, 110 mCi/mmol; Amersham) and DCA (100 mM mehanolic solution), and taken to dryness at room temperature under nitrogen. To the dried residue distilled water was added and was followed by sonication for 1 min in a Bransonic bath sonicator. For the experiments shown in Fig. 4, CA, CDC, TDCA, or Triton X-100 was added by 760 nM of chloroform:methanol (1:2, v/v) and 100 nM of 2 M KCl. After mixing, 220 nM of chloroform and 220 nM of 2 M KCl were added and mixed, and the phases were separated by centrifugation. The organic phase was dried under nitrogen. The dried residue distilled water was added and was followed by sonication for 1 min in a Bransonic bath sonicator. For the experiments shown in Fig. 4, CA, CDC, TDCA, or Triton X-100 was added by 760 nM of chloroform:methanol (1:2, v/v) and 100 nM of 2 M KCl. After mixing, 220 nM of chloroform and 220 nM of 2 M KCl were added and mixed, and the phases were separated by centrifugation. The organic phase was dried under nitrogen. In the paper to disolve in a small amount of chloroform:methanol (2:1, v/v), chromatographed on silica gel G plates by developing in hexane/diethyl ether-acetic acid (50:50:3 by volume) and subjected to AR. Under the TLC-AR conditions, degradation of PC (Rf = 0) and formation of free FA (Rf = 0.37), DAG (Rf = 0.29) and MAG (Rf = 0.08) could be readily detected.
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Preparation of Fecal Homogenates. For the preparation of fecal homogenates, fresh fecal specimens from healthy adults were homogenized with 4 volumes (w/v) of 0.1 M potassium phosphate buffer (pH 7.0) by shaking vigorously with glass beads. Dietary debris was removed by centrifugation at 500 × g for 1 min.

Preparation of [14C]DAG. Radiolabeled DAG was prepared from L-3-phosphatidylcholine, 1,2-di[1-14C]palmitoyl by incubation with phospholipase C (from Clostridium perfringens, type IX, Sigma) as described previously (13). [14C]DAG was purified by TLC on silica gel plates developed under the same conditions as described above.

Quantification of Total DAG in Fecal Specimens. Lipids were extracted by the method of Bligh and Dyer (14) from portions of the same fecal specimens (5 and 10 mg equivalent to wet weight) used for the assays of DAG-producing activity shown in Fig. 5. The total amount of DAG in the fecal extract was analyzed by using Escherichia coli DAG kinase (Lipidex; Middleton, WI), using a previously described procedure (15, 16) and expressed as nmol per g wet weight of original feces. Control studies indicated that the extraction procedure for DAG was quantitative and that the enzymatic assay was linear.

RESULTS

To test the hypothesis that DAG might be produced from phospholipids by the human intestinal microflora, 14C-labeled dipalmitoyl phosphatidylcholine (labeled in the palmitoyl residue) was incubated with fecal homogenates containing intact bacteria, and the production of DAG was monitored by TLC-AR. In our initial studies, we found that accumulation of DAG in the reaction mixture was observed, but only in the presence of the bile acid DCA (Fig. 1), although the release from PC of free FA occurred in the absence of DCA (data not shown here). DCA produced a concentration-dependent stimulation of DAG production when tested at 2 to 10 mM and the reaction continued for at least 2.5 h (Fig. 1). The effects of varying the pH from 5 to 8 on degradation of PC and the formation of DAG were also examined, as a function of DCA concentration (Fig. 2). The extent of PC degradation increased with increasing pH and was maximum at 5 mM DCA. Curiously, increasing the DCA concentration from 5 to 10 mM resulted in a significant decrease in PC degradation (Fig. 2a). On the other hand, the formation of DAG was highest at the 10 mM concentration of DCA and at a pH of about 6 (Fig. 2b). Fig. 3 compares the time course of degradation of PC to that of the formation of FA, DAG, and MAG, when the incubation was done in the presence of 10 mM DCA at pH 7. The degradation of PC and the accumulation of FA, the major product of PC degradation, were found to be approximately linear over a 2-h period (Fig. 3a and b). During this time period about 22% of the PC was converted to FA. The accumulation of MAG was also approximately linear during this time period. On the other hand, the accumulation of DAG was curvilinear and tended to plateau with time (Fig. 3c; see also Fig. 1). The time course results obtained in Figs. 1 and 3 suggested that with time some of the DAG that is synthesized is subsequently degraded to MAG and free FA. Indeed, when [14C]DAG (1.6 mM, 2 × 10⁴ cpm) was incubated in the same reaction system containing fecal bacteria and 10 mM DCA, 16.1% of the added DAG was converted to MAG (1.0%) and free FA (15.1%) after a 120-min incubation period.

No differences were seen when the incubations were performed under aerobic or anaerobic conditions in terms of PC degradation and the formation of DAG, FA, and MAG. In addition, more than 90% of all the above metabolic activities were found in the sedimentable fraction of the fecal homogenate obtained after low-speed centrifugation, indicating that they reflected the activity of intact bacteria rather than soluble enzymes (data not shown).

We also examined the effects of different types of bile acids, and of the detergent Triton X-100, on the conversion of PC to its various metabolites in the above system. PC degradation and the formation of FA, the major product of PC degradation, were enhanced by the addition of several bile acids; CA showed the highest enhancing activity, producing an 8-fold increase (Fig. 4, a and b). On the other hand, DCA showed the highest enhancing activity with respect of the formation of both DAG and MAG (Fig. 4, c and d). In all of these assays Triton X-100 had only a modest enhancing effect (Fig. 4).
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Fig. 3. Time-course study of degradation of PC. The assays were performed at pH 7 with 10 mM DCA. Mean values and SD of the four assays are shown; (a) percentage of PC remaining; (b), (c), and (d), percentage of conversion to FA, DAG, and MAG, respectively.

Fig. 4. Effects of different bile acids and Triton X-100 on degradation of PC. The conditions of the assay were similar to those described in Fig. 1. Fecal homogenates from 3 healthy volunteers were incubated individually with PC in the presence of the indicated bile acid (10 mM) or Triton X-100 (TX; 0.3 or 3 mg/ml) for 60 min at pH 7.0. Significance of the data was analyzed by Student's t test. *, significantly different from control (no addition), P < 0.05; **, significantly different from control (no addition), P < 0.01. bars, range of SD values obtained with the three different samples. (a) percentage of PC degradation; (b), (c), and (d), percentage of conversion to FA, DAG and MAG, respectively.

Fig. 5 shows the DAG-producing activity of 10 fecal specimens obtained from different normal individuals, as well as day-to-day variations in one individual, when assayed by the method described in Fig. 1 in the presence of 10 mM DCA. Fig. 6 shows the absolute amounts of 1,2-sn-DAG present in the same fecal specimens, when assayed by the 1,2-sn-DAG-specific DAG kinase method. The donors of the specimens were healthy male volunteers (age, 33.1 ± 6.6 (SD); range, 23–45) working in research laboratories at this university. They are from China, Columbia, Ecuador, Japan, India, and the United States. Their diets were quite variable and this aspect was not controlled or monitored in this pilot study. We found that both the DAG-producing activity of fecal bacteria and the absolute amount of fecal DAG differed considerably (over 27-fold) among individuals. Replicate assays of the same sample, however, showed little variation (less than 10%) and a time course study on one individual extending over a period of 115 days displayed less than a 4-fold variation for both activities (Figs. 5 and 6).
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review see Refs. 2 and 3), although the significance of this
association in terms of causal mechanisms is not known. The
results of the present study suggest that dietary lipid and bile
acids might act together to influence the production of DAG
bacteria present in the lumen of the colon, since we were
able to demonstrate this type of effect with homogenates of
human fecal specimens. Among the bile acids examined in the
present study, DCA showed the highest activity, and CDCA
the next highest activity with respect to stimulating DAG
formation from PC (Fig. 4c). DCA and CDCA have an uncon-
jugated carboxylic head group and a hydrophobic domain con-
taining two hydroxyl groups, which are not present in CA and
TDCA. Thus, steric features of the hydrophobic domain and the
amphiphilic character of DCA and CDCA may play impor-
tant roles in this enhancing effect. Triton X-100 showed little
effect on the bacterial conversion of PC to DAG (Fig. 4), even
though this detergent and all of the bile acids tested readily
solubilized the PC present in the reaction medium, since the
medium became clear with the addition of these agents. Our
results could be of biological significance since bile acids are
present in the lumen of the colon at mM concentrations (17-
19), i.e., the concentration range in which we find that they
enhance DAG formation by fecal bacteria. In previous studies
we reported that at mM concentrations specific bile acids can
also act directly to enhance or inhibit PKC activity (20).

The present findings are consistent with a recent report (21)
that DAGs are present at relatively high levels in human feces,
although these authors did not identify the precise sources of
the DAGs and the factors that might influence their levels. It
is of interest that these authors also demonstrated that DAGs
induce DNA synthesis in colon adenoma and colon carcinoma
cell cultures, but do not have this effect in normal primary
colon epithelial cell cultures (21). The authors suggested that
DAG is generated from the partial breakdown of dietary tri-
glycerides in the intestine (21). Experimental studies indicate
that PKC is specifically stimulated by DAGs that have the 1,2-
-sn configuration (22). However, DAGs derived from triglycerol
by the action of digestive lipases such as pancreatic lipase and
liver lysosomal lipase do not show stereospecificity (23–25).
Furthermore, DAGs derived from triacylglycerol by the action
of a heparin-releasable hepatic lipase or a lipoprotein lipase
have a 2,3-sn configuration (23–25). It seems more likely,
therefore, that the 1,2-sn-DAGs produced by the phospholipase
C enzyme present in specific intestinal bacteria would stimulate
PKC activity in colonic epithelial cells. We should emphasize,
however, that it remains to be determined whether DAGs
produced by bacteria in the lumen of the colon are actually
taken up by the colonic epithelium and influence the PKC
activity and proliferation of these cells. This does not seem
unlikely in view of the evidence that exogenously added DAGs
stimulate PKC activity when added to cultured human epider-
moid carcinoma cells, human colon adenoma or carcinoma
cells, fibroblasts, and platelets (10, 21). Furthermore, Craven
et al. (26) found that intracolonic instillation of 5 mM 1-oleoyl-
2-acetylglycerol activated colonic epithelial PKC and induced
ornithine decarboxylase and [3H]thymidine incorporation in
rats. We should also emphasize that in the present studies we
used 14C-labeled dipalmitoyl PC as a convenient substrate, but
we assume that other phospholipids present in the lumen of
the intestine would also serve as a source of DAG. Indeed, in recent
studies we have found that phosphatidyl inositol and phospha-
tidyl ethanolamine also serve as substrates for the production
of DAG by human fecal bacteria. Studies are in progress to
determine the effects of using substrates that contain various
types of fatty acids. It is known that in humans a high fat diet
increases fecal excretion of phospholipids to levels as high as
400 mg/day. The components include PC, phosphatidyl glyc-
erol, phosphatidyl ethanolamine, and phosphatidyl inositol
(27). They are derived from dietary sources and also from
synthesis by the intestinal bacteria. In view of this diversity of
potential substrates, it is not unreasonable to assume that some
of the DAGs produced would have structures that favor cell
uptake and activation of PKC. Studies are in progress to verify
this assumption. It will also be of interest to isolate the specific
strains of fecal bacteria that are the most active in producing
DAG. We are currently attempting to identify which of the

DISCUSSION

Epidemiological data and experimental animal studies have
shown a positive association among dietary lipid intake, fecal
bile acid excretion, and the development of colon cancer (for
review see Refs. 2 and 3), although the significance of this
association in terms of causal mechanisms is not known. The
results of the present study suggest that dietary lipid and bile
acids might act together to influence the production of DAG
in the lumen of the colon, since we were able to demonstrate this type of effect with homogenates of
human fecal specimens. Among the bile acids examined in the
present study, DCA showed the highest activity, and CDCA
the next highest activity with respect to stimulating DAG
formation from PC (Fig. 4c). DCA and CDCA have an uncon-
jugated carboxylic head group and a hydrophobic domain con-
taining two hydroxyl groups, which are not present in CA and

Fig. 5. DAG-producing activity of fecal specimens from individuals and day-
to-day variation in one individual. The assay conditions were; pH 7, 10 mM DCA,
and incubation time, 60 min; (a) mean values of duplicate assays of 12 stool
specimens collected from 10 healthy volunteers. The same symbols represent
specimens from the same subject; (b) day-to-day variation of DAG producing
activity in one individual.

Fig. 6. Quantitation of total DAG in fecal specimens from 10 individuals and
the day-to-day variation in one individual. The total amount of DAG in feces was
expressed as nmol per g wet weight of original feces; (a) amount of DAG in 12
stool specimens from 10 healthy volunteers; (b) day-to-day variation in one
individual.

was no correlation, however, between a given individual’s DAG-
producing activity and the absolute level of DAG present in the
same fecal specimen (P > 0.05). This may reflect the fact that
multiple factors influence the steady state level of DAG, as
discussed above.

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large number of bacterial species in human feces produce DAG. This is a difficult task because of the large number of species (over 200) and the difficulty in growing them. Using purified ATCC type strains we have found that Clostridium perfringens does show DAG-producing activity in our assay, but we have not yet established which strain or strains of bacteria are responsible for the activity we detect in human fecal samples.

The results obtained in the present study suggest that the levels of specific DAGs in the lumen of the colon are a function of multiple factors, including the dietary intake of specific lipids; the lipid metabolizing activity of specific bacteria in the intestinal microflora, including the relative levels of DAG-producing and DAG-degrading enzymes; and the levels of specific bile acids, which are also a function of dietary lipid. If the present hypothetical framework is correct, then the interplay between these variables could play an important role in colon cancer causation and the design of new approaches to its prevention.

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