Follow-up of tumor development in the colons of living rats and implications for chemoprevention trials: Assessment of aspirin-difluoromethylornithine combination

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Abstract. We aimed to establish a reliable procedure allowing the follow-up of tumor development by computed tomographic (CT) colonography in an animal model of colon carcinogenesis in order to assess the chemopreventive efficacy of aspirin and difluoromethylornithine (DFMO) given in combination. Fischer rats received an intraperitoneal injection (25 mg/kg) of di-methylhydrazine (DMH) once a week for two weeks in order to initiate colon carcinogenesis. Five months after the last injection of DMH, a first CT colonography was performed and rats were then randomly separated into two groups (control and experimental). The experimental group received a 0.1% mixture of aspirin and DFMO in drinking water. CT colonography was performed at 6, 7 and 8 months. Data showed a precise correlation between location and size of tumors found at autopsy and those detected by CT colonography at 8 months. All tumors were also detected on the CT views obtained previously. Animals of the aspirin/DFMO group exhibited an inactivation of ornithine decarboxylase, a key enzyme in polyamine biosynthesis, and a two-fold reduction in the prostaglandin E_2 content of the colonic mucosa ($p<0.01$). In rats with tumors at the start of the aspirin/DFMO treatment, a significant slow-down of tumor development was observed. In contrast, in rats where no tumors were detected at the start of the treatment, tumor formation was inhibited. Our data show that CT colonography represents a reliable method to assess in a living animal the efficacy of chemopreventive agents.

Introduction

Computed tomographic (CT) colonography has been used in humans for more than ten years for colorectal neoplasia detection. This technique offers the advantage of a non-invasive cross-sectional imaging examination that can reliably detect colorectal lesions (1). This technology has the potential to be of substantial influence in colorectal polyp detection and colon cancer screening (2). Current knowledge regarding the role of CT colonography in diagnosing cancer is making the use of the technique in an animal model of colon carcinogenesis very attractive especially for the evaluation of chemopreventive interventions.

Approximately 80% of colorectal cancers are sporadic and the high mortality is due to the late stage at which many cases are detected. Attention is therefore being focused on preventive strategies given that polyps appear to be identifiable and treatable precursor lesions of this disease (3). A systematic review of colon chemoprevention in rodents and humans has recently shown that the carcinogen-induced rat model is a good predictor of chemopreventive efficacy in humans (4). In this model, aberrant crypt foci (ACF) were regarded as putative preneoplastic lesions for colon cancer and ACF were used as biomarkers to evaluate the efficacy of potential chemopreventive agents (5). These lesions may be precursors of adenomatous polyps and colorectal cancer in humans (6-10).

Chemopreventive studies with preclinical models have been hindered by the impossibility to follow in the same living animal the formation of polyps and their transition to carcinoma. In this regard, the use of CT colonography for the follow-up of tumorigenesis may offer a new tool for chemoprevention research. Up to now, only one preliminary study aimed to assess the feasibility of CT colonography in a rodent polyp model has been realized (11). This is the first report establishing a reliable procedure allowing the follow-up by CT colonography of tumor development for several months in a carcinogen-induced colon cancer model, and assessing in living rats the chemopreventive efficacy of aspirin and difluoromethylornithine (DFMO). These compounds were found to be more efficient chemopreventive agents when associated than when administered separately (5,12). In addition, clinical data support the use of DFMO and aspirin in combination as a strategy for colon cancer prevention in humans (13).

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Materials and methods

Animal model and treatments. All animal experiments were performed in accordance with the institutional guidelines of the French Ethics Committee (authorization no. A67-480, French Ministry of Agriculture). Male Fischer (F344) rats (5 weeks old; Charles River Laboratories, Les Oncins, France) with a mean body weight of 135 g were housed under standardized conditions (22˚C, 60% relative humidity, 12:12-h light:dark cycle, 20 air changes/h) and fed a standard diet with free access to drinking water. All animals were administered the chemical carcinogen dimethylhydrazin (DMH; Sigma-Aldrich, Saint Quentin Fallavier, France) by i.p. injection (25 mg/kg body weight) once a week for two weeks. Five months following the last DMH injection, at a stage where tumors may already be present, a first CT colonography was performed and rats were then randomly separated into a control group (3 animals, group C) and an experimental group (6 animals, group E). Thereafter group E received daily at 5 pm a freshly prepared mixture of 0.1% soluble aspirin (acetylsalicylate of DL-lysine; Sanofi-Synthelabo, Paris, France) and 0.1% DFMO (Ilex oncology Inc., San Antonio, USA) in drinking water. Rats consumed daily 22 to 24 ml during the whole experimental study. The daily intake of aspirin and DFMO per rat was 55-60 mg/kg body weight for each compound.

CT colonography was performed on each rat once a month from 6 to 8 months. All animals were sacrificed at 8 months after the last CT-scan and the colon was collected for histo-morphological and biochemical analyses. The tumor volume was evaluated at autopsy based on the formula $V = 4/3\pi \times (A/2)^2 \times B/2$, where A is the width of the tumor in millimeters and B the length. Two different readers reviewed the CT colonography scan prior to autopsy which was performed by an analyst unaware of the CT scan results.

Animal preparation before CT colonography. Three days before CT colonography, rats were allowed to drink only a 7% Klean-Prep (Helsinn Birex Pharmaceuticals Ltd, Dublin, Ireland) solution in water containing polyethylene glycol and sodium salts. At 2 pm, on the day before CT-scan, rats were deprived of food and received 3 ml of the Klean-Prep solution by gavage and had free access to the same drinking fluid (7% Klean Prep in water). Before imaging, the animals were anesthetized with isofluran (2-3%) in O_2/N_2O (30/70%). An intrarectal injection of a contrasting agent was performed in the anesthetized animal. The colon was filled with 20 ml of a 25% solution of meglumine and sodium ioxaglate (Hexabrix 320, Guerbet, Roissy CdG, France) in 0.9% NaCl. The rectal tube was locked with a mineral wax stopper to prevent leakage during examination and the animal was immediately proccessed for CT colonography.

CT colonography and image analysis. CT colonography was performed with a micro-CT scanner Micro Cat II (Imtek Inc., San Diego, USA). Rats were scanned in supine position under anesthesia with isofluran (2-3%) in O_2/N_2O (30/70%). Bio-monitoring during acquisition allowed synchronization with an automated breath tracking system in order to avoid breathing movement interferences. The acquisition duration was 10 min

for each animal. Parameters were as follows: 500 μA, 80 kV, 150-μm slice thickness.

The micro-scanner differs from a conventional scanner used in humans by the fact that during a scan, 2D-X ray images are acquired as the source (X-ray tube) and the CDD detector completes only one rotation of 360˚ around the animal. The Microcat™ software allows a 3D reconstruction process from the 2D-X ray images. Axial, sagital and coronal views are obtained. We show only coronal views since they allow an excellent visualisation of the entire colon and provide the best views for measuring tumor location using pubic sympheses as a reference mark.

Assessment of aberrant crypts and tumors in the colon. The colon was collected, washed with saline, and laid out flat with the mucosa on the upper side in order to record the number, size and localization of tumors. The mucosa of the proximal part of the colon was scraped off with a glass slide and frozen in liquid nitrogen for biochemical analyses. A 6-cm-long segment corresponding to the distal part of the colon was fixed in 10% buffered formalin. For aberrant crypt assessment, the segment was stained with 0.2% methylene blue for 5 min, rinsed in Krebs-Ringer buffer, placed on a glass slide and examined under blinded conditions using a microscope with a low-power objective (x5).

Measure of ornithine decarboxylase (ODC) activity. Enzyme activity was measured in mucosal samples of the proximal colon. Mucosal samples were homogenized in 100 mmol/l Tris-HCl buffer, pH 7.4 (1 mmol/l EDTA, 1 mmol/l dithio-threitol, 0.5 μmol/l leupeptin, 0.5 mmol/l phenylmethylsulfonyl fluoride). ODC assay was performed by measuring $^{14}CO_2$ formation from [1-^{14}C]L-ornithine (55 mCi/mmol; Amersham Pharmacia Biotech, Orsay, France) (14).

Determination of PGE_2 concentrations. A sample of the mucosal scraping was homogenized in 100 mmol/l Tris-HCl buffer, pH 7.8, containing 1 mmol/l EDTA. After centrifug-ation at 10,000 x g for 15 min at 4˚C, the supernatants were collected for PGE_2 measurements using the PGE_2 assay kit (R&D Systems Inc., Lille, France). The assay was performed according to the manufacturer's procedure.

Statistical analysis. Data are reported as mean ± standard error of the mean (SE). Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using the Student's t-test or the Student Neuman-Keuls multiple comparison test.

Results

Follow-up of tumor development. As shown on the views obtained after CT colonography, at 8 months after the last DMH injection all controls exhibited tumors in the colon (Fig. 1). Their locations found by post-mortem examination corresponded to those measured on the CT scan views, using pubic sympheses as a reference mark.

As shown for rat C1 (Fig. 1), 5 months after DMH, one tumor was detected in the distal part of the colon by CT colonography. The volume of the tumor increased progressively
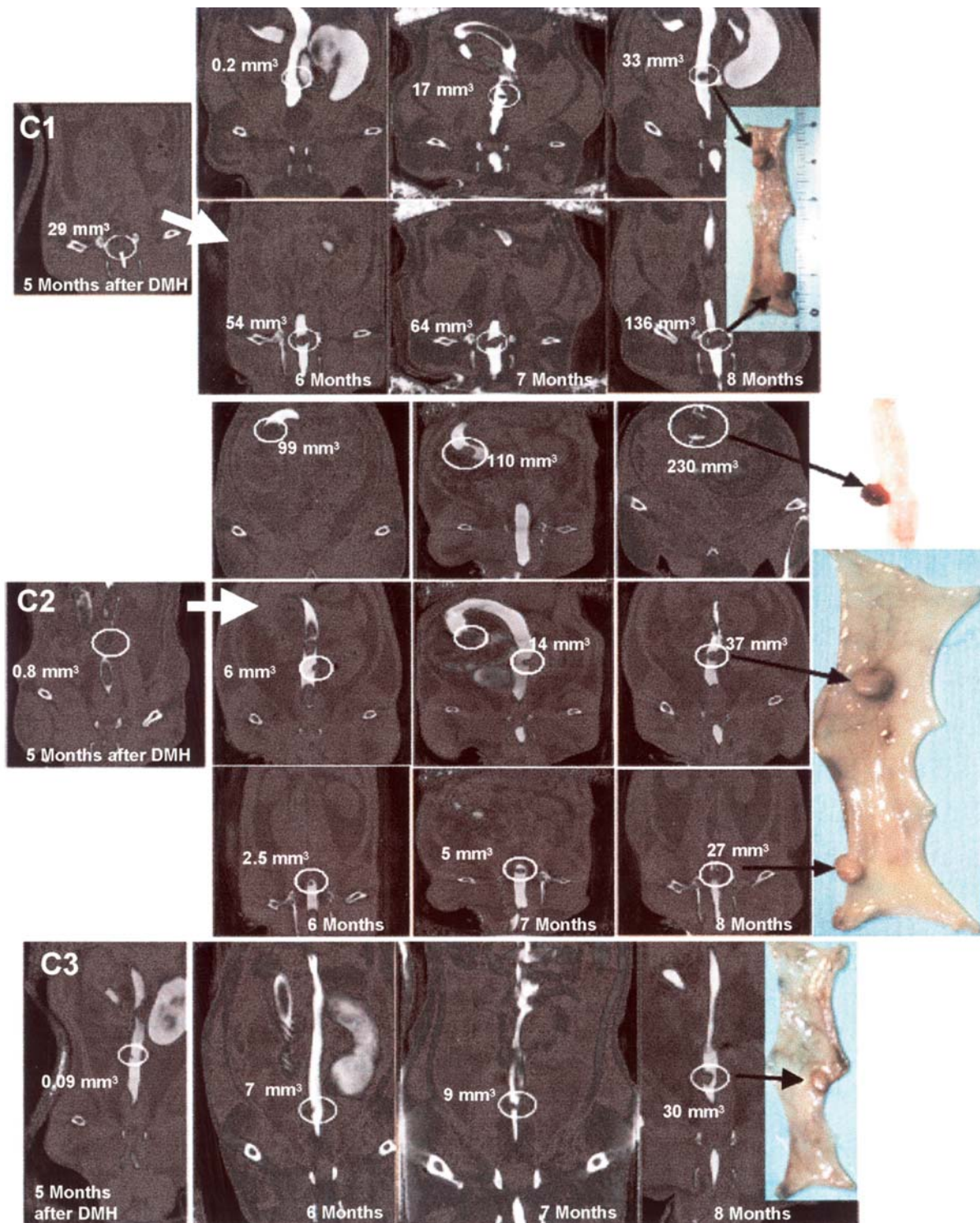
Figure 1. Anterior CT colonographic coronal views obtained in three anesthetized rats (C1, C2, and C3) 5, 6, 7, and 8 months after chemical carcinogen injection. White circle indicates polyp and tumor location. Pubic symphese was used as a reference mark for tumor location. Photographic inserts show tumor location in the proximal (only in C2) or distal colon found at post-mortem examination 2 weeks after the last CT colonography. Arrows show correspondence between tumors detected on CT views and those found at autopsy. The tumor volume was evaluated based on the formula $V = 4/3\pi \times (A/2)^2 \times B/2$, where A is the width of the tumor in millimeters and B the length.

at 6 and 7 months reaching 136 mm³ at 8 months. At autopsy (after the last CT colonography) the shape and volume of the tumor was similar to the spot observed on the CT scan view (see photographic insert). Another tumor of 33 mm³ was also detected by CT colonography and confirmed at autopsy in the same animal but situated in the upper part of the distal

colon. This tumor was retrospectively also found at 7 months. At 6 months, at the same location, a tiny polyp (0.2 mm³) was barely detected by CT colonography.

At autopsy, the rat C2 (Fig. 1) exhibited three tumors after 8 months: one voluminous tumor (230 mm³) was detected in the proximal colon and two others respectively of 37 and
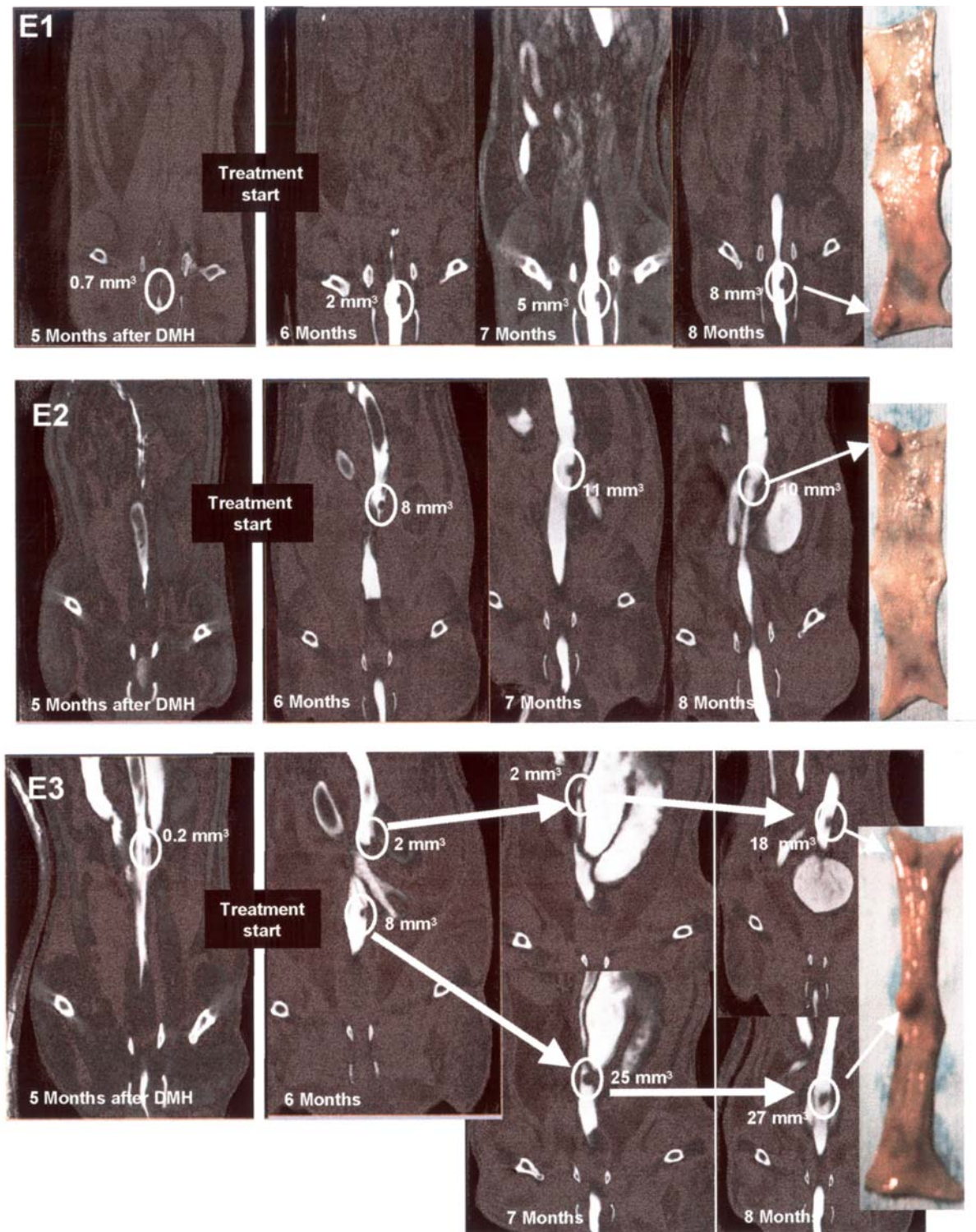
Figure 2. Anterior CT colonographic coronal views of three anesthetized rats (E1, E2, E3) obtained 5, 6, 7, and 8 months after chemical carcinogen induction. After the first CT colonography at 5 months, all rats received daily at 5 pm a freshly prepared mixture of 0.1% soluble aspirin and 0.1% difluoromethylornithine (DFMO) in drinking water. White circle indicates polyp and tumor location. For other details see legend of Fig. 1.

27 mm^3, were found in the distal half of the colon. All these tumors were also identified by CT colonography at 7 and 8 months. At earlier stages the tumor located in the proximal colon was only detected retrospectively on the CT scan views at 6 months and could not been seen at 5 months, due to the presence of residual fecal material in the segment. The polyps which evolved to form the two tumors present in the distal half of the colon were also detected on the scan at 6 months:

a small polyp (2.5 mm^3) was identified as the precursor of the most distal tumor, whereas the upper tumor located in the distal colon was clearly originating from the polyp measuring 6 mm^3. At the same location a small polyp (0.8 mm^3) was also found retrospectively 5 months after DMH injection despite the presence of residual stool in the colonic lumen. The CT colonography of rat C3 (Fig. 1) allowed the detection of one tumor (30 mm^3) at 8 months in the distal colon which
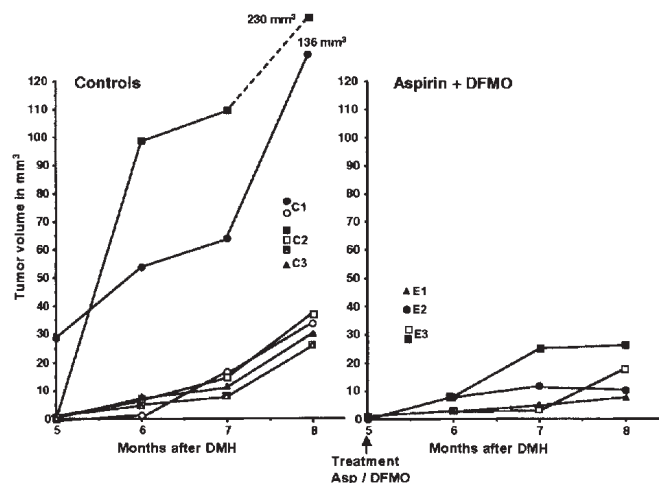
Figure 3. Evolution of tumor growth from 5 to 8 months after DMH-injection in controls and aspirin/DFMO-treated rats based on tumor volume measured on the CT views.
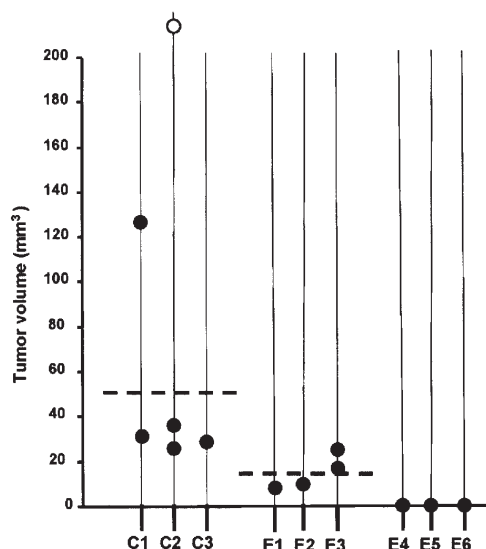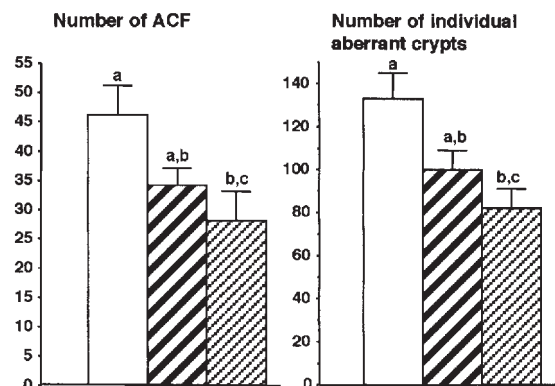

Figure 5. Number of aberrant crypt foci (ACF), total number of individual aberrant crypts in the distal colon (6 cm in length); open columns, DMH-injected controls; hatched columns, aspirin/DFMO-treated rats (large strips: rats E1, E2, E3; narrow strips: rats E4, E5, E6). Values are means ± SE. Columns not sharing a common superscript letter differ significantly (p<0.05).

Figure 4. Size (in mm³) of each tumor found at autopsy 8 months after DMH injection in control or aspirin/DFMO-treated rats. Black circle, tumor located in the distal half of the colon; open circle, tumor located in the proximal half of the colon. The dotted lines correspond to the mean tumor volume measured in the distal colon of control and treated rats.

was also found at autopsy. The tumor was also detected at months 6 and 7 at the same location. Retrospectively we found, on the CT views obtained at 5 months, that its precursor was a small polyp (0.09 mm³).

In animals treated with the aspirin/DFMO combination following the first CT colonography at 5 months, two different situations occured:

The first situation was observed in rats with apparent (E1 and E3) or suspected (E2) polyps detected at 5 months and confirmed on the CT views at 6 months (Fig. 2). The data obtained by CT colonography showed that aspirin/DFMO treatment did not favor regression of the polyps. However the treatment caused a slow-down of tumor growth when compared to DMH-injected controls (Fig. 1).

In rat E1, one small polyp (0.7 mm³) was detected at 5 months reaching a volume of 8 mm³ three months later. Location and size was confirmed at autopsy.

In rat E2, one tumor of 10 mm³ was found by CT colonography at 8 months and was confirmed at autopsy. The tumor was also observed on the CT views at 6 and 7 months, and at 5 months the presence of residual stool hindered a correct evaluation of its size.

In rat E3, two tumors were detected on the CT views at 8 months and confirmed two weeks later at autopsy. These two tumors were also detected by CT colonography at 6 and 7 months, but only the upper one was also retrospectively detected at 5 months, before the initiation of the aspirin/DFMO treatment (Fig. 2).

The second situation was observed in 3 rats (E4, E5, E6) where no tumors were detected by CT colonography and at autopsy. However, ACF and aberrant crypts were present in the colons of all animals indicating that the aspirin/DFMO treatment inhibited the evolution of these preneoplastic lesions towards the formation of polyps and tumors (not shown).

Fig. 3 illustrates the time-evolution of tumor volume based on the CT scan data for each control rat (C1, C2,C3) and for each rat treated with aspirin/DFMO (E1, E2 and E3). A clear reduction of tumor growth was observed in the aspirin/DFMO group. In addition, the volume of each tumor measured at autopsy in each animal (Fig. 4) was reduced in the aspirin/DFMO-treated rats with at least one polyp at the initiation of the treatment (E1-E3). No tumors were found at autopsy in rats where no polyps or tumors were detected by CT colonography (E4-E6).

Measure of ACF and aberrant crypt foci. As observed at autopsy, all rats injected with the chemical carcinogen developed aberrant crypts in the distal half of the colon regardless of the treatment (Fig. 5). However, a 25-30% reduction in the number of ACF and of the total number of aberrant crypts was observed in rats treated with aspirin/DFMO. The distribution of aberrant crypts in the ACF (ACF size) was not significantly modified in aspirin/DFMO-treated rats versus controls (Fig. 5). There was no significant difference
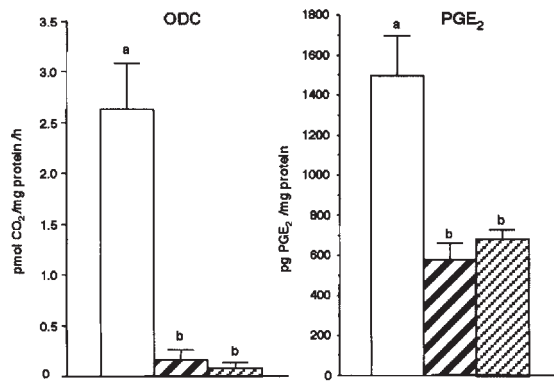
Figure 6. Effects of aspirin/DFMO treatment on the activity of ODC and on the mucosal PGE_2 concentration in samples of the proximal colon. Open columns, DMH-injected controls; hatched columns, aspirin/DFMO-treated rats (large strips: rats E1, E2, E3; narrow strips: rats E4, E5, E6). Values are means ± SE. Columns not sharing a common superscript letter differ significantly (p<0.001).

in the total number of aberrant crypts, in the number of ACF between aspirin/DFMO-treated rats which bore tumors (E1-E3) and those which remained tumor-free (E4-E6).

Mucosal ODC activity and PGE_2 content. The colonic mucosa of rats receiving aspirin and DFMO (60 mg/kg body weight for each compound) starting at 5 months after DMH injection exhibited at 8 months a profound depletion of ODC activity caused by DFMO, the selective inactivator of ODC. In addition, aspirin, a known cyclooxygenase inhibitor, caused a 2-fold diminution of the PGE_2 content in the colonic mucosa (Fig. 6). ODC and PGE_2 content were affected similarly in aspirin/ DFMO-treated rats bearing tumors (E1-E3) and those which remained tumor-free (E4-E6).

Discussion

This is the first study showing that CT colonography represents a reliable method for the follow-up of tumor promotion/ progression in the colons of individual rats. A significant correlation was found between the locations and sizes of tumors found at autopsy and those revealed by CT colonography. All tumors found at autopsy were already identifiable on the CT views obtained several months before autopsy.

We report also an easy procedure for colon preparation before acquisition, which represents a crucial step for good quality imaging. Three days before CT colonography, rats recieved an oral administration of a gut cleaning solution administered routinely to patients undergoing colonoscopy. After a 24-h starvation period, just before CT colonography, a contrasting solution (20 ml) was administered intrarectally to the anesthetized rat. This commercially available solution contains meglumine and sodium ioxaglate and is used routinely by intravenous route in patients undergoing radiography. In contrast to CT colonography in humans (1,15) no intrarectal air insufflation is needed, but in order to prevent fluid leakage the rectum was locked by a mineral wax stopper. Furthermore, in order to avoid breathing movement interference during acquisition, a bio-monitoring device with an automated breath tracking system allowed correct synchronisation.

The carcinogen-induced model used in the present study is well established (4). Most published studies were performed in rats injected with DMH or with its metabolite azoxymethan (AOM). In this model the induced tumors share many histopathological and genetical characteristics with sporadic colon tumors in humans (4). DMH or AOM initiates a multistep carcinogenic process that transforms the normal colonic epithelia into a carcinoma with an adenomatous polyp as an intermediate step in the process (12). The distribution of carcinogen-induced colorectal tumors resembles human colon carcinoma where the great majority of tumors (80%) are recorded in the distal half of the colon (16-18). In humans, colon carcinogenesis is a long, chronic process that is thought to take up to 10 to 20 years (19). In the DMH or AOM-induced rat model, preneoplastic lesions (aberrant crypts) appear 3 to 4 weeks after carcinogen injection, and tumors are detected by 5 to 6 months (20), making this model very attractive for the screening of potential chemopreventive agents.

In the present study we aimed to evaluate by CT colonography the efficiency of two chemopreventive agents: aspirin and DFMO, known to exert chemopreventive properties in carcinogen-induced colon carcinogenesis in rats (5,12,21).

Several studies have consistently shown a beneficial effect of aspirin on polyp recurrence in humans. Aspirin, by inactivating cylooxygenase activities and lowering prostaglandin concentrations in colorectal mucosa (22) may have a meaningful impact on polyp development by decreasing both size and number of polyps (23,24) since prostaglandins are promotors of tumorigenesis (25). DFMO is a well-known irreversible inhibitor of ODC, a key enzyme in polyamine synthesis. ODC and the polyamine pathway have an important role in colon carcinogenesis (26). Previous clinical cancer chemoprevention trials indicate that DFMO can be given over long periods of time at doses (0.2-0.4 g/m^2/day) that lower polyamine contents in gastrointestinal tissues but cause no detectable hearing loss or other side effects (27,28). DFMO is currently being evaluated in combination with other COX inhibitors (23). Genetic polymorphisms in the ODC gene are also of relevance, supporting the use of ODC inhibitors and aspirin in combination as a strategy for colon cancer prevention (13). This approach is in accordance with previous data obtained in animal models of colon cancer in which the combination of DFMO and aspirin yielded synergistic inhibition (21).

In the present study, aspirin and DFMO were administered together in the drinking water 5 months after the last DMH injection. The daily amount of aspirin or DFMO given to the rats was 60 mg/kg corresponding to 360 mg/m^2, a non-toxic well-tolerated dose in rats (21) and also in humans (24,27). We show that these doses were sufficient to cause the inactivation of ODC by DFMO and a two-fold diminution of the PGE_2 content in the colonic mucosa of treated rats. Our data based on CT colonography show that in rats in which tumors are already present at the start of the treatment, a significant slow-down of tumor growth was observed during individual follow-up by CT colonography when compared to DMH-injected controls. In contrast, in rats where no tumors were detected at the start of the treatment, there was a complete inhibition of tumor formation as revealed by CT colonography and confirmed at autopsy. Furthermore, we

showed that a 30% reduction of ACF and of the total number of aberrant crypts was observed in aspirin/DFMO-treated rats when compared to DMH-injected controls. However, in rats treated with aspirin/DFMO no differences were recorded in the number or in the size of ACF between rats bearing tumors and those which remained tumor-free. These observations are in accordance with studies reporting an absence of correlation between the number and size of ACF and the formation of tumors in carcinogen-treated rodents (29,30). The present study is also in accordance with reports showing that in carcinogen-treated rodents and in patients the number of tumors is only marginal compared with the huge number of aberrant crypts (29-31), indicating that only a very small fraction of the ACF has the potential to progress to the stage of a tumor. In this regard, our findings suggest that the combination of aspirin and DFMO avoid such a progression.

The present study shows that CT colonography is a very attractive method for the follow-up of chemopreventive strategies in individual living rats. This approach which can be performed on a small number of animals, and repeated several times so that each animal serves as its own control, has a very high potential for the evaluation of chemopreventive preclinical protocols.

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