Long-term administration of aspirin inhibits tumour formation and triggers anti-neoplastic molecular changes in a pre-clinical model of colon carcinogenesis

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Abstract. Despite numerous studies aimed at verifying the anti-tumour activity of aspirin on colon carcinogenesis little is known on the molecular targets involved in the anti-carcinogenic properties of this drug. We investigated the long-term administration of low dose of aspirin in a model of experimental colon carcinogenesis in rats. Adult Wistar rats received an intraperitoneal injection of azoxymethane (AOM) once a week for two weeks in order to initiate colon carcinogenesis. One week after AOM injection, rats received daily 0.01% aspirin (6 mg/kg body weight) in drinking water for 10 months. Compared to AOM control rats, aspirin treatment for 10 months caused a 50% reduction of the number of aberrant crypt foci associated with a 50% reduction of prostaglandin E2 (PGE2) concentration and suppressed by 80% tumour formation in the colon. RT-PCR quantitative analysis showed that aspirin treatment reduced significantly (P<0.01) the AOM-triggered increase in mRNA levels of soluble inflammatory mediators (TNFα and IL-1β) and metalloproteinases (MMP3 and MMP7). Conversely, we detected an increased expression level of α-defensin-5 (Rd-5, 2 fold) and lipocalin-2 (LCN2, 4 fold), two markers of the innate immunity system. The expression of apoptosis-related genes such as death receptors and their ligands were reduced by aspirin and the Bcl-2/Bax transcript ratio dropped, Bcl-2 expression being reduced to the level found in saline control rats. The present study identifies new molecular targets triggered by aspirin in the colonic mucosa and may support the use of non-toxic low dose of aspirin in long-term treatments as a prophylactic approach against colon carcinogenesis.

Introduction

There are basically two strategies for the prevention of colorectal cancer (CRC): early diagnosis of the disease and chemoprevention. Treatment of pre-malignant or early stage malignant lesions decreases the incidence and mortality of CRC (1-3). In recent years, the non-steroidal anti-inflammatory drugs (NSAIDs) have attracted a great interest as potential candidates for colorectal cancer chemoprevention. There is now a great body of evidence supporting the role of NSAIDs, particularly of aspirin, in tumour chemoprevention through their anti-inflammatory properties. Especially by targeting the cyclooxygenase 2 (COX-2) enzyme linked to inflammation and tumour growth through its effects on cell survival and angiogenesis (4,5). Although the epidemiologic data in humans and the experimental data in rodents are generally consistent and show a strong relationship between aspirin use and the reduction of colorectal cancer incidence (6), they are not conclusive regarding the dose to be used, the duration of treatment and the route of administration (7-10). For example, aspirin has been shown to inhibit azoxymethane (AOM)-induced colon cancer in rats when provided in the diet before colon cancer initiation and given throughout the study (9). It was also reported that aspirin prevented aberrant crypt foci (ACF) formation when administered 4 weeks after AOM injection (11). However, others have shown that similar concentrations of aspirin (200 and 400 mg/kg diet corresponding to a daily aspirin intake of ~15-30 mg/kg body weight) did not prevent ACF formation in the colonic mucosa of AOM-treated rats (12). In the 1,2-dimethylhydrazine model of colon cancer, aspirin was effective in preventing colon tumours when administered during the initiation phase (before and during treatment with the chemical carcinogen); however, it was ineffective when aspirin was given after colon cancer initiation (4 weeks after carcinogen injection) (13). Other studies (14-18) have reported that treatment of normal rats with aspirin or indomethacin at doses that suppress prostaglandin production in the colonic mucosa, increased thymidine incorporation into mucosal DNA of colon, gastric fundus, small intestine and duodenum. This enhanced proliferative activity was associated with an increased risk of colon tumour formation (19,20). Clearly, the influence of NSAIDs on colon
carcinogenesis is complex and may be dependent on the nature of the NSAIDs, the doses used and/or the duration of the treatment. Furthermore, the molecular mechanisms and targets involved in the chemopreventive activity of NSAIDs are not yet clearly identified.

A systematic review of colon chemoprevention in rodents and humans has shown that the chemical carcinogen-induced rat model represents a good predictor of chemopreventive efficacy in humans (21). In this model, ACF are considered as putative pre-neoplastic lesions and ACF can be used as biomarkers to assess the efficacy of potential chemopreventive agents (22). Some of these lesions are precursors of adenomatous polyps and colorectal adenomas in rats and humans (23).

The goal of the present study was to determine whether the long-term treatment of rats with aspirin at a low dose (6 mg/kg body weight corresponding to a daily intake of 36 mg/m² in humans) given in the drinking fluid after colon cancer initiation by AOM is able to prevent ACF and colon tumour formations. Treatment with aspirin was started one week after AOM injection and was continued for 10 months. In order to get more insight into the molecular targets involved in the anti-neoplastic effects of low dose of aspirin the expression of several key genes involved in the inflammatory, immune and apoptotic responses were measured in the colonic mucosa.

Materials and methods

Animals and treatments. All animal experiments were performed in accordance with the institutional guidelines of the French Ethics Committee (authorization n°: 67-49, French Ministry of Agriculture). Male Wistar rats (5 weeks old) obtained from Charles River Laboratories (Les Oncins, France) and weighing 240–250 g were housed under standardized conditions (22°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes/h) and fed a standard diet with free access to drinking water. One group of rats (n=12) received intra-peritoneal injections of azoxymethane (AOM) (Sigma-Aldrich, Saint Quentin Fallavier, France), 15 mg/kg body weight, once a week for 2 weeks. One week after the last injection rats received aspirin (6 mg/kg body weight) given in the drinking fluid after colon cancer induction by AOM was performed according to the manufacturer's instructions. All samples were run in triplicate, in a 25 μl reaction volume. Real-time RT-PCR was performed by using TaqMan Universal PCR master mix (Applied Biosystems) and Applied Biosystems Prism 7500 Sequence Detection System in triplicate wells. The data were analyzed by a comparative threshold cycle (Ct) method. Ct values were calculated using the 7500 SDS software (Applied Biosystems). Total RNA from colonic mucosa from the saline rats was used as internal reference to normalize the data; mRNA expression assays for MMP7, MMP3 (assay ID: Rn00563467; RN01478512; RN00685720; RN00563754; RN00686175; RN01753393; RN00591740; RN00591740; RN00563754; RN00591740), and Bcl2, Bax, Fas (CD95), FasL, TRAIL, DR5, LCN2 (assay ID: RN09999125; RN02532082; RN01478512; RN00685720; RN00563754; RN00686175; RN01753393; RN00590612) according to the manufacturer's instructions.

Determination of PGE2 concentration in colonic mucosa. A sample of the mucosal scraping was homogenized in 100 mmol/l Tris-HCl buffer, pH 7.8, containing 1 mmol/l EDTA. After centrifugation at 10,000 × g for 15 min at 4°C, the supernatants were collected for PGE2 measurements using the PGE2 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 ± SE. Statistical differences between control and treated groups were evaluated using the Student's t-test. Differences between groups are considered significant at P<0.01.
the colon of rats receiving aspirin showed a significant reduction (P<0.01) in the number of pre-neoplastic lesions. The number of ACF and the total number of aberrant crypts were reduced by 50% in AOM rats receiving aspirin when compared with AOM control rats (Table I). No aberrant crypts or tumours were present in the colon of saline rats.

Striking differences were observed in the number and size of tumours present in the colon of AOM rats and aspirin-treated AOM rats (Fig. 1). In the AOM rats, a total number of 21 tumours were recorded, in this group no rats were found tumour-free and 5 of 6 animals exhibited multiple tumours. Among these tumours, 5 had a size between 300 and 450 mm$^3$, 5 between 50 and 300 mm$^3$ and 11 tumours were <50 mm$^3$. In contrast, only 3 tumours were found in 3 of the 6 animals receiving aspirin, 2 small tumours not exceeding 15 mm$^3$ and 1 tumour had a size of 50 mm$^3$. No tumour was detected in the colon of saline rats.

Aspirin modulated the expression of inflammatory and host-defence genes. IL-1β is an important pro-inflammatory cytokine, which together with tumour necrosis factor (TNFα) are acting as primary initiators of the complex inflammatory response which is suspected to be positively associated with the formation of colorectal adenoma in humans (25).

In the colonic mucosa of AOM rats a significant (P<0.01) up-regulation of IL-1β (12-fold) and TNFα (8-fold) were observed when compared with the saline-rats. The colonic mucosa of aspirin-treated AOM rats exhibited a significant (P<0.01) reduced expression of these inflammatory cytokines with a level close to that measured in the mucosa of saline rats (Fig. 2A).

In humans an increased expression of metalloprotease (MMP) genes has been associated to the early stages of cancer (26). We showed that the expression of MMP3 and MMP7 were significantly enhanced respectively by 8- to 10-fold in the colonic mucosa of AOM rats (Fig. 2A). Long-term treatment with aspirin maintained the gene expression of these MMPs near the level found in the mucosa of saline rats (Fig. 2A). We found also that two biomarkers of the innate response which is suspected to be positively associated with the formation of colorectal adenoma in humans (25).

Aspirin is a non-selective inhibitor of COX activities leading to a lowering of prostaglandin (PG) concentrations in the colorectal mucosa. Accordingly, we found that long-term aspirin treatment was associated with a reduction of 50% of PGE2 concentration in the colonic mucosa when compared to AOM rats (Fig. 2B).

Aspirin modulated the expression of apoptotic genes. The ability of aspirin to prevent AOM-induced ACF and tumour formation may be correlated to regulatory effects on genes implicated in the control of cell survival and/or cell death. We measured the effects of aspirin treatment on the expression of genes involved in the control of two main cell death pathways: the extrinsic apoptotic pathway triggered by specific death receptors located at the cell surface Fas (CD95) or TRAIL receptors (DR5) and the intrinsic apoptotic pathway induced following mitochondrial injury. This intrinsic pathway is controlled by members of the Bcl-2 family, and more specifically by the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins. Here, we show that the colonic mucosa of AOM rats exhibited an up-regulation of both pro-apoptotic (Fas, DR5, FasL, TRAIL, Bax, p53) and anti-apoptotic (Bcl-2) genes, with a major effect on Bcl-2 and DR5 which were respectively enhanced by 8- and 12-fold when compared to saline rats (Fig. 3A and B). Aspirin treatment did not modify significantly the expression level of Bax and p53 in the mucosa of AOM rats. However, aspirin treatment caused a drastic down-regulation of the Bcl-2 transcripts which were reduced to the level found in saline rats. The down-regulation of all apoptotic genes of the extrinsic pathway (Fas, DR5, TRAIL) was also observed in the mucosa of aspirin-treated AOM rats (Fig. 3B). Thus, these data suggested that long-term use of aspirin might reduce colonic pre-neoplastic lesions and other cellular events relevant to colon carcinogenesis through an

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**Table I. Effect of low dose of aspirin on pre-neoplastic lesions in distal colon.**

<table>
<thead>
<tr>
<th></th>
<th>Total AC/cm</th>
<th>ACF/cm</th>
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<tbody>
<tr>
<td>Control</td>
<td>68.4±5.0</td>
<td>21.0±1.6</td>
</tr>
<tr>
<td>Aspirin</td>
<td>30.3±3.1*</td>
<td>9.8±0.7*</td>
</tr>
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Total number of aberrant crypts (AC) and number of aberrant crypt foci (ACF) per cm distal colon (6 cm in length) in AOM rats and in rats receiving 0.01% aspirin in the drinking fluid. Values are the mean ± SE. *P<0.01.

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**Figure 1. Number and size (in mm$^3$) of tumours found at autopsy 10 months after AOM injection in each AOM control rat (C1 to C6) and aspirin-treated AOM rat (A1 to A6).**
increased Bax/Bcl-2 transcript ratio favouring the intrinsic (mitochondrial) apoptotic pathway.

**Discussion**

The AOM-induced colon cancer rat model used in the present study is well established (21). In this model the induced tumours share many histopathological and genetical characteristics with sporadic colon tumours in humans. AOM initiates a mutistep carcinogenic process that transforms progressively the normal colonic epithelia into a carcinoma with pre-neoplastic lesions (aberrant hyperproliferative crypts) and adenomatous polyps as intermediate steps in the process (28). In humans, colon carcinogenesis is a long, chronic process that is thought to take up to 10 to 20 years (29). In the AOM-induced rat model, pre-neoplastic lesions (hyperproliferative aberrant crypts) appear 3 to 4 weeks after AOM injection and tumours are detected by 6 months, making this model very attractive for the screening of potential chemopreventive agents (30). As in the AOM-induced rat model, aberrant crypt foci (ACF) in humans represent an early pre-neoplastic step appearing before colorectal adenoma and are one of the earliest changes that can be seen in the colon of humans that may lead to cancer (31). The ACF/adenoma transition is favoured by the appearance of additional mutations and epigenetic alterations (32). During the process of carcinoma formation from normal colonic mucosa, pre-cancerous cells release various growth factors and cytokines into their surroundings, recruit and re-program various other cell types to establish a 'tumour microenvironment' (33). There is now growing evidence that tumours are sustained and promoted by inflammatory signals from the surrounding microenvironment. Inflammation is implicated in all stages of carcinogenesis, i.e., initiation, promotion and progression (34,35).

The non-steroidal anti-inflammatory drug aspirin has shown repeatedly anti-carcinogenic properties on colon carcinogenesis through its anti-inflammatory properties by inhibiting prostaglandin synthesis (6). However, due to increased risk of associated gastrointestinal toxicity the benefit of these drugs has to be weighed against the risk in long-term treatments. Therefore, in order to evaluate the anti-carcinogenic effects of long-term aspirin we used a very low dose of aspirin, corresponding to a 5- to 10-fold lower daily intake of aspirin reported in previous animal studies (9-11,36) and corresponded in humans to a daily aspirin intake of 36 mg/m². This dose used over the 10 months period showed no apparent toxicity. Both AOM rats and aspirin-treated AOM rats exhibited similar body weight evolution reaching respectively 660±22 g and 642±28 g (mean value ± SD). In order to get more insight into the molecular targets involved in the anti-neoplastic effects of aspirin the expression of several key genes involved in the inflammatory, immune and apoptotic responses were measured in the colonic mucosa.

The present study showed that the long-term administration of aspirin given after colon cancer initiation suppressed...
AOM-induced colon tumorigenesis and reduced significantly ACF formation. The best known molecular target for aspirin is cyclooxygenase (COX). COX is a key regulatory enzyme involved in prostaglandin (PG) biosynthesis, which is strongly implicated in the induction of inflammation and cell proliferation (37). Inhibitors of the enzyme are associated with a reduction of up to 50% of colorectal cancer morbidity and mortality (38). Our data showed that the anti-carcinogenic effects of aspirin were associated with a 50% reduction in prostaglandin PGE₂ concentration and to changes in the expression of key genes involved in the control of inflammation, host defence and apoptotic responses.

It was recently reported in humans that ACF formation is closely related to tumorigenesis and that inflammatory genes of the MMP family such as MMP7 were activated during the early period of ACF formation (26). Several studies have reported on the major role of the MMPs in carcinogenesis transcriptional regulation (39). Here we showed that MMP3 and MMP7 expression were significantly enhanced in the colonic mucosa of AOM rats. The expression levels of these genes were significantly (P<0.01) lowered by aspirin treatment near the level found in the colonic mucosa of saline rats. Furthermore, we showed also that the AOM-induced overexpression of pro-inflammatory cytokine IL-1ß and TNF-α were suppressed by aspirin treatment. Thus, we observed an inverse correlation between the genetic expression of the pro-inflammatory cytokines and the genetic expression of host-defence components of the innate immune system such as α-defensin 5 (Rd-5) and lipocalin 2 (LCN2), these genes being up-regulated in aspirin-treated AOM rats. The functions of α-defensin 5 and lipocalin 2 are currently thought to be not only involved in anti-microbial action, but also in the modulation of both the inflammatory and immune responses (27,40) and these components are considered as active weapons against several cancer cell types (41,42).

Furthermore, the impaired expression of α-defensin 5 and lipocalin 2 and the overproduction of the pro-inflammatory cytokine IL-1ß have been closely associated with inflammatory bowel disease (43,44). It was also shown that α-defensins have pro-apoptotic properties of lipocalin 2 and may favour defence mechanisms against mucosal inflammation (27). A tight regulatory mechanism is involved in the control of cell proliferation and cell death to maintain structural and functional integrity of the colonic mucosa. We demonstrated that AOM perturbated the genetic expression of several mediators involved in the two main apoptotic pathways. Here also aspirin treatment was able to 'normalize' the genetic expression of mediators involved in the control of the extrinsic apoptotic pathway (Fas, FasL, DR5, TRAIL). Interestingly, an important change

regulation of inflammatory genes and up-regulation of host-defence mediators may favour a rebalanced regulation between proliferation and death of colonic epithelial cells. In this regard, pro-apoptotic properties of lipocalin 2 and α-defensins have been reported (46,47).

Several lines of evidence suggest that the wide range of anti-proliferative potencies of aspirin does not correlate exclusively with its anti-inflammatory effects, since NSAIDs can induce apoptosis in colon cancer cell lines that lack detectable expression of COX-2 protein (48). The balance between cell survival and apoptosis is dependent upon the relative expression of specific genes, such as p53, whose products interact to determine the final outcome of the apoptotic signal. Target genes regulated by p53 include pro-apoptotic Bax, Fas, DR5, TRAIL and also the anti-apoptotic gene Bcl-2 (49). Indeed, our study showed that AOM initiated an up-regulation of the pro-apoptotic Bax, DR5/TRAIL and Fas/FasL genes, but also of the anti-apoptotic Bcl-2 gene. Aspirin treatment reduced significantly the high expression level of death receptor genes and their ligands observed in AOM rats. More specifically, we showed that the expression of the anti-apoptotic Bcl-2 gene was dramatically down-regulated, whereas the pro-apoptotic Bax gene remained up-regulated when compared with saline rats and unchanged when compared to AOM rats.

Induction of programmed cell death may result from the activation of two main pathways: the extrinsic apoptotic pathway which is triggered by specific cell surface death receptors: Fas or TRAIL receptors (DR4, DR5), and the intrinsic apoptotic pathway which is induced following mitochondrial injury and is controlled by members of the Bcl-2 family, and more specifically by the anti-apoptotic Bcl-2 protein and the pro-apoptotic Bax protein. The Bcl-2/Bax ratio is a determinant factor for apoptosis and the decline of this ratio favours mitochondria alterations leading to apoptosis (50). Therefore, our present data suggest, that long-term treatment with low dose of aspirin may favour apoptosis through the activation of the intrinsic mitochondrial pathway as supported by the observed decrease of the Bcl-2/Bax transcript ratio when compared with AOM rats.

Our study demonstrates that the long-term (10 months) daily administration of a non-toxic low dose of aspirin after colon cancer initiation by AOM in rats suppressed tumour formation and reduced by 50% the formation of pre-neoplastic lesions (ACFs). This is the first study showing that these effects were associated with a 'normalization' in the colonic mucosa of several biomarkers of the inflammatory response such as IL-1ß, TNFα, MMP3 and MMP7 gene expression. In addition, aspirin treatment activated the genetic expression of important mediators of the innate immune system such as α-defensin 5 (Rd-5) and lipocalin 2 which may favour defence mechanisms against mucosal inflammation (27). A tight regulatory mechanism is involved in the control of cell proliferation and cell death to maintain structural and functional integrity of the colonic mucosa. We demonstrated that AOM perturbated the genetic expression of several mediators involved in the two main apoptotic pathways. Here also aspirin treatment was able to 'normalize' the genetic expression of mediators involved in the control of the extrinsic apoptotic pathway (Fas, FasL, DR5, TRAIL). Interestingly, an important change
of the Bcl-2/Bax transcript ratio was observed in aspirin-treated AOM rats caused by a dramatic and specific down-regulation of the anti-apoptotic Bcl-2 transcript. These changes may support an activation of the intrinsic (mitochondrial) apoptotic pathway by aspirin in mucosal cells countering the imbalance between cell proliferation and cell death initiated by AOM which favoured the activation of proliferative signals.

In conclusion, the present study identifies new molecular targets triggered by aspirin in the colon and supports the use of non-toxic low dose of aspirin in long-term treatments as a prophylactic approach against colon carcinogenesis. The use of low dose of aspirin single or in association with other chemopreventive agents should be re-assessed in long-term protocols designed for populations (familial predispositions, polypectomized patients, ageing population) at risk to develop the disease.

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