Aspirin inhibits endothelial nitric oxide synthase (eNOS) and Flk-1 (vascular endothelial growth factor receptor-2) prior to rat colon tumour development

Marta ESCRIBANO*, Laura MOLERO†, Antonio LÓPEZ-FARRÉ†, Cynthia ABARRATEGUI*, Carolina CARRASCO†, Antonio GARCÍA-MENDEZ*, Félix MANZARBEITIA‡, María Jesús MARTÍN*, Marian VÁZQUEZ*, Paloma SÁNCHEZ-FAYOS*, Luis RICO† and Juan Carlos PORRES CUBERO*

*Digestive Research Laboratory, Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain,
†Cardiovascular Research Laboratory, Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain, and
‡Department of Anatomopathology, Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain

ABSTRACT

Formation of blood vessels is a fundamental element in the control of tumour growth in which vascular endothelial growth factor (VEGF) and nitric oxide (NO) have been demonstrated to be involved. Our aim was to analyse whether changes in the expression of endothelial NO synthase (eNOS) and VEGF in colonic tissue could be detected early and even before the identification of colon tumour-associated morphological modifications in azoxymethane-treated rats. We studied further whether aspirin treatment changed these parameters. An increased expression of both eNOS and VEGF in colonic tissue from azoxymethane-treated rats compared with that from control rats was found. Aspirin treatment (10 mg/kg of body weight per day) reduced eNOS expression, but failed to modify the expression of VEGF in the colonic tissue of azoxymethane-treated rats. No evidence of aberrant crypt formation or changes in the number of blood vessels were observed in the colon of any of the animals studied. Expression of the VEGF receptor Flk-1, but not Flt-1, was increased in colonic tissue of azoxymethane-treated rats compared with control rats. The expression of Flk-1 was mainly localized in the epithelial cells, particularly in the lower part of the crypt. Aspirin treatment reduced Flk-1 expression in both control and azoxymethane-treated rats. Caspase-3 activity, which has been considered as an apoptotic index, was almost undetectable in azoxymethane-treated rats. Aspirin treatment stimulated caspase-3 activity. Overexpression of eNOS, VEGF and its receptor Flk-1 occurred early after azoxymethane administration in rat colonic tissue, even before morphological changes associated with tumour generation were observed, and aspirin prevented the overexpression of both eNOS and VEGF receptor Flk-1.

INTRODUCTION

Nitric oxide (NO) is generated in endothelial cells by the endothelial NO synthase (eNOS) via the metabolic conversion of L-arginine into L-citrulline [1,2]. Although eNOS was initially considered to be constitutive, in recent years it has been demonstrated that the incubation of cultured endothelial cells with vascular endothelial

Key words: aspirin, colon tumour, endothelial nitric oxide synthase (eNOS), Flk-1, vascular endothelial growth factor (VEGF).

Abbreviations: COX-2, cyclo-oxygenase-2; NO, nitric oxide; eNOS, endothelial NO synthase; NSAID, non-steroidal anti-inflammatory drug; PARP, poly(ADP-ribose) polymerase; VEGF, vascular endothelial growth factor.

Correspondence: Dr Juan Carlos Porres Cubero (e-mail blarrea@fjd.es).
growth factor (VEGF) up-regulated eNOS expression, whereas cytokines down-regulated eNOS expression through destabilization of eNOS mRNA [3–5].

NO is recognized as a pluripotential molecule with a wide spectrum of effects, including protection of endothelial cells from apoptosis (for reviews, see [6–8]).

Formation of blood vessels, i.e. angiogenesis, is a fundamental element in the control of tumour growth. VEGF-induced angiogenesis is essential for the formation of the vasculature during embryogenesis and plays a central role in pathophysiological neovascularization in human disease providing nourishment for growing tumours and establishing continuity between the tumour cell and the host vasculature [9–11]. The presence of VEGF has been demonstrated in several human cancer cell lines in vitro and in surgically resected tumours of the human gastrointestinal tract, ovary, brain and kidney and in the serum of patients with lung and gynaecological cancers [12–15]. However, most of these studies have identified the presence of VEGF when the tumour was well established.

On the basis of these considerations, our first aim was to analyse whether modifications in the expression of eNOS and VEGF in colonic tissue may be detected early and even before identifying morphological modifications associated with colon tumours in azoxymethane-treated rats.

Azoxymethane-induced tumours represent a well-characterized animal model of colon carcinogenesis associated with the metabolic activation of azoxymethane which takes place in two steps, namely, the hydroxylation of methylazoxymethanol in the liver and the oxidation of methylazoxymethanol to methylazoxy-formamide in the liver and colon [16–18]. Numerous studies have demonstrated the presence of aberrant crypt foci several weeks after the first exposure to azoxymethane (12–36 weeks); such foci have been associated with an increased expression of the cyclo-oxygenase-2 (COX-2) isoform [19,20].

There is compelling evidence that non-steroidal anti-inflammatory drugs (NSAIDs) and, more particularly, aspirin have a protective effect against colorectal cancer [21]. Interestingly, NSAIDs substantially inhibit in vitro angiogenesis [22]; however, their detrimental side-effects limit their potential use. Therefore we need to understand the mechanisms by which aspirin exerts its preventive effect in order to allow the development of safer alternatives. Thus, in a second set of experiments, we studied the effect of aspirin treatment on VEGF and eNOS expression in colonic tissue.

METHODS

Animal procedures

The protocol was approved by the Institutional Ethics Committee for Animals and was performed according to international conventions on animal experimentation.

The studies were performed in 7-week-old male Wistar-Kyoto rats. The rats were divided into four experimental groups. The first group (n = 8) was treated with subcutaneous injections of azoxymethane (15 mg/kg of body weight; Sigma, St Louis, MO, U.S.A.) once a week for 2 weeks and killed 7 weeks after the last injection. The second group, the control group (n = 14), was treated with weekly saline injections for 2 weeks in the same manner as azoxymethane-treated rats and killed 7 weeks after the last injection. The third experimental group (n = 8) was treated with azoxymethane (15 mg/kg of body weight) and aspirin (10 mg/kg of body weight per day) dissolved in the drinking water. The aspirin concentration was adjusted for daily water intake and body weight to obtain an average daily dose of 10 mg/kg of body weight per day. Aspirin was also administered during the 7 weeks after the last azoxymethane injection. An additional group of control rats (n = 7) treated with saline injections for 2 weeks and with aspirin in the drinking water (10 mg/kg of body weight per day) during 7 weeks after the last saline injection was also included. All rats were housed in individual cages and were fed with standard rat chow.

At 16 weeks of age, rats were anaesthetized with sodium pentobarbital (30 mg/kg of body weight) and distal colonic samples were then removed. The samples were analysed histopathologically by a pathologist to detect any early aberrant crypt formation and then submitted to the following studies.

Determination of eNOS and VEGF expression

eNOS and VEGF expression levels were analysed by Western blot, as described previously [23,24]. The colonic tissue was homogenized and solubilized in Laemmli buffer containing 2-mercaptoethanol [25]. Proteins (20 µg/lane) were separated in a denaturing SDS/10% (w/v) polyacrylamide gel. Western-blot analysis was performed using monoclonal antibodies against eNOS (Transduction Laboratories, Lexington, KY, U.S.A.) and VEGF (R & D Systems, Minneapolis, MN, U.S.A.) respectively. eNOS and VEGF antibodies were used at a concentration of 1:2500 and 1:5000 respectively. eNOS and VEGF were detected by enhanced chemiluminescence (ECL®; Amersham Biosciences, Little Chalfont, Bucks., U.K.) after 5 min of film exposure and evaluated by densitometry (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Western-blot analysis of a parallel gel with identical samples was performed with a monoclonal antibody against the constitutively expressed protein β-actin (1:2000; Calbiochem, Bad Soden, Germany). Prestained markers were used to measure the molecular mass (Sigma).
**Immunohistochemical studies**

Immunohistochemical studies were used to locate eNOS and Flk-1 expression in colonic tissue. Rats were perfused with 50 ml of fixative solution containing 4% paraformaldehyde in saline (1:1, v/v) as reported previously [26]. Paraffin blocks were sectioned at 4 μm on a standard rotatory microtome (Microm HM 325; Microm, Walldorf, Germany). The sections were stained with haematoxylin and eosin using an automated staining system (Techmate 500; Dako, Carpinteria, CA, U.S.A.). Exposure of the antigens to the antibodies was facilitated by submitting the sections to a pressure cooker for 2 min [27] and subsequently incubating with polyclonal antibodies against eNOS and Flk-1 at a dilution of 1:100 and 1:700 respectively, overnight at 4°C. A mouse anti-(rabbit IgG) antibody (Dako) was applied for 30 min at room temperature and preformed alkaline phosphatase–anti-alkaline phosphatase) immune complexes were added for another 30 min as described previously [28]. The sections were washed in PBS containing 1% Tween-20 before each incubation. The colour was finally developed with Fast Red substrate (Dako). After washing in deionized water, the sections were briefly counter-stained with haematoxylin and a coverslip was added.

Parallel samples were used to analyse the morphology of the crypts. Aberrant crypts were distinguished from the normal crypts by an increased size, increased distance between the crypts and the easily discernible pericrystal zone. Samples were always evaluated in a blinded manner by a pathologist.

**RESULTS**

**General observations**

The body weights of the rats treated with saline, saline + aspirin, azoxymethane and azoxymethane + aspirin were comparable throughout the study period (body weights at the end of the study were 320 ± 7 g, 310 ± 9 g, 314 ± 9 g and 317 ± 7 g respectively: P value, not significant). No evidence of aberrant crypt formation in the colon of any of the studied animals was observed. In haematoxylin/eosin-stained samples, no significant differences were observed in the number of vessels counted in the colonic samples of the different experimental groups of rats.

**eNOS and VEGF expression in colonic tissue**

Colonic tissue isolated from azoxymethane-treated rats had an increased expression of eNOS compared with that of control rats (Figure 1, upper panel). Treatment with aspirin prevented the increased eNOS expression in azoxymethane-exposed rats, although it was still higher than in control rats (Figure 1, upper panel). A higher dose of aspirin (20 mg/kg of body weight per day) failed to produce a greater inhibition of eNOS expression than that obtained with 10 mg/kg of body weight per day (results not shown). No changes in β-actin expression were observed between the three experimental groups, supporting the specificity of the observed modifications in eNOS expression (Figure 1, upper panel). Positive immunostaining for eNOS was only localized in the capillary endothelium (Figure 1, lower panel), whereas epithelial cells were negatively stained for eNOS.

The expression of VEGF was found to be enhanced in colonic tissue from azoxymethane-treated rats when compared with control rats (Figure 2). However, aspirin treatment failed to modify VEGF expression in the colonic tissue of azoxymethane-treated rats (Figure 2). VEGF binds to two specific receptors, Flt-1 (also known as VEGF-receptor-1) and Flk-1 (also known as VEGF-receptor-2). Therefore we analysed whether azoxymethane administration changed the expression of these different VEGF receptors in the colonic tissue. Western-blot analysis using a specific monoclonal antibody against Flt-1 (1:400; Santa Cruz Biotechnology) showed that the expression of Flt-1 was not changed in the colonic tissue of azoxymethane-treated and azoxymethane + aspirin-treated rats compared with that of control rats (Figure 3, top panel). However, the expression of Flk-1 (1:400; Santa Cruz Biotechnology) was markedly increased in the colonic tissue of azoxymethane-treated rats when compared with control rats (Figure 3, middle panel). Aspirin reduced Flk-1 expression in the colonic tissue of azoxymethane-treated rats,

**Caspe-3 protease activity**

During apoptosis, caspase-3 cleaves the 116 kDa substrate poly(ADP-ribose) polymerase (PARP) into a stable 85 kDa fragment containing the C-terminal and a 25 kDa fragment [29,30]. We thus determined the level of the 85 kDa form as an index of apoptosis, as reported previously [31]. The proteins obtained from the distal colon of the different groups of rats were separated on a SDS/12.5% (w/v) polyacrylamide gel, and the 85 kDa form was detected by immunoblotting, as described above, using an anti-PARP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The anti-PARP antibody (1:500) corresponded to amino acids 764–1014 at the C-terminus of PARP.

**Statistical methods**

Results are expressed as means ± S.E.M. The comparison between treatments was made using ANOVA or the paired or unpaired Student’s t test, as appropriate. Bonferroni’s correction for multiple comparisons was used to measure the significance of the P values. A value of $P < 0.05$ was considered significant.
Figure 1 Effect of azoxymethane alone or in combination with aspirin on the expression of eNOS in colonic tissue

Upper panel, representative Western blot demonstrating the expression of eNOS in colonic tissue from control (C), azoxymethane-treated and azoxymethane + aspirin-treated (ASA) rats. The expression of β-actin is also shown. The densitometric analysis of the Western blots is also shown. Results are presented as means ± S.E.M. *P < 0.05 compared with control; **P < 0.05 compared with azoxymethane-treated rats. Lower panel, cross-section of paraffin-embedded colonic tissue from azoxymethane-treated rats showing the expression of eNOS (as indicated by the arrowheads). eNOS was mainly detected in the capillary endothelium. Epithelial cells did not appear to be stained for eNOS. Magnification, ×120.

although it remained elevated compared with that of control rats (Figure 3, middle panel). The expression of Flk-1 was localized in the epithelial cells, particularly in the lower parts of the crypt, and it was almost undetectable in the luminal part of the crypt (Figure 3, bottom panel). The endothelium of the microvessels was negatively stained for Flk-1 (Figure 3, bottom panel).

Caspase-3 activity and apoptotic index in the colonic tissue

The 85 kDa form of PARP was used as an index of apoptosis. In colonic samples of azoxymethane-treated rats, the 85 kDa form of PARP was almost undetectable and PARP was found in its intact form (116 kDa; Figure 4). Aspirin treatment significantly increased the level of 85 kDa PARP, thereby reducing the level of the intact 166 kDa form (Figure 4). The modification of the apoptotic state of the colonic tissues was analysed further by determining the ratio of the expression of Bcl-2 and Bax, two proto-oncogenes that protect and promote apoptosis respectively. Western-blot analysis showed a marked and significant reduction of Bax expression in the colonic tissue in azoxymethane-treated rats (Figure 5, top panel). However, aspirin administration significantly increased the level of Bax expressed in the colonic tissue of azoxymethane-treated rats (Figure 5, top panel).

The expression of Bcl-2 tended to be reduced in the colonic tissue of azoxymethane-treated rats, although this did not reach statistical significance compared with control (Figure 5, middle panel). A significant reduction in Bcl-2 expressed in colonic tissue was observed by the administration of aspirin to azoxymethane-treated rats (Figure 5, middle panel). The Bcl-2/Bax ratio, which has been considered as apoptotic index, was significantly enhanced in colonic tissue from azoxymethane-treated rats compared with that of control rats (Figure 5, bottom panel), whereas aspirin administration significantly reduced the Bcl-2/Bax ratio in these rats (Figure 5, bottom panel).

Effect of aspirin in control rats

Fischer et al. [32] have shown that aspirin treatment alone affects eNOS in the gastric mucosa. Therefore we tested further whether aspirin administration modified VEGF, Flk-1 and eNOS expression in colonic tissue from control rats. Aspirin alone did not modify the content of VEGF nor the expression of eNOS in the colonic tissue of control rats (Figure 6, upper- and lower left-hand panels). However, the expression of Flk-1 was markedly reduced in the colonic tissue of aspirin-treated control rats (Figure 6, upper right-hand panel). There were no changes...
in β-actin expression in colonic tissues obtained from control and aspirin-treated control rats (Figure 6, upper right-hand panel). In addition, aspirin treatment alone did not modify the Bcl-2/Bax ratio in the colonic tissue from control rats (Figure 6, lower right-hand panel).

**DISCUSSION**

In the present study we have demonstrated that the expression of eNOS, VEGF and its receptor Flk-1 were up-regulated early on in the colon of azoxymethane-treated rats. This modification in the expression of these parameters occurred before the morphological appearance of the tumours was detected and was associated with inhibition of colonic apoptosis. Aspirin prevented the up-regulation in the expression of eNOS and Flk-1 elicited by azoxymethane administration.

We [3] and others [4] have demonstrated an overexpression of eNOS in vitro in growing endothelial cells. However, few studies have evaluated the presence of eNOS in consolidated colon tumours in vivo. Takahashi et al. [33] have demonstrated an increased expression of eNOS in endothelial cells of blood vessels from colon carcinomas of azoxymethane-treated rats. Other NOS isoforms have also been reported to be overexpressed in a variety of neoplasms [34,35]. However, to our knowledge, the present study is the first to demonstrate an up-regulation of eNOS before any morphological changes associated with tumour formation could be detected in the colon.

NO plays an important role in the regulation of vascular tone and blood flow. Therefore it is possible that the production of NO by eNOS in endothelial cells may cause vasodilation and an increased blood
Figure 5 Effect of azoxymethane alone or in combination with aspirin on the expression of Bax and Bcl-2 in colonic tissue

Representative Western blots demonstrating the expression of Bax (top panel) and Bcl-2 (middle panel) in colonic tissue from control (C), azoxymethane-treated and azoxymethane + aspirin-treated (ASA) rats. Bottom panel, ratio of Bcl-2 and Bax expression. Results are presented as means ± S.E.M. *P < 0.05 compared with control (in top panel) and azoxymethane alone (in middle and bottom panels); †P < 0.05 compared with azoxymethane alone (in top panel).

flow to the colon tumour, which could promote tumour growth further, as has been reported by Maeda et al. [36]. Indeed, it has been demonstrated [37] that inhibitors of NOS activity reduce blood flow in tumour-associated neovascularature. Interestingly, although NO seems to be a weak inhibitor of endothelial proliferation, it has been recognized that NO regulates matrix protein synthesis [38,39], a closely related cell growth phenomenon.

Expression of VEGF has been associated with tumour metastasis and proliferation of human colon cancer [40]. In our present study, we also observed that the expression of VEGF was enhanced before detecting morphological modifications in the colonic tissue.

The cellular responses to VEGF are primarily mediated by its receptors Flt-1 and Flk-1 [41,42]. In the present study, we observed that Flk-1, but not Flt-1, was up-regulated in the colon after azoxymethane exposure. In this regard, whereas Flt-1 has been suggested as a decoy receptor rather than as a signal-transducing molecule, Flk-1 appears to be more intimately involved in a variety of cellular actions related to VEGF, including up-regulation of eNOS and protection from endothelial apoptosis [41,43].

It is noteworthy that inhibition of cell elimination by apoptosis seems to facilitate tumorigenesis. In our present study, up-regulation of eNOS and Flk-1 was associated with a marked reduction in the level of the 85 kDa fragment of PARP in colonic tissue of azoxymethane-treated rats, suggesting that caspase-3 activity and, therefore, apoptosis was reduced.

In an attempt to study the association between the modifications in Flk-1, eNOS expression and caspase-3 activity, we performed experiments in the presence of aspirin, a drug that has been reported previously [44] to reduce the incidence of colon carcinogenesis. Aspirin decreased the expression of Flk-1 in colonic tissue, which was accompanied by the down-regulation of eNOS and an increased fragmentation of PARP, suggesting increased caspase-3 activity. However, aspirin did not modify the expression of VEGF, but reduced the expression of the Flk-1, suggesting that the relationship between eNOS and caspase-3 activity with VEGF could be coupled to Flk-1. Nevertheless, we bear in mind that, although eNOS was expressed in the capillaries of the colonic tissue, Flk-1 was only detected in the epithelial crypts. It is plausible that activation of Flk-1 in the epithelial cells may induce the release of other mediators from these cells that stimulated the expression of eNOS in the capillary. We cannot rule out the fact that the observed changes in the expression of Flk-1 and eNOS in colonic tissue from aspirin-treated and control rats are unrelated phenomena. Indeed, aspirin reduced the expression of Flk-1 in colonic tissue from control healthy rats, whereas the level of expression of eNOS was not modified.

As mentioned, several studies have demonstrated the presence of aberrant crypt foci several weeks after exposure of rats to azoxymethane (12–36 weeks). In our animal model, we killed the rats 9 weeks after the first azoxymethane injection in an attempt to detect molecular modifications before the appearance of morphological changes in the colon. Indeed, we did not observe the appearance of aberrant crypts in any of the rats from the different experimental groups. Bedi et al. [45] have reported that the transformation of colorectal epithelium to carcinomas is associated with a progressive inhibition of apoptosis. Moreover, Chang et al. [46] have
Early markers of colon malignant tumours

Figure 6  Effect of aspirin on the expression of VEGF, eNOS, Flk-1 and Bcl-2/Bax ratio in colonic tissue

Representative Western blot showing the expression of VEGF (upper left-hand panel), eNOS (lower left-hand panel) and Flk-1 (upper right-hand panel) and Bcl-2/Bax ratio (lower right-hand panel) in colonic tissue from control (C) and aspirin-treated (ASA) rats. In the upper right-hand panel, the expression of β-actin is also shown. The densitometric scanning of the corresponding Western blots is also shown. Results are presented as means ± S.E.M. *P < 0.05 compared with untreated controls.

Our experimental design in the present study did not allow us to assess the molecular mechanisms involved in the reduced expression of Flk-1 and eNOS by aspirin administration. Jones et al. [22] have demonstrated that selective COX-2 inhibitors and non-selective NSAIDs reduced angiogenesis in different endothelial cell types. Moreover, it has been suggested that aspirin may exert its protective effect against colonic tumours by inhibiting COX-2. In this regard, elevated prostaglandin levels have been found in colon tumours [48], which have been associated with an increased expression of COX-2. Interestingly, tumour growth was attenuated when colon tumour cells were implanted in COX-2 knock-out mice, due to a decreased vascular supply to the tumours (for a review, see [49]). Therefore it could be speculated that COX-2 inhibition by aspirin may be involved in the early reduced expression of Flk-1 and eNOS in the colonic tissue of azoxymethane-treated rats reported in the present study. Although further studies are needed to explore this hypothesis, a recent study by Matsumoto et al. [50] has shown that mice with a null mutation for the gene encoding COX-2 have attenuated uterine angiogenesis, primarily due to defective VEGF signalling through Flk-1. However, other mechanisms independent of COX-2 inhibition could also be involved in the modulation of Flk-1 and eNOS expression by aspirin, since it has also been reported [22] that NSAIDs could exert their anti-angiogenic effect via prostaglandin-independent pathways.

In conclusion, the results of the present study demonstrate that overexpression of eNOS, VEGF and its receptor Flk-1 occur early in the process and even before any morphological changes associated with tumour generation can be detected in the colonic tissue, suggesting that these factors may contribute to the initiation and progression of colonic tumours. Aspirin prevented the overexpression of both eNOS and Flk-1 VEGF receptor. Further studies are needed to assess...
whether these findings could be involved in the beneficial effects of aspirin against malignant tumours in the colon.

**ACKNOWLEDGMENTS**

This work was supported by grants from Laboratorios Bayer. M. E., L. M., A. G.-M. and C. A. are fellows of Fundacion Conchita Rábago de Fundación Jiménez Díaz. C. C. is a fellow of Fondo de Investigaciones Sanitarias de la Seguridad Social (PI 020347). The authors thank Begoña Larrea for secretarial assistance.

**REFERENCES**


© 2004 The Biochemical Society