Bettina Scholtka, Dana Kühnel, Felicitas Taugner, Pablo Steinberg

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GENOTOXICITY AND CARCINOGENICITY

Inflammation does not precede or accompany the induction of preneoplastic lesions in the colon of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-fed rats

Dana Kühnel · Felicitas Taugner · Bettina Scholtka · Pablo Steinberg

Abstract Heterocyclic aromatic amines (HCAs) are formed in meat cooked at high temperatures for a long time or over an open flame. In this context 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant HCA in cooked meat, has been suggested to be involved in colon and prostate carcinogenesis. In the latter case it has been reported that: (1) roughly 50% of Fischer F344 male rats treated with PhIP develop carcinomas in the ventral prostate lobe at 1 year of age; (2) inflammation precedes prostatic intraepithelial neoplasia in PhIP-fed rats; (3) inflammation specifically occurs in the ventral prostate lobe of PhIP-fed rats. To test whether PhIP by itself leads to inflammation in the colon and whether a human-relevant concentration of PhIP is able to induce preneoplastic lesions in the colon, male F344 rats were fed 0.1 or 100 ppm PhIP for up to 10 months and thereafter the colon tissue was analyzed histochemically. In none of the experimental groups signs of acute or chronic colonic inflammation were observed. 0.1 ppm PhIP leads to the development of hyperplastic and dysplastic lesions in the colon of single animals, but the incidence of these lesions does not reach a statistical significance. In contrast, in rats fed 100 ppm PhIP for 10 months hyperplastic and dysplastic colonic lesions were induced in a statistically significant number of animals. It is concluded that: (1) the induction of preneoplastic lesions in rat colon by PhIP is not preceded or accompanied by an inflammatory process; (2) a human-relevant concentration of PhIP alone is not sufficient to initiate colon carcinogenesis in rats.

Keywords Colorectal cancer · Heterocyclic aromatic amines · Inflammation

D. Kühnel · B. Scholtka · P. Steinberg
Chair of Nutritional Toxicology, Institute of Nutritional Science,
University of Potsdam, Arthur-Scheunert-Allee 114-116,
14558 Nuthetal, Germany

D. Kühnel
Department of Cell Toxicology,
Helmholtz Centre for Environmental Research UFZ,
Permoser Str. 15, 04318 Leipzig, Germany

F. Taugner
Fraunhofer Institute for Toxicology and Experimental Medicine,
Nikolai-Fuchs-Straße 1, 30625 Hannover, Germany

P. Steinberg (✉)
Institute for Food Toxicology and Analytical Chemistry,
University of Veterinary Medicine Hannover,
Bischofsholer Damm 15, 30173 Hannover, Germany
e-mail: pablo.steinberg@tiho-hannover.de
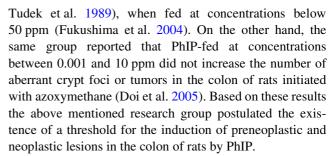
Introduction

The role of food in general and of certain food constituents in particular in the development of colorectal cancer has been a matter of considerable debate for a number of years now. Ten years ago a scientific panel of the World Health Organisation concluded that consumption of red meat and processed meat was probably associated with an increased colorectal cancer risk (Scheppach et al. 1999). In the meantime various epidemiological studies have shown that this is in fact the case (Norat et al. 2002; Chao et al. 2005; Norat et al. 2005; Sinha et al. 2005). As to the specific components in processed meat responsible for the induction of sporadic colon carcinomas it has been suggested that heterocyclic aromatic amines (HCAs), which are formed in meat when it is cooked at high temperatures for a long time or over an open flame, might be involved in this process (Norat et al. 2002).

Among the ten HCAs, which have proven to be carcinogenic in experimental animals (Wakabayashi et al. 1992) 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is considered to be a major risk factor for colon cancer, since PhIP is usually the most abundant HCA in cooked meat (Layton et al. 1995), is mutagenic in bacterial and mammalian cell-based genotoxicity assays (Thompson et al. 1987) and induces colon tumors in rats (Ito et al. 1991). Moreover, PhIP–DNA adducts have been detected after random sampling in human colon DNA (Friesen et al. 1994). The binding of PhIP to DNA has also been demonstrated in colon tissue samples from patients, which had undergone surgery to remove colon carcinomas and which were exposed to an amount of PhIP similar to that present in cooked food prior to surgery (Dingley et al. 1999).

For a number of years now it has been postulated that chronic inflammation, which occurs in patients with inflammatory bowel disease, predisposes to colorectal cancer (Rhodes and Campbell 2002; Itzkowitz and Yio 2004). Furthermore, the development of precursor lesions of colorectal cancer such as polyps or adenomas is often accompanied by histological features of inflammation (Rhodes and Campbell 2002; Higaki et al. 1999). In this context it is interesting to note that a recent global gene expression analysis of rat colon cancers induced by PhIP revealed a strong up-regulation of inflammation-associated genes such as those encoding interleukin 1β, small inducible cytokine subfamily A20 precursor and Mob-1 (Fujiwara et al. 2004). However, up to the present time the induction of inflammation in colon mucosa in response to PhIP has not been described, whereas PhIP is known to cause chronic inflammation together with myocyte necrosis, myofibrillar dissolution and disarray as well as dilation of T-tubules in rat heart (Davis et al. 1994). It also has been reported that roughly 50% of Fischer F344 male rats treated with PhIP develop carcinomas in the ventral prostate lobe at 1 year of age (Shirai et al. 1997). In this context Borowsky et al. (2006) have shown that inflammation precedes prostatic intraepithelial neoplasia in PhIP-fed rats. Furthermore, Nakai et al. (2007) have demonstrated that inflammation specifically occurs in the ventral prostate lobe of PhIP-fed rats.

In the early carcinogenicity studies, in which PhIP was shown to be a colon carcinogen in male rats, it was added to the rodent chow in extremely high (i.e. for humans in totally irrelevant) concentrations (100–400 ppm) (Ito et al. 1991; Hasegawa et al. 1993). Up to the present time only two studies, published by Fukushima et al. (2004) and Doi et al. (2005), have dealt with the effects of PhIP-fed at human-relevant concentrations (i.e. below 0.1 ppm) on the rat colon. On the one hand, it has been shown that PhIP alone does not induce the formation of so called aberrant crypt foci, preneoplastic lesions in the rat colon (Bird 1987;

Tudek et al. 1989), when fed at concentrations below 50 ppm (Fukushima et al. 2004). On the other hand, the same group reported that PhIP-fed at concentrations between 0.001 and 10 ppm did not increase the number of aberrant crypt foci or tumors in the colon of rats initiated with azoxymethane (Doi et al. 2005). Based on these results the above mentioned research group postulated the existence of a threshold for the induction of preneoplastic and neoplastic lesions in the colon of rats by PhIP.

In order to test whether inflammation precedes or accompanies the PhIP-mediated development of preneoplastic lesions in the colon, F344 rats were fed 0.1 or 100 ppm PhIP for up to 10 months and thereafter the colon tissue was analyzed histochemically.

Materials and methods

Chemicals

All chemicals were of reagent grade and from commercial sources. PhIP was obtained from Albrecht Seidel (Biochemisches Institut für Umweltcarcinogene, Großhansdorf, Germany) and buffer salts from AppliChem (Darmstadt, Germany) as well as Carl Roth (Karlsruhe, Germany).

Animals and diets

Six-week-old male F344 rats were purchased from Charles River WIGA GmbH (Sulzfeld, Germany) and allowed to acclimatize to the housing conditions for 2 weeks before the start of the experiment. Two animals per cage were held under specific pathogen-free conditions at a room temperature of 22°C, 40–60% air humidity, a fixed 16/8 h day and night cycle and free access to food and water. PhIP was dissolved in vegetable oil (Brökelmann + Co.-Oelmühle GmbH + Co., Hamm, Germany), added to the standard powdered rodent lab chow Altromin 1321 FORTI (Altromin Gesellschaft für Tierernährung mbH, Lage, Germany) and then pelleted to give a final PhIP concentration of 0.1 or 100 ppm. The mixing and the pelleting were performed by the first author of this report at the animal housing facility of the German Institute for Human Nutrition (Nuthetal, Germany). The animal study was approved by the Ministerium für Wissenschaft, Forschung und Kultur des Landes Brandenburg, Germany (approval number 32/48-3560-1/27).

Experimental design

Rats were randomly divided into three groups of 40 animals each. The first group received a standard rodent lab chow,

the second group a standard rodent lab chow supplemented with 0.1 ppm PhIP and the third group a standard rodent lab chow supplemented with 100 ppm PhIP. Body weight and food consumption were recorded weekly for up to 10 months. After 2, 4, 6 and 10 months, ten animals per group were sacrificed. The large intestines were flushed with cold saline solution, macroscopically examined, cut into three segments of equal length (defined as proximal, middle and distal colon) and fixed in 4% buffered formalin for at least 24 h. Each segment was totally submitted as multiple transverse sections for histological processing and comprised in average 5–6 pieces.

Histology

Sections of formalin-fixed tissue samples were stained with haematoxylin/eosin for the light microscopic examination of tissue structure. Infiltration of the colonic epithelium by inflammatory cells was used as evidence of active inflammation, crypt architectural distortion as evidence of chronic inflammation. Colonic lesions were defined as being hyperplastic (i.e. dilated crypts with normal epithelium) or dysplastic (i.e. lesions showing a distorted crypt structure, a decrease in the number of goblet cells, nuclear stratification and enlarged nuclei).

Immunohistochemistry

As mentioned above infiltration of the colonic epithelium by inflammatory cells was one of the criteria used as an evidence of active inflammation. In order to quantify the number of T-lymphocytes present in the colonic tissue samples, CD3 expression was analyzed. CD3 is known to be expressed by T cells in thymus, bone marrow, peripheral lymphoid tissue and blood (Campana et al. 1987). The staining of the colon sections was performed by making use of a polyclonal rabbit anti-human CD3 antibody from Dako (Glostrup, Denmark) according to the instructions of the antibody supplier.

Statistics

Statistical analysis of the results was performed by using Dunnett's test for multiple comparisons with a control (Dunnett 1964).

Results

No statistically significant differences in weight gain or in food consumption were observed between control animals and rats fed either 0.1 or 100 ppm PhIP (data not shown). No animal in the three experimental groups died prema-

turely. Based on the daily food consumption rats fed 0.1 and 100 ppm PhIP took up a mean amount of 1.53 μg and 1.55 mg PhIP/rat/day, respectively.

The histopathological analysis of 842 haematoxylin/eosin stained slides revealed no signs of acute or chronic inflammation in any colon section. A slight lymphangiectasis, a very slight mononuclear cell infiltration or an acute haemorrhage was observed in individual rats (Table 1), but they were detected in control as well as PhIP-treated animals and were regarded as incidental. In accordance with the results of the haematoxylin/eosin staining the analysis of CD3 expression in the colon sections revealed that there were no differences in the number of T-lymphocytes detected in the colon epithelium of control and PhIP-treated rats (data not shown).

Furthermore, hyperplastic and dysplastic colonic crypts were observed in rats fed either 0.1 or 100 ppm PhIP, but not in controls (Table 2). Hyperplastic crypts are enlarged due to an increase in the number of cells within the crypt without changes in cell size or cell composition. Dysplastic crypts are characterised by changes in cell composition due to increased mitotic activity and dedifferentiation of epithelial cells. In two out of ten rats fed 0.1 ppm PhIP for 6–10 months hyperplastic and dysplastic lesions were detected, whereas in animals fed 100 ppm PhIP for 6 or 10 months three and six out of ten rats, respectively, showed areas of hyperplasia and dysplasia in the colon (Table 2). The difference in the number of hyperplastic and dysplastic lesions in the colon of control and PhIP-fed was statistically significant only in the case of animals fed the higher concentration of PhIP. Dysplastic lesions were first observed in the two PhIP-treated rat groups after 6 months and in the case of animals fed 100 ppm PhIP the number of

Table 1 Histopathological findings other than hyperplastic or dysplastic lesions in the colon of rats fed 0.1 or 100 ppm PhIP for up to 10 months

Group	Histopathological findings[a]
Controls	Multifocal slight lymphangiectasis, diffuse slight mucosal and submucosal oedema (2 out of 10, after 4 months, in the colorectum)
0.1 ppm PhIP	Focal very slight submucosal mononuclear cell infiltration (1 out of 10, after 4 months, in the distal colon)
	Multifocal very slight lymphangiectasis, diffuse very slight mucosal oedema (1 out of 10, after 4 months, in the colorectum)
	Multifocal very slight submucosal mononuclear cell infiltration (1 out of 10, after 6 months, in the colorectum)
100 ppm PhIP	Multifocal severe submucosal haemorrhage (1 out of 10, 10 months, in the proximal colon)

[a] The number of animals affected after a certain time and the localization of the histopathological finding are given in parentheses

Table 2 Incidence of hyperplastic or dysplastic lesions in the colon of rats fed 0.1 or 100 ppm PhIP for up to 10 months

Group	Number of rats with hyperplastic or dysplastic lesions in the colon after			
	2 months	4 months	6 months	10 months
Controls	0/10[a]	0/10	0/10	0/10
0.1 ppm PhIP	0/10	0/10	2/10 (1 × HP, 1 × DP)	2/10 (1 × HP, 1 × DP)
100 ppm PhIP	1/10 (1 × HP)	1/10 (1 × HP)	3/10 (1 × HP, 2 × DP)	6/10 (1 × HP, 5 × DP)[b]

HP hyperplastic lesion, *DP* dysplastic lesion

[a] Data are presented as the number of rats with hyperplastic or dysplastic lesions/total number of rats in the corresponding group

[b] Significantly different from the corresponding value in the control group ($P < 0.05$, analysis of variance and Dunnett's post-test)

animals with dysplastic lesions increased with time (Table 2). In 9 out of 15 animals showing hyperplastic or dysplastic lesions these were restricted to the distal colon, in five cases they were observed in the colorectum and in one case they were observed in the proximal as well as in the distal colon.

Discussion

An earlier report by Davis et al. (1994) as well as two more recent reports by Borowsky et al. (2006) and Nakai et al. (2007) demonstrated that inflammation develops in the heart and prostate of rats treated with high concentrations of PhIP. The importance of inflammation in the development of PhIP-induced tumors in the rat prostate is supported by two observations. First, inflammation precedes prostatic intraepithelial neoplasia in PhIP-fed rats (Borowsky et al. 2006). Second, inflammation specifically occurs in the ventral prostate lobe of PhIP-fed rats (Nakai et al. 2007) and roughly 50% of F344 male rats treated with PhIP develop carcinomas in the ventral prostate lobe (Shirai et al. 1997). In the present study it is shown that PhIP does not induce inflammation in the rat colon. If one takes into account that hyperplastic/dysplastic lesions were detected in up to six out of ten rats treated with 100 ppm PhIP, one must conclude that in the colon, in contrast to the situation in the prostate, inflammation is not a prerequisite in order that hyperplastic/dysplastic lesions develop.

While in the present study PhIP itself does not lead to colonic inflammation, a number of studies show that in rodents, in which colon inflammation was chemically induced (e.g. by treating the animals with dodecyl sodium sulphate), the carcinogenicity of PhIP is enhanced (Nishikawa et al. 2005; Tanaka et al. 2005; Nakanishi et al. 2007). As to the role of inflammation in PhIP-induced colon carcinogenesis various possibilities should be taken into account. Due to inflammation colonic epithelial cells could die off and colonic epithelial cells having been initiated by the genotoxic agent PhIP could proliferate in order to substitute the dead cells, thereby leading to preneoplastic and

neoplastic lesions. In this case inflammation would have a tumor promoting character by stimulating the growth of previously initiated cells. It is also conceivable that increased mucosal proliferation in the inflamed tissue could increase the sensitivity of colonic epithelial cells to fixation of DNA damage (i.e. mutations). Furthermore, inflammation could lead to an enhanced absorption of PhIP by increasing the permeability of colon mucosa. Thereafter, PhIP could either be metabolized in the colon epithelium to one or more genotoxic metabolites capable of initiating the stem cells situated at the bottom of the colonic crypts or be transported via the blood circulation, e.g. to the liver, where it could be activated, and the genotoxic metabolites could then be further transported via the blood stream to the bottom of the colonic crypts, where they could initiate single stem cells. A third possibility is that the transport of polar PhIP metabolites (e.g. glucuronides) from the colon epithelial cells into the gut lumen is reduced, i.e. the potentially toxic PhIP metabolites are "retained" within the inflamed colon mucosa. It is known, e.g. that the breast cancer resistance protein (Bcrp1/Abcg2), which is expressed in the apical membranes of epithelial cells in the colon (Maliepaard et al. 2001), is able to pump out PhIP and its polar metabolites from the colon mucosa into the gut lumen (van Herwaarden et al. 2003). Whether the expression of the breast cancer resistance protein is down-regulated in the colon of PhIP-fed rats remains to be investigated.

In the early studies by Ito et al. (1991) and Hasegawa et al. (1993) aberrant crypt foci and carcinomas developed in the proximal as well as in the distal colon of male F344 rats fed with PhIP, while in the present study hyperplastic/dysplastic lesions were detected almost exclusively in the distal colon and the colorectum (1 out of 15 rats showed such lesions in the proximal and the distal colon). The discrepancy between the different studies remains unexplained at the present time.

The fact that feeding 0.1 ppm PhIP for up to 10 months did not lead to a statistically significant increase in the number of rats with hyperplastic/dysplastic lesions in the colon when compared to the corresponding control group is in accordance with previous results obtained by Fukushima

et al. ([2004](#)) and Doi et al. ([2005](#)). In this context Fukushima et al. ([2004](#)) postulated that a threshold of 50 ppm for the PhIP-mediated induction of aberrant crypt foci in the rat colon can actually be defined. Taking into account that in the present study, although not statistically significant, two out of ten rats fed 0.1 ppm PhIP developed hyperplastic/dysplastic lesions, the question whether a threshold for the induction of preneoplastic lesions in the rat colon by PhIP does in fact exist remains open at the present time.

It should be pointed out that hyperplasic lesions are not considered to be a preneoplasic stage in rat colon carcinogenesis, whereas dysplasia in the rat colon is a preneoplastic lesion (Nakagama et al. [2002](#); Ochiai et al. [2003](#)). The data presented in Table [2](#) for rats fed 100 ppm PhIP is in accordance with this assumption. Although the number of rats per group is low, it is evident that hyperplasia precedes dysplasia (i.e. in rats fed 100 ppm PhIP for up to 4 months only hyperplastic lesions were detected) and that the number of dysplastic lesions increases with time (i.e. the number of rats showing dysplasia in the colon was two and five after feeding 100 ppm PhIP for 6 and 10 months, respectively).

Whereas it is evident from this and previous studies (Fukushima et al. [2004](#); Doi et al. [2005](#)) that a human-relevant concentration of PhIP alone is not sufficient to initiate colon carcinogenesis, a mixture of food contaminants (e.g. HCAs, polycyclic aromatic hydrocarbons, nitrosamines, acrylamide) could very well overcome the threshold to induce preneoplastic lesions in the colon. Further experiments are needed to clarify this important open question in chemically induced colon carcinogenesis.

In conclusion, the data obtained in the present study show that the induction of preneoplastic lesions in rat colon by PhIP is not preceded or accompanied by an inflammatory process and that a human-relevant concentration of PhIP alone is not sufficient to initiate colon carcinogenesis in rats.

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