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review

Use of preneoplastic lesions in colon and liver in experimental oncology

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The present article gives a brief overview on the use of altered hepatic foci (AHF) and aberrant crypt foci (ACF) in the colon in experimental cancer research. These foci are easily detectable preneoplastic lesions, which have been discovered approximately 30 years ago. AHF and ACF are valuable tools for the detection of cancer - initiating and promoting compounds, and for the detection of chemoprotective agents. They were also successfully used in numerous studies aimed at elucidating the molecular mechanisms of early neoplasia, such as alterations of the expressions of oncogene and tumor suppressor genes, and changes in the activities of cancer associated enzymes.

Key words: preneoplastic lesions; liver neoplasms; colonic neoplasms

Introduction

Preneoplastic lesions are used in experimental research since more than thirty years. They consist of morphologically or functionally altered populations of cells that are precursors of neoplasms. In contrast to long term experiments in which tumor formation is used as an endpoint, they have the advantage that they can be detected after comparatively short time periods (after 2-5 months) and that the number of animals, which are required are relatively small (usually 8-10 animals are used per experimental group). Preneoplastic lesions have been identified in a number of organs, for example in the skin (epidermal dysplasia and hyperplasia, epithelial papillomas), lung (alveolar and focal hyperplasia, nodular lesions), pancreas (atypical acinar foci), kidney (tubules with irregular epithelium), mammary gland (hyperplastic alveolar nodules) and also in liver and colon (for overview see[2]). The present article is focused on hepatic altered foci (AHF) and aberrant crypt foci (ACF) in the colon, which have been used extensively in the last years for the detection of carcinogens, for the identification of chemoprotective agents and also in mechanistic studies. It describes their mor-

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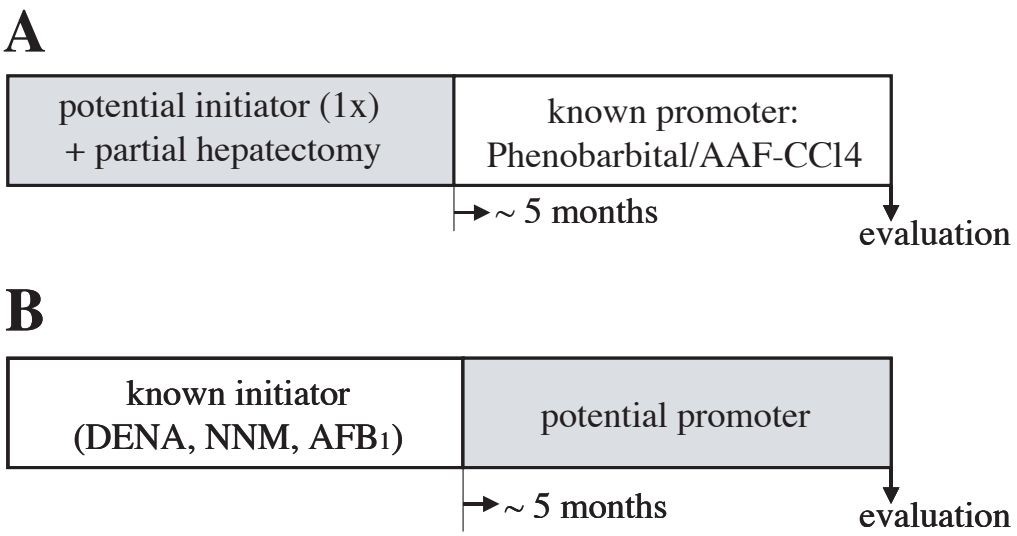

Figure 2A,B. Different treatment schedules for the detection of initiating and promoting carcinogens for experiments in which AHF are used as biological endpoint.

phology and molecular characteristics and their use in the identification of initiating, promoting and protective agents and the development of new techniques.

Altered hepatic foci – morphology and phenotypes

The use of altered hepatic foci started in the 1970's. In the early years, a classification system was developed, which was based on the staining behaviour and included clear, acidophilic, intermediate, tigroid, basophilic and also mixed cells of AHF.[2] In subsequent years, it was shown that the expression of a variety of enzymes of AHF differs from that of the normal tissue, and based on this observations, histochemical methods were developed which enable the detection of enzymatically altered AHF (for review see [1]). An overview on the different markers is given in the article of Pitot.[3] At present, the most widely used endpoint is the expression of the placental form of glutathione-S-transferase ($GSTp^+$), which can be detected by immunohistochemistry. About 80% of all foci stained positive for $GSTp^+$.[3] Another frequently used marker is γ-glutamyltranspeptidase. Figure 1 depicts a GSTp+ foci.

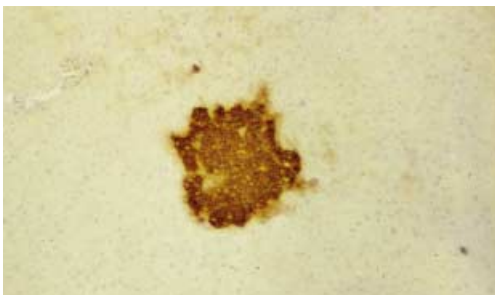

Figure 1. A GSTp+ focus.

Methodological aspects

AHF can be used to detect tumor initiating (Figure 2A) and promoting properties (Figure 2B) of chemicals. To distinguish between these characteristics, the test animals are treated with the compounds according to different schedules.[4]

Initiators and promoters of AHF

Numerous synthetic and natural compounds have been identified, which either initiate or promote the formation of AHF.[4] Typical examples for initiators are nitrosamines (which are the most frequently used carcinogens in mechanistic studies), urethane, aflatoxin B1, heterocyclic aromatic amines, and haloethans.[5] Also polycyclic aromatic hydrocarbons such as benzo(a)pyrene cause formation of AHF in rats, although the liver is not a target organ for tumor induction of this compound.

Typical examples for compounds which promote the growth of AHF in the liver are barbiturates (phenobarbital etc.), steroid hormones such as dexomethazone and testosterone, hypolipidemic drugs and polychlorinated biphenyls (for review see[4]).

Inhibition of foci formation

Numerous investigations have been conducted to identify compounds, which prevent the formation of liver foci. These agents were either protective at the initiation level (*i.e.* when administered before and/or simultaneously with the carcinogen) or at the promotion level (after carcinogen treatment). Examples for anti-initiators are food additives such as butylated hydroxyanisole, which protects against AFB_1[6] and butylated hydroxytoluene, which inhibited the foci formation caused by 2-acetyl-aminofluorene.[7] Also glucosinolates, contained in cruciferous vegetables were found protective towards AFB_1 and cruciferous plants themselves inhibited foci formation induced by the heterocyclic aromatic amine (HAA) IQ.[8-13]

A number of compounds were identified which prevent the development of foci when administered after the carcinogen treatment. For example acetaminophen and aminophenol were protective against formation of foci that had been induced by a nitrosamine in the liver[4] and flavonone reduced significantly areas of placental $GSTp^+$ foci induced by aflatoxin B1 during the phenobarbital- induced promotion step.[14]

A very interesting observation was made in experiments with rats in which the restriction of dietary calories reduced the number and volume of AHF by 85% in 3 month; food restriction lowered DNA replication but increased apoptosis. When treated with a tumor promoter (nafenopin) after food restriction, only half as many hepatocellular adenomas were found as in animals fed ad libitum throughout their lifetime. The authors concluded that restricted calorie intake preferentially enhances apoptosis of preneoplastic cells.[15]

Mechanistic aspects

It is well documented that AHF increase in number and size with continued exposure to both, genotoxic and non-genotoxic carcinogens.[16-18] Some of the phenotypical abnormalities of AHF are stable, however under specific conditions some phenotypical characteristics are lost ("phenotypic reversion").[19] In rats, it is well documented that AHF develop by the clonal expansion of individual cells.[19] As a result of sustained growth, AHF develop into nodular lesions.[20,21] If these nodules are neoplasms, as suggested by some studies,[22] AHF truly represent preneoplastic lesions.

A number of studies have been conducted in which the ratio between cell division and programmed cell death during development of liver cancer was investigated. It was shown, that the cell division rates are increased in AHF compared to normal tissue; in adenomas and carcinomas even higher division rates were observed. Also the death rates (apoptosis) increased gradually from normal to preneoplastic to adenoma and carcinoma tissue.[23] Further studies showed, that the prenoplastic tissue is more susceptible to stimulation of cell replication and cell death;[24,25] and that tumor promoters evidently act as survival factors by inhibiting apoptosis in

preneoplastic liver cells, thereby stimulating growth of preneoplastic lesions. Interestingly, withdrawal of tumor promoters led to excessive elimination of preneoplastic lesions, whereas normal tissue was less affected.[24]

New developments

Grasl-Kraupp and coworkers[25] developed recently an *ex vivo* cell culture model, with initiated rat hepatocytes. Following treatment of the rats with a nitrosamine (N-nitrosomorpholine), hepatocytes were isolated after 22 days (maximal occurrence of $GSTp^+$-cells) and cultivated *in vitro.* Then the cells were either treated with the mitogen cypoterone acetate or with transforming growth factor (TGF-β) for 1-3 days. In culture, the rate of DNA-replication of $GSTp^+$-cells was compared to that of normal hepatocytes. It was found, that $GSTp^+$-cells show an inherent growth advantage and a preferential response towards the effects of TGF-β and cypoterone acetate as in the *in vivo* situation. Based on these results, the authors stress that this *ex vivo* system may provide a useful tool to elucidate biological and molecular changes during the initiation stage of carcinogenesis.

Aberrant crypts in the colon – morphology

In 1987[27], Bird discovered that the treatment of rats with a colon carcinogen (dimethylhydrazine, DMH) leads to formation of morphologically aberrant foci, which can be visualized with methylene blue stain. ACF consist of altered cells, which exhibit cytoplasmic basophilia, a high nuclear to cytoplasmic ratio, prominent nucleoli, loss of globlet cells, loss of polarity, and in the upper part of the crypt they exhibit increased proliferative activity.[28] Figure 3A and 3B depict typical aberrant crypts, which are abnormally large, darkly

Table 1. Biochemical and immunohistochemical alterations of ACF.[30,33-42]

Endpoint	Comment	Reference
Hexosaminidase increased	gene closely located to the APC gene 95% of ACF in rats stain positive, not a marker for human ACF	Boland *et al.*, 1992 Pretlow *et al.*, 1993
Carcinoembryonic antigen (CEA)	intracellular adhesion molecule in human ACF altered (93%) but not a marker for dysplasia	Pretlow *et al.*, 1994
P-Cadherin E-Cadherin	cell adhesion molecules P-c expressed in ACF prior to and independent from E-c and β-catenin	Hardy *et al.*, 2002
β-Catenin	transcriptional activator in ACF nuclear expression increased (see also chapter: development for new markers)	Hao *et al.*, 2001
Inducible nitric oxide synthase (iNOS)	increased in dysplastic but not in hyperplastic ACF	Takahashi *et al.*, 2000
Cyclooxygenase 2 (COX-2)	overexpression in ACF	Takahashi *et al.*, 2000
Cell proliferation markers Ki-67, proliferating cell nuclear antigen (PNCA) $P16^{INK4a}$	several studies show altered patterns in ACF	Renehan *et al.*, 2002 Cheng *et al.*, 2003
Placental form of GST	might be associated in humans with K-ras expression, induced in human ACF and CRC	Miyanishi *et al.*, 2001
Changes in mucin production	alteration of mucin-patterns seen in ACF in rats and in humans	Uchida *et al.*, 2001 Bara *et al.*, 2003

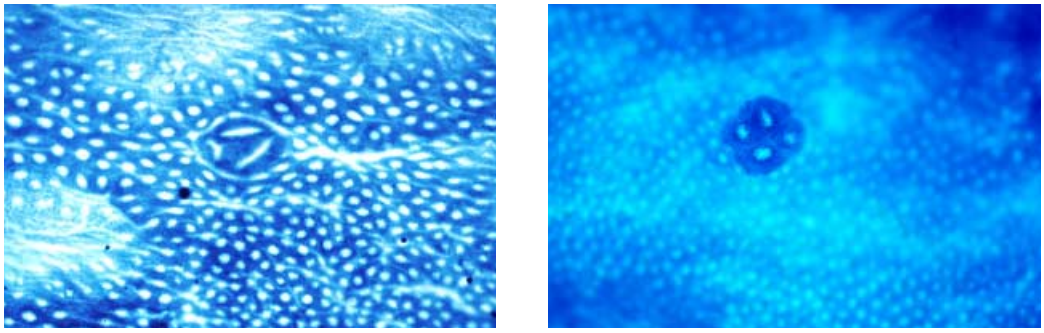

Figure 3A,B. A- An aberrant crypt focus with a high level of dysplasia , which is microscopically elevated with a slit-shaped luminal opening. B- A crypt with oval openings. A GSTp+ focus.

staining and slightly elevated. Dysplastic crypts with a slit-shaped luminal opening are shown in Figure 3A; Figure 3B depicts non-dysplastic crypts with a larger pericryptical zone.[29]

ACF show variable features – ranking from mild hyperplasia to dysplasia, and are generally divided into three groups, namely dysplastic, non-dysplastic (atypic) and mixed type (for details see[30]). In ACF without dysplasia, the crypts are enlarged (up to 1,5-fold) and have slightly enhanced nuclei, no mucin depletion and crypt cells staining positive for PNCA and Ki-67 remain in the lower part of the crypts. In ACF with dysplasia, crypts are more elongated, and the nuclei enlarged. PNCA and Ki-67 stain is extended to the upper part of the crypts. Mixed type ACF show combinations of the features of pure adenomatous pattern (with dysplasia) and hyperplastic characteristics.

In humans, ACF were first described in 1991.[31,32] They resemble those seen in rodents induced by carcinogens[27] and several lines of evidence support the assumption that they are precursors of colorectal tumors (for details see Cheng et al.).[30]

Biochemical and immunohistochemical alterations of ACF

A number of biochemical alterations are typical for ACF. The most important features are listed in Table 1.

Table 2. Epigenetic and genetic alterations in ACF.[43-50]

Alteration	Remarks	Reference
K-ras mutation	in ACF in rats, identified in many studies also in humans	Stopera *et al.*, 1992 Losi *et al.*, 1996
APC mutation	deleted in human ACF – but lower rates as in adenomas/carcinomas	Smith *et al.*, 1994 Nascimbeni *et al.*, 1999
hMSH2 mutation	mismatch repair gene alteration in ACF in mice colons	Reitmair *et al.*, 1996
CpG island methylation	in 53% of ACF of humans with sporadic CRC but only in 11% of FAP patients	Chan *et al.*, 2002
Microsatellite instability	detected in animal models and in humans in ACF	Augenlicht *et al.*, 1996
Fragile histidine triad (FHIT) candidate tumor suppressor gene	lost in CRC (40%) – only few ACF showed reduced expression; the loss correlated with the extent of dysplasia	Hao *et al.*, 2000

Table 3. Compounds, which act as tumor promotors in the colon and cause increased formation of ACF.[58-66]

Compound	Result	Reference
Thermolysed protein	increasing thermolysis of casein increases AOM induced foci numbers and size	Zhang *et al.,* 1992
Thermolysed sucrose (5-hydroxymethyl-2-furaldehyde)	increases the size of AOM induced foci weakly initiating carcinogens	Zhang *et al.,* 1993
Fat (beef tallow)	AOM experiments with mice:increases 3-5 times the size of chemically induced foci	Corpet *et al.,* 1990
Refined sugars (sucrose, fructose, dextrin) induced foci in rats	increased formation of AOM induced foci sucrose and dextrin enhance no. of AOM	Stamp *et al.,* 1993 Poulsen *et al.,* 2001
Progastrin (PG)	ACF significantly more common in AOM treated mice overexpressing PG	Cobb *et al.,* 2004
Haemoglobin, haemin	especially haemin but also haemoglobin were potent ACF promoters in AOM treated rats, when fed a low-calcium diet	Pierre *et al.,* 2003
Chenodeoxycholic acid (CDCA)	AOM induced foci as well as crypt multiplicity significantly increased in rats	Ghia *et al.,* 1996 Sutherland *et al.,* 1994

Genetic and epigenetic alterations

Different genetic alterations have been identified in ACF in humans and also in chemically induced ACF in rats; a detailed overview is given in the article of Cheng *et al.*[30] Many genes, which are considered to be involved in colon carcinogenesis, were found to be altered in ACF; this supports the assumption that they (ACF or specific subpopulations) represent indeed preneoplastic lesions. Table 2 lists up different alterations which were identified in ACF.

Methodological aspects

As in AHF-experiments, ACF-studies allow to discriminate between initiating and promoting compounds. The treatment schedule is more or less identical as that used for the detection of liver foci, but other model chemicals are used.

Only a few compounds have been detected, which are initiators of colon cancer and aberrant crypts. The most frequently used agents are DMH and its metabolite azoxymethane (AOM).[51] Both compounds lead to DNA methylation and to formation of ACF, which become apparent[5-7] weeks after the administration.[52] Also heterocyclic aromatic amines (HAs), which are found in fried meat cause formation of ACF[28,53,54] and were used in a number of chemoprevention studies (for review see Dashwood[55] and Schwab *et al.*[56]). Other agents which cause ACF are N-methyl-N-nitrosurea (MNU) and 3,2-dimethyl-4-aminobiphenyl (DMABP), but these compounds were hardly ever used in mechanistic and chemoprevention studies.[57]

Use of the ACF-model to detect factors which act as tumor promoters in the colon

The ACF-model was intensely used in studies aimed at detecting dietary factors which cause tumor promotion in the colon. Table 3 lists up a number of studies.

Use of the ACF-model for the detection of chemoprotective compounds

Numerous studies have been conducted aimed at identifying compounds which are protective towards colon cancer with the ACF model. Recently, Corpet and Tache[57] have published a review on this topic. They

found in total 137 articles and results for about 186 complex mixtures and individual compounds are available (the data can be downloaded from: http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html). The establishment of a ranking order of protective potency showed, that the most potent were pluronic, polyethylene glycol, perilla oil containing β-carotene and indole-3-carbinol (for details see[57]). In addition, many other dietary constituents were found protective, for example vitamins, lactobacilli in fermented foods, different glucosinolates in Brassica vegetables, carotinoids and fibers to name only a few.[57]

In most of the studies, DMH or AOM were used to cause foci formation and the putative protective compounds were added either before or after administration of the carcinogen. The prevention during the foci "initiation" phase might be either due to inactivation of DNA-reactive molecules, inhibition of metabolic activation or induction of DNA-repair processes[67] and is compound specific. Since humans are not exposed to DMH and its metabolite AOM, chemoprotective effects seen in such experiments cannot be extrapolated to the human situation. On the other hand, it is assumed that the further development of preneoplastic cells (promotion, progression) is triggered by molecular processes which are independent from the chemical carcinogen used.[68] Therefore antipromoting effects seen in the AOM/DMH ACF model might be considered relevant for humans.

HAs are formed during cooking of meats.[69] They cause cancer in the colon of rodents, and in other organs as well[70] and evidence is accumulating that HAs are involved in the etiology of colon cancer in humans.[71] HAs were used in a number of chemoprevention studies in which inhibition of ACF formation was used as an endpoint[55,56,] and a number of dietary components such as fibers, chlorophyllins, Brassica vegetables and lactobacilli were found protective. In this context it is interesting that epidemiological studies indicate that consumption of these factors is also inversely related with the incidence of colon cancer in humans.

One of the problems of the use of HAs in ACF studies is that the foci yield is relatively low, even when the animals are treated with high doses (up to 100 mg/d for several days). The foci frequency could be substantially increased by feeding the animals a high fat and fiber free diet, which facilitates the detection of putative protective effects.[72] In contrast to AOM or DMH it is not possible to induce ACF with a single HA-dose, therefore it is not possible to distinguish clearly between anti-initiating and anti-promoting effects in these experiments.

Corpet and Pierre[51] published an article on the correlation between the results of chemoprevention studies using ACF as an endpoint, and data from experiments with the *Apc*$^{Min/+}$ mouse model (these animals have a mutated *Apc*-gene and therefore highly increased rates of intestinal spontaneous tumors, and are often used as a model for human hereditary colon cancer). Comparison of the efficacy of protective agents in the *Apc*$^{Min/+}$ mouse and in the ACF rat model showed a significant correlation ($p<0,001$). Furthermore, the authors also compared the results of rodent studies with clinical intervention studies. For a number of compounds, which were protective in the animal models, also chemopreventive properties were seen in humans.

New developments

Although numerous studies show that ACF detect colon carcinogens and have been used extensively for the identification of dietary factors enhancing or reducing the risk for colorectal cancer, some results suggest that misleading results may be obtained with certain compounds.[73] For example it is well documented that cholic acid, a primary bile acid, is a strong tumor promoter in the colon, whereas it significantly decreases the number of

ACF.[74,75] A similar contradiction was seen with the xenoestrogen genistein.[76-78]

It was repeatedly postulated by Japanese groups[79-82] that β-catenin acculmulating crypts (BCAC), which are independent from ACF, are more reliable biomarkers for colon cancer development. They show that cholic acid increases the frequency of AOM-induced BCAC in rats. In a critical comment of Pretlow and Bird[83] it is stated that BCAC represent in fact specific dysplastic ACF. In a subsequent paper of Hao *et al.*[37], human ACF were analyzed for β-catenin expression and in approximately 56% of dysplastic ACF, cytoplastic β-catenin was increased, whereas in ACF with atypia, β-catenin in the cytoplasm was only seen in 2% of the total number.

As mentioned above, Magnuson and coworkers[74] also found that the number of ACF at early time points did not predict tumor incidence in rats treated with cholic acid. Therefore the authors suggest that crypt multiplicity should be measured in future studies, due to the fact that it was a consistent predictor of tumor outcome in their study.

Another potential short-term endpoint for colon cancer might be mucin-depleted foci (MDF). In AOM-treated rats such foci could be visualised with high-iron diamine Albicon blue.[84] Their frequency was lower than that of ACF and they were histological more dysplastic than mucinous ACF. In a recent article, it was shown that the number of MDF-foci declined in AOM treated rats, after piroxicam (a colon cancer inhibiting drug) administration, whereas their frequency increased after treatment with cholic acid.[84]

Conclusions

In the last years, highly effective molecular techniques (e.g. microarray based methods) have been developed, which can be employed to elucidate the mechanisms of carcinogenesis. These approaches can be used to analyze gene expression patterns *in vitro* in cell culture models, and also in tumors and can be compared with histological endpoints related to neoplasia. The predictive values of results obtained in in vitro models is often restricted, since the indicator cells which are used lack often characteristic features which are important for the in vivo situation. Typical examples are chemoprevention studies in which metabolically incompetent cell lines may give misleading results, as they do not reflect the activation/detoxification of DNA-reactive carcinogens.[85] On the other hand, the use of tumor formation in animal experiments as endpoints is hampered by the high costs and the time requirement and in case of human studies additionally by the limited availability of material. These shortcomings underline the value of preneoplastic foci models, which represent early stages of the neoplastic process. It has been shown that many compounds, considered as human carcinogens, can be detected with these models in rodents and also that protective agents which were identified in such foci experiments prevent specific forms of cancer in humans. Furthermore, the foci models are also useful to monitor the time course of biochemical and genetic alterations in neoplasia. On the basis of the important information created by the use of these foci models, it is likely that they will be also important tools in future research activities.

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