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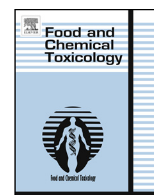

Induction of an altered lipid phenotype by two cancer promoting treatments in rat liver

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ABSTRACT

Changes in lipid metabolism have been associated with tumor promotion in rat liver. Similarities and differences of lipid parameters were investigated using the mycotoxin fumonisin B_1 (FB_1) and the 2-acetylaminofluorene/partial hepatectomy (AAF/PH) treatments as cancer promoters in rat liver. A typical lipid phenotype was observed, including increased membranal phosphatidylethanolamine (PE) and cholesterol content, increased levels of C16:0 and monounsaturated fatty acids in PE and phosphatidylcholine (PC), as well as a decrease in C18:0 and long-chained polyunsaturated fatty acids in the PC fraction. The observed lipid changes, which likely resulted in changes in membrane structure and fluidity, may represent a growth stimulus exerted by the cancer promoters which could provide initiated cells with a selective growth advantage. This study provided insight into complex lipid profiles induced by two different cancer promoting treatments and their potential role in the development of hepatocyte nodules, which can be used to identify targets for the development of chemopreventive strategies against cancer promotion in the liver.

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1. Introduction

Investigations into alterations of lipid metabolism in hepatocarcinogenesis have been conducted utilising a variety of animal models, which form the basis of fatty acid (FA) based chemopreventive approaches (Abel et al., 2004; Calviello et al., 1998; Ramesh and Das, 1995). However, only few studies distinguish between nodular lipid profiles and surrounding tissue to provide insight into the changes occurring during nodule development. Characterisation of lipid changes in nodules and hepatoma tissue induced by dietary 2-acetylaminofluorene (AAF) in Wistar rats indicated that sphingomyelin (SM) content decreased, while phosphatidylethanolamine (PE) and phosphatidylcholine (PC) increased in plasma membranes (Tamiya-Koizumi et al., 1985). The relative levels (% of total FA) of certain fatty acids, such as C18:1 and C18:2, increased, while C18:0 and C20:4ω6 decreased. In nodules and hepatomas induced by diethylnitrosamine (DEN) and a two week dietary AAF treatment in combination with partial hepatectomy (PH) as promoting stimulus, cholesterol decreased in microsomes and increased in mitochondria, while PE increased in the mitochondria (Canuto et al., 1989). The saturated FA (SFA), C16:0 and monounsaturated FA (MUFA) increased, while C18:0 and C20:4ω6 decreased in PC of microsomes and mitochondria. Nodules induced by dietary AAF for 25 weeks in Wistar rats showed an increase in C16:0, while C18:0 and C20:4ω6 content decreased in PC and PE in microsomes, mitochondria and lysosomes (Olsson et al., 1991). However, no change in PE or cholesterol levels in these nodules was reported.

A study on the kinetics of lipid alterations in hepatocyte nodules induced by the resistant hepatocyte model (Solt and Farber, 1976) with DEN as initiator and AAF/PH as cancer promoter was conducted over a period of 9 months and compared changes to normal regenerating liver following PH (Abel et al., 2001). The PE phospholipid fraction, cholesterol content and the SFA C16:0 increased, while C18:0 decreased in PC in the hepatocyte nodules. The MUFA C18:1 was increased in nodular PC and PE, while the absolute content of the polyunsaturated FA (PUFA) C20:4ω6 tended to decrease in PC, but increased in PE. Corresponding changes were noticed in regenerating liver following PH, although they reverted back within 4 weeks. Similarities in the reported lipid patterns associated with the development of precancerous and cancerous lesions and regenerating liver therefore imply a critical role during hepatocarcinogenesis. However, the mechanisms of how cancer promoters induce the lipid changes associated with selective growth of pre-neoplastic cell populations in the liver are not known at present.

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Cancer promotion by fumonisin B_1 (FB_1), a secondary metabolite of *Fusarium verticillioides* commonly occurring on maize, has been associated with altered lipid metabolism in the liver of rats and mice (Gelderblom et al., 2001; Howard et al., 2001). FB_1-induced changes in the phospholipid and fatty acid patterns in rat liver closely mimic those observed in hepatocyte nodules generated with cancer initiation/promotion models described above. These include increased cholesterol and PE content with a concomitant increase in absolute fatty acid content of C18:1ω9, C18:2ω6 and C20:4ω6 in PE, while long-chain polyunsaturated fatty acids (LCPUFA) decreased in PC (Abel et al., 2001; Gelderblom et al., 2001). It was suggested that these lipid changes induced by FB_1 selectively create a growth differential stimulating the outgrowth of a population of initiated cells into hepatocyte foci and nodules, while the growth of normal hepatocytes is inhibited (Gelderblom et al., 2001). The role of specific changes in lipid homeostasis in the liver during cancer promotion therefore became of interest based on their involvement in growth regulatory signals within normal, pre-neoplastic and cancerous hepatocytes (Serhan, 2005; Yamashita et al., 2009). A better understanding of the role these lipid changes play during cancer promotion would facilitate a specific fatty acid targeted approach in cancer prevention.

In order to characterise the involvement of lipid changes during cancer promotion resulting in the development of pre-neoplastic lesions, altered lipid and FA profiles of two different cancer promotion regimen were compared, i.e. the dietary FB_1 treatment (Gelderblom et al., 1996b), the AAF/PH protocol (Semple-Roberts et al., 1987), as well as a sequential combination of both in non-initiated rat liver. This will provide insight into lipid profiles in rat liver cancer promotion models that will assist in defining specific lipid targets for counteracting and/or prevention of this critical phase of hepatocarcinogenesis.

2. Materials and methods

2.1. Chemicals and reagents

2-Acetylaminofluorene (AAF), 2,5-bis-(5′tert-Butylbenzoxazolyl-[2′])-thiophene (BBOT), butylated hydroxy-toluene (BHT), malachite green and the free fatty acid standards were obtained from Sigma Chemical Corporation, Johannesburg, South Africa. Fumonisin B_1 was isolated according to the method described previously (Cawood et al., 1991) to a purity of approximately 95%. Cholesterol oxidase and esterase, peroxide-free Triton-X-100 and Tween-20 as well as the cholesterol standards were purchased from Boehringer Mannheim (Roche Applied Science, Randburg, South Africa). All other chemicals, solvents as well as the silica gel 60 plates for lipid analysis were obtained from Merck, Cape Town, South Africa. Organic solvents (methanol, chloroform and hexane) used for lipid extractions and analyses were glass distilled prior to use. All glassware was cleaned with a phosphate free soap (Contrad concentrate, Merck, Cape Town, South Africa) and rinsed with glass-distilled methanol.

2.2. Animals and diets

The use of laboratory animals in this study was approved by the Ethics Committee for Research on Animals of the MRC and experiment was conducted in compliance with policies and standards detailed in the MRC's principles and guidelines for use of animals in biomedical research. Male Fischer 344 rats, weighing between 150 and 180 g, were provided by the Primate Unit of the Medical Research Council, Tygerberg, South Africa and fed the AIN-76A diet (AIN, 1977; Bieri, 1980) using sunflower oil (5%) as fat source after weaning. Rats were housed individually in wire-bottomed cages in a controlled environment with a constant temperature of 23–25 °C, a 12 hour light/dark cycle and free access to drinking water. The FB_1 was dissolved in methanol, applied to a subsample (200 g) of the AIN-76A diet and dried in a fume cupboard for 4 hours, mixed with the standard AIN-76A diet to achieve the desired concentration (250 mg FB_1/kg diet) and stored under nitrogen at 4 °C. A control diet was prepared using methanol and stored under similar conditions.

2.3. Experimental design

Rats were divided into 4 groups of 5 rats each and treated according to the protocol summarised in Fig. 1, as published previously (van der Westhuizen et al., 2004). Treatments include rats receiving the (i) control diet (Ctrl), (ii) the FB_1 feeding treatment (250 mg/kg diet for 14days), (iii) AAF/PH cancer promoting regimen (20 mg/kg body weight AAF by gavage for 3 days, partial hepatectomy on the 4th day) and (iv) the combined AAF/PH and FB_1 promoting treatments introduced in a sequential manner. The animals were sacrificed after the 14 day FB_1 treatment period and 28 days after the AAF/PH treatment using sodium pentobarbital (200 mg/kg body weight; intra peritoneal). Liver tissue was collected, snap frozen in liquid nitrogen and stored at −80 °C.

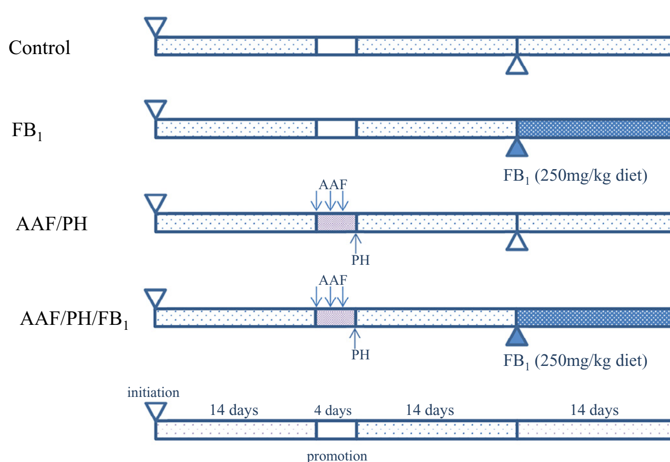

Fig. 1. Outline of the experimental groups and treatments. Male Fischer-344 rats, divided into four groups (n = 5) were fed the AIN 76A diet containing sunflower oil as fat source (5% of diet) throughout the experiment. Treatments were as follows: For this study, no rats were initiated; instead, they were acclimatised to the control diet for 14 days. Rats in the control group were fed the control diet throughout the experiment (46 days). Rats in the FB_1 promotion group were not subjected to the AAF/PH treatment and fed the control diet for 32 days; thereafter rats received 250 mg FB_1/kg diet for 14 days. The AAF/PH cancer promoting treatment included AAF (20 mg/kg body weight) by gavage on 3 consecutive days and partial hepatectomy on the 4th day. Thereafter rats received the control diet. Rats in the AAF/PH/FB_1 group were treated sequentially as described above for the AAF/PH and FB_1 promoting treatments. Abbreviations: AAF, 2-acetylaminofluorene; FB_1, fumonisin B_1; PH, partial hepatectomy.

2.4. Lipid analyses

The collected tissue samples were cut into fine pieces, ground to powder in liquid nitrogen using a mortar and pestle and approximately 100 mg were weighed into glass tubes and kept on ice. For lipid extraction these samples were suspended in saline and extracted with chloroform:methanol (CM 2:1, v/v, containing 0.01% butylated hydroxytoluene) ensuring a CM:saline ratio of 20:1 according to the method described by Folch et al. (1957) and as modified by Smuts et al. (1992). Extracts were stored in CMS (chloroform:methanol:saline, 86:14:1, v/v/v, containing 0.01% BHT) at 4 °C under nitrogen in the dark prior to thin layer chromatography.

The extracted lipids were separated using TLC as described by Gilfillan et al. (1983). FA analyses were performed on the PE and PC fractions following transmethylation with 11.6N sulfuric acid in methanol (5:95, v/v) at 70 °C for 2 hours as described by Smuts et al. (1994). The FA methyl esters were analysed on a Varian model 3300 Gas Chromatograph equipped with fused silica megabore DB-225 columns (0.53 mm internal diameter, 30m length, 1 µm film thickness, catalogue number 125–2232, J&W Scientific/Agilent Technologies, Folsom, California, USA). A standard mixture consisting of free FA (C14:0 to C22:6) was used to identify the FA methyl esters in the samples by comparison of retention times. An internal standard (C17:0) was used for quantification and results were expressed in µg FA per mg protein (absolute FA content) and as % FA of total FA (relative FA content). The phospholipid content of the lipid extracts was determined spectrophotometrically using a malachite green dye as described by Itaya and Ui (1966). Results were expressed as µg inorganic phosphate per mg protein. Total cholesterol content was determined using an enzymatic iodide method based on the Preciset Cholesterol kit (catalogue number 125512; Indianapolis, IN, USA) and Richmond (1973), and expressed as µg cholesterol per mg protein. Protein content was determined according to the modified Lowry method described by Markwell et al. (1978) using bovine serum albumin as an external standard and calculated using the Lowry Basic computer program (Grant, 1990).

The molar PC/PE ratio was calculated with the M_r of 787, and 744 for PC and PE, respectively, and the M_r of 386.7 for cholesterol was used for the molar cholesterol/total phospholipid ratio (Chol/TPL), with PC and PE combined as the major cellular phospholipids. The fatty acid parameters in Table 3 are defined as follows: The absolute FA content (µg/mg protein) was converted to nmol/mg protein for the calculation of the ω6/ω3, polyunsaturated to saturated (P/S) and the C18:0/C18:1 FA ratios. The latter was used as an indication of delta-9 desaturase activity (D9D).

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