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The level of aryl hydrocarbon (Ah) receptor and of 4S polycyclic aromatic hydrocarbon (PAH) binding protein in diploid and polyploid hepatocytes of 2-acetylaminofluorene-treated rats

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Sequential treatment of partially hepatectomized male Wistar rats with diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) induces the emergence of diploid hepatocyte populations. These carcinogen-induced hepatocytes are thought to include the precursor cells of liver carcinomas that arise later in this treatment protocol. The growth of the diploid hepatocytes is promoted by AAF and it has been suggested that the action of the arylamine may be receptor-mediated. AAF has been shown to bind specifically to the aryl hydrocarbon (Ah) receptor and the so-called 4S polycyclic aromatic hydrocarbon (PAH) binding protein. The present study addresses the question of whether the concentrations of the two binding proteins differ in diploid and polyploid hepatocytes from DEN/AAF-treated rats. Hepatocytes from carcinogen-treated rats were isolated and diploid, and tetraploid hepatocytes separated by means of centrifugal elutriation. Whereas Ah receptor concentrations in diploid hepatocytes were insignificantly lower (21.8 ± 5.9 versus 29.2 ± 6.6 fmol/mg cytosolic protein; $n = 4$; $P = 0.1$), levels of the 4S PAH binding protein in diploid hepatocytes were twice as high as in tetraploid hepatocytes (252.3 ± 93.6 versus 124.0 ± 18.5 fmol/mg cytosolic protein; $n = 4$; $P = 0.04$). We conclude from our results that the differences in growth control in polyploid and carcinogen-induced diploid hepatocytes are not associated with changes in the levels of the Ah receptor. The role of the 4S PAH binding protein in the process of hepatocarcinogenesis remains to be established.

Introduction

The sequential treatment of two-thirds hepatectomized rats with diethylnitrosamine (DEN*, 1 × 50 mg/kg body wt) and 2-acetylaminofluorene (AAF, 0.02% in the diet for 4–5 weeks) induces a dramatic increase in the ratio of diploid/tetraploid hepatocytes (1,2). The newly arising diploid hepatocytes are thought to include the precursor cells for liver carcinomas (1,3). Seglen and co-workers showed that AAF exerts its effects, e.g. stimulation of non-polyploidizing growth and promotion of the outgrowth of large liver nodules, by non-cytotoxic mechanism (4,5). Neither post-regenerative liver growth nor the histological appearance of liver sections was affected by AAF in the foregoing protocol (5). Thus, they suggested that AAF may act via distinct cellular receptors (5,6). Also Neumann and co-workers suggested on the basis of their findings that promotion of tumor formation by AAF may be mediated by a receptor, possibly by the cytosolic aryl hydrocarbon (Ah) receptor (7,8). The Ah receptor is a regulatory protein that, either directly or together with other factors, controls the expression of two phase I genes and of four phase II genes: the so-called Ah gene battery (9). Nebert suggested that the Ah receptor participates in tumor initiation and tumor promotion by the induction of these enzymes that generate mutagenic and toxic intermediates (9). In addition, it was proposed that the Ah receptor may be involved in the regulation of cell proliferation and differentiation by the expression of a second battery of genes in some tissues or cells (10), e.g. epidermal cells in the skin (11). Recently, it was shown that AAF binds specifically to the Ah receptor (12). Besides the Ah receptor, liver cytosol contains another protein with high affinity for polycyclic aromatic hydrocarbons (PAH). This second protein is referred to as 4S PAH binding protein according to its sedimentation behavior in sucrose density gradients (13). The 4S PAH binding protein displays some characteristics of a cellular 'receptor': (i) saturable, high-affinity binding of PAHs (14); (ii) translocation to the nucleus after ligand binding (15); (iii) binding to 5′ upstream sequences of *CYP1A1* gene (13). This protein has been detected in various tissues of 'responsive' and 'non-responsive' mice and in the liver of diverse animal species (16). Its physiological role, e.g. its participation in gene expression is a matter of controversy (13,15,17,18). AAF also binds specifically to the 4S PAH binding protein, though with a lower affinity than 3-methylcholanthrene (MC) (12). In the present study we have addressed the question of whether the level of the Ah receptor differs in carcinogen-induced diploid and polyploid rat hepatocytes isolated by means of centrifugal elutriation. Furthermore, we have also determined the level of the 4S PAH binding protein which possibly interacts with AAF *in vivo* and thus may play a role in its biological activity.

*Abbreviations: DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; Ah, aryl hydrocarbon; PAH, polycyclic aromatic hydrocarbon; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin; TCDF, 2,3,7,8,-tetrachlorodibenzofuran.

Materials and methods

Materials

[^{3}H]MC (1.26 TBq/mmol) was from Amersham (Braunschweig, Germany). [^{3}H]2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, 0.96 TBq/mmol) was a generous gift of Dr A.Poland (McArdle, Laboratory, WI, USA) and was further purified by HPLC before use. DEN and AAF were from Serva (Heidelberg, Germany); 2,3,7,8-tetrachlorodibenzofuran (TCDF) was from Promochem (Wesel, FRG).

Animals and treatment

Male Wistar rats (4 weeks old, 70–80 g body wt) from the GSF breeding colonies, were subjected to a two-thirds hepatectomy; 20 h later they received DEN (1 × 50 mg, by gastric intubation). After 1 week the animals were fed a standard pellet diet (Altromin, Lage, Germany) containing 0.02% AAF for a period of 4–5 weeks.

Preparation of diploid and polyploid hepatocytes and of cytosol

About 5 weeks after finishing treatment with AAF, liver cells were isolated by collagenase perfusion as described recently (19) except that no second collagenase treatment was performed prior to centrifugal elutriation. Diploid and polyploid hepatocytes were isolated by centrifugal elutriation (2). In brief: 125 × 10^6 vital hepatocytes were loaded into the Beckman JE-6B rotor (standard elutriation chamber) at a flow rate of 19 ml/min, 1700 r.p.m. and 10°C. By increasing the flow rate the following fractions were obtained: dead cells (26 ml/min), diploid cells (33 ml/min), 'mixed cell fraction' (50 ml/min) and polyploid cells (60 ml/min

and decreasing the r.p.m. to 1500). The DNA content of hepatocytes in the parent cell suspension and the elutriated cell fractions was analyzed using a FACS-Analyser (Becton-Dickinson). Flow cytometry and correction of the data was performed as described elsewhere (19). Cytosol was prepared with 25 mM HEPES, 1.5 mM EDTA, 1 mM DTT, 10% (w/v) glycerol, 150 mM NaCl (HEDGN), pH 7.2 (4°C) according to standard procedures.

Assay of Ah receptor

The Ah receptor was determined by a single-point assay based on the use of an excess of ligand concentration. The single-point assay yields quantitatively accurate data. To 500 μl cytosol, 1.5 μl [^{3}H]TCDD (in DMSO) was added to give a final TCDD concentration of 5 nM. This mixture was immediately divided into two equal parts. A 500-fold excess of TCDF (in DMSO) was added to one half, an equal amount of DMSO to the other. After 2 h of incubation at 4°C, the unbound TCDD was removed by charcoal–dextran treatment (0.1 mg charcoal–dextran/mg cytosolic protein) according to Manchester *et al.* (20). Labeled cytosol samples (200 μl) were analyzed by the density gradient centrifugation technique described by Tsui and Okey (21). Cytosol was used either undiluted or was diluted by a factor of up to 1.5 with HEDGN buffer. The cytosolic protein concentrations were as follows: parent cell suspension and polyploid hepatocytes, ~10 mg/ml; diploid hepatocytes, ~5 mg/ml; and liver of untreated rats, ~12 mg/ml. The Ah receptor concentration increases linearly with the protein concentration in the concentration range between 5 and 10 mg/ml.

Assay of 4S PAH binding protein

Cytosol was diluted by a factor of 1.25 with HEDGN buffer. Two incubations with 500 μl cytosol were performed consecutively, each mixture containing 10 nM [^{3}H]MC (in ethanol) and either a 500-fold excess of unlabeled MC (in DMSO) or an equal amount of DMSO. The next steps followed the procedure of the Ah receptor assay. Labeled cytosol (400 μl) was applied to a HPLC gel filtration column Superose 6 (Pharmacia, Sweden) and the column was eluted with the HEPES buffer without glycerol at 4°C at a flow rate of 0.2 ml/min. Starting with the void volume of the column (6 ml), 50 fractions of 0.4 ml were collected.

Results

Sequential treatment of two-thirds hepatectomized rats with DEN and AAF induced the emergence of diploid hepatocytes. Whereas the percentage of diploid hepatocytes amounts to only ~5% in liver cell suspensions isolated from untreated animals (19), it increases to >40% in hepatocyte suspensions obtained from the carcinogen-treated animals (Table I). Diploid and polyploid hepatocytes were isolated by means of centrifugal elutriation. The ploidy distribution of the cell fractions obtained is shown in Table I; the purity of the 'diploid' and 'polyploid' cell fractions was 84 and 87% respectively. The viability of the hepatocytes was ~90% as determined by trypan blue exclusion.

For the determination of the PAH binding proteins two different methods were applied. While the Ah receptor was determined by sucrose density gradient centrifugation using [^{3}H]TCDD as a ligand (Figure 1), the 4S PAH binding protein was analyzed with labeled MC by HPLC gel filtration (Figure 2). This latter technique allows not only the determination of the 4S PAH binding protein but also, in addition, of the Ah receptor. Binding of labeled TCDD to cytosolic proteins (Figure 1) resulted in two peaks; however, only the radioactivity of the second peak could be displaced by a 500-fold excess of unlabeled TCDF, indicating specific binding of TCDD. Since peak II was absent in hepatic cytosol prepared without molybdate from the non-responsive mouse strain DBA/2J (data not shown), this peak represents the cytosolic Ah receptor. MC was bound specifically to two cytosolic proteins (Figure 2): the Ah receptor (peak I) and to another protein which is most likely identical with the 4S PAH binding protein described by Zytkovicz (14) according to its Stokes' radius (R_S = 3.0 nm, Figure 2) and binding characteristics of PAH ligands (data not shown). The latter protein was present in higher concentration than was the Ah receptor (Figure 2) assuming one ligand binding site per protein. The determination of the Ah receptor by the two methods yielded qualitatively identical results, but higher values were obtained with TCDD as a ligand (data not shown). The level of the Ah receptor and the 4S PAH binding protein in the parent cell suspension and the two elutriated cell fractions are given in Table II. The amount of the Ah receptor was lower in the elutriated hepatocyte fractions than in the parent cell suspension. Hepatocytes of the 'diploid' cell fraction contained ~25% less receptor per mg cytosolic protein than did hepatocytes of the 'polyploid' cell fraction. However, this difference was not

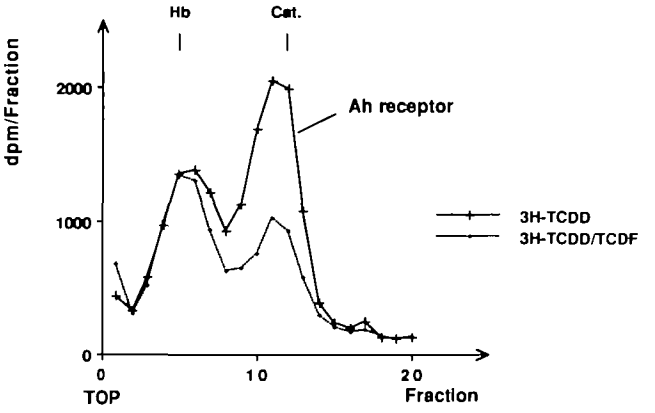

Fig. 1. Typical elution pattern of [^{3}H]TCDD-labeled cytosol of hepatocytes from carcinogen-treated rats analyzed by sucrose density gradient centrifugation for the determination of Ah receptor. Binding in the presence of a 500-fold excess of TCDF is indicated by a dashed line. Catalase (11.3S) and hemoglobin (4.4S) were taken as marker proteins.

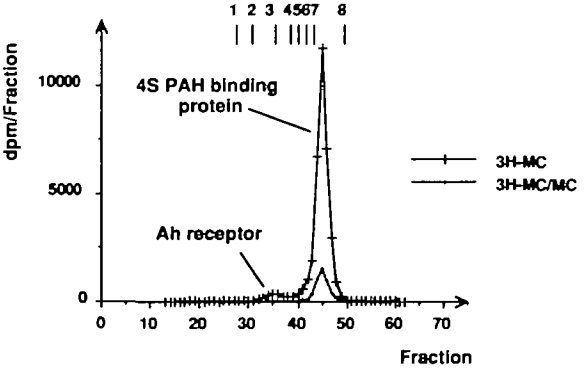

Fig. 2. Typical elution pattern of [^{3}H]MC-labeled cytosol of hepatocytes from carcinogen-treated rats analyzed by gel permeation HPLC for the determination of 4S PAH binding protein. Binding in the presence of a 500-fold excess of MC is indicated by a dashed line. Vertical lines indicate the elution of marker proteins (Stokes radius, nm): **1**, dimer of apoferritin (9.02); **2**, threoglobulin (8.6); **3**, apoferritin (6.71); **4**, catalase (5.23); **5**, alcohol dehydrogenase (4.55); **6**, bovine serum albumin (3.62); **7**, ovalbumin (2.83); **8**, cytochrome *c* (1.63).

Table I. Isolation of diploid and polyploid hepatocytes from carcinogen-treated rats by centrifugal elutriation: determination of the ploidy distributions and viabilities in the parent cell suspensions and the elutriated hepatocyte fractions

Hepatocytes[a]	Viability[b] (% of unstained cells)	Ploidy 2c	4c	8c
Parent cell suspension	88 ± 3	43 ± 6	53 ± 5	4 ± 2
'Diploid' cell fractions	91 ± 3	84 ± 7	16 ± 7	0
'Polyploid' cell fractions	90 ± 4	13 ± 2	82 ± 3	4 ± 2

[a]Data represent the ploidy distributions of the hepatocyte fractions used for the determination of receptor levels (Table II). Values represent the means ± SD of four different cell isolations.
[b]Viability of hepatocytes was determined with 0.4% trypan blue.

statistically significant. In contrast to the Ah receptor, the concentration of the 4S PAH binding protein was twice as high in the 'diploid' than in the 'polyploid' hepatocyte fraction. Intermediate levels of the 4S PAH binding protein were determined in hepatocytes of the parent cell suspension. The values for the two PAH binding proteins shown in Table II reflect cellular concentrations because both the amount of protein and the cell volumes differ by a factor of 2 in carcinogen-induced diploid and tetraploid hepatocytes (22). To substantiate this claim we also determined the water space of diploid and polyploid hepatocytes using tritiated water according to Baur *et al.* (23). The cellular water space was similar in the two cell fractions when expressed per mg protein and amounted to ~4 μl/mg protein.

Discussion

In the present study the levels of the Ah receptor and the 4S PAH binding protein were determined in carcinogen-induced diploid and polyploid hepatocytes isolated by centrifugal elutriation. Both proteins are known to bind AAF. The question has been raised whether biological activities of the aromatic amine, such as the induction of tumor formation, which has been shown to take place in the carcinogen regimen used in this study, may be mediated via these receptors.

The concentration of the Ah receptor in the parent hepatocyte suspension, 42.2 fmol/mg cytosolic protein, is similar to the concentration in liver cytosol of untreated rats, 42.6 ± 2.5 fmol/mg cytosolic protein. Centrifugal elutriation decreases the level of the Ah receptor (Table II). The reason for this reduction is not known. As determined by the trypan blue exclusion test, centrifugal elutriation does not affect the integrity of the cell membrane as a barrier for small molecules. In addition several other findings indicate viability of elutriated hepatocytes: after centrifugal elutriation hepatocytes can be cultured in monolayers, respond to the growth factor EGF (2), and are competent in carrier-mediated transport of organic compounds (24). In contrast to the Ah receptor, the level of 4S PAH binding protein is not decreased in the elutriated cell fractions.

The concentration of the Ah receptor did not differ significantly in diploid and polyploid hepatocytes. Thus our results indicate that the differences in growth control in polyploid and carcinogen-induced diploid hepatocytes is not associated with changes in the levels of the Ah receptor (4). However, other differences in the Ah receptor of the two cell populations cannot be excluded at present, e.g. the nuclear receptor complex itself may differ in carcinogen-induced diploid and polyploid hepatocytes. Since no exchange assays are known for the activated nuclear receptor after dissociation of 90 kDa heat shock protein (25) and since only very small amounts of cells are obtained by centrifugal elutriation, these questions were not studied. However, it is also possible that the receptor does not participate in the non-ploidizing growth of diploid hepatocytes.

To the best of our knowledge the level and physicochemical characteristics of the Ah receptor have not been determined in human or animal tumors. Gasiewics *et al.* studied the ontogeny of the Ah receptor in rat liver (26). The receptor increases perinatally and remains at a high level from days 2 to 21. After this time the concentration of the receptor decreases markedly. During the first 2–3 weeks, rat liver is almost exclusively diploid (27,28), thereafter the percentage of polyploid hepatocytes increases rapidly. Polyploidization has been regarded as an irreversible aspect of hepatocellular differentiation (28,29). Thus the ontogeny of the Ah receptor might suggest that the high level of the Ah receptor during the first weeks of life may be due to the high percentage of diploid cells in the liver and that the decrease of the Ah receptor is a result of the increasing polyploidization of the liver. However, the present data indicating no difference in the receptor concentration of the diploid and polyploid hepatocytes argue against this notion, though it is not known whether the receptor levels differ in carcinogen-induced and normal diploid hepatocytes. Adult rat liver contains only ~5% diploid hepatocytes (2,19), a percentage that is too low to be enriched by centrifugal elutriation to a degree and purity which would allow the analysis of diploid hepatocytes from untreated animals.

In contrast to the Ah receptor, the level of the 4S protein differs significantly in 'diploid' and 'polyploid' hepatocyte fractions. The concentration of the 4S protein in hepatocytes of the 'polyploid' cell fraction, 124 ± 18.5 fmol/mg cytosolic protein, corresponds well with the concentration of this binding protein in the cytosol of untreated adult Wistar rats, 129.9 ± 25 fmol/mg cytosolic protein. In agreement with this finding we showed recently that hepatocyte suspensions isolated from adult Wistar rats contain ~95% polyploid (2,19). The level of the 4S PAH binding protein was twice as high in the 'diploid' as in the 'polyploid' cell fraction of carcinogen-induced hepatocytes. There has been some controversy about the physiological function of the 4S PAH binding protein. Various compounds with different functional groups and biological activities bind to the 4S PAH binding protein. Thus, it has been suggested that the 4S PAH binding protein may act as an intracellular carrier protein for hydrophobic PAHs and may enhance their metabolism because of their increased transport to the microsomal fraction. The oxidized products formed during microsomal metabolism were also bound to the protein (30). On the other hand it has been shown that the 4S PAH binding protein is translocated from cytosol to the nucleus (15), which has led to other suppositions about its

Table II. The level of Ah receptor and of 4S PAH binding protein in 'diploid' hepatocyte and 'polyploid' hepatocyte fractions from carcinogen-treated rats compared to the parent cell suspensions before elutriation

Cell fraction	Ah receptor[a] (fmol/mg cytosolic protein)	4S PAH binding protein[a] (fmol/mg cytosolic protein)
Parent cell suspension	42.2 ± 5.7	203.1 ± 29.5
'Diploid' hepatocyte fraction	21.8 ± 5.9	252.3 ± 93.6*
'Polyploid' hepatocyte fraction	29.2 ± 6.6	124.0 ± 18.5
Hepatic cytosol (untreated)	42.6 ± 2.5[b]	126.9 ± 25.0[c]

[a]Data are the mean ± SD of four animals.
[b]$n = 3$.
[c]$n = 6$.
*Significantly increased ($P < 0.05$) compared to polyploid hepatocytes.

biological role. (i) It has been proposed that the 4S PAH binding protein functions as a regulatory transcriptional factor of *CYP1A1* expression because of its specific binding to the 5′-upstream region of the gene (31). However, another study did not confirm a significant role in CYP1A1 induction (17). (ii) It has been speculated that the 4S PAH binding protein may modulate carcinogenesis because ligands of the protein have been shown to alter the carcinogenic process in the mouse skin tumor model (32).

Which of the above mentioned hypotheses prove to be right and whether the differences in the concentrations of the 4S PAH binding protein are important for the promoting activity of AAF in the present treatment protocol remain to be established.

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