Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 466-472

PRESENCE OF CYTOCHROME P-450- AND CYTOCHROME P-448-DEPENDENT MONOOXYGENASE FUNCTIONS IN HEPATOMA CELL LINES

F.J. Wiebel[a], T. Wolff[a] and M. Lambiotte[b]

[a]Gesellschaft f. Strahlen- u. Umweltforschung, Abteilung Toxikologie, Institut f. Toxikologie u. Biochemie, D-8042 Neuherberg b. München

[b]Department Unité de Génétique Cellulaire, Institut de Recherches en Biologie Moleculaire du C.N.R.S. Université Paris VII, Place Jussieu - 75221 Paris Cedex 05, France

Received March 25,1980

SUMMARY

Cell lines derived from Reuber H-4-II-E hepatoma cells and their hybrids that differ in the expression of liver-specific functions are shown to contain different forms of monooxygenases. According to 1) the specificity toward the substrates benzo(a)pyrene, aldrin and chenodeoxycholic acid, 2) the kinetics of the epoxidation of aldrin, 3) the response to inducers, such as benz(a)anthracene and dexamethasone, and 4) the in vitro modifier 7,8-benzoflavone, the monooxygenases predominating in differentiated cell lines belong to the cytochrome P-450-dependent enzyme(s), those in the less differentiated lines belong to the cytochrome P-448-dependent form(s).

The usefulness of cell cultures in toxicological studies largely depends on their expression of microsomal monooxygenases which are the key enzymes in the conversion of numerous chemicals to their reactive intermediates (1, 2). The majority of established cells in culture have been shown to contain monooxygenase activity (2-4). However, this activity was found to represent only specific forms of the family of monooxygenases i.e., forms of the "cytochrome P-448"-type (4,5) that are inducible by polycyclic hydrocarbons and predominate in extrahepatic tissues (6,7). Other monooxygenases, the "cytochrome P-450"-dependent forms that predominate in the liver of rodents (6,7) and most likely of man (8), appeared to be lacking in established cell cultures. To date, the lack of these monooxygenase forms poses the most serious obstacle to the general applicability of established cell cultures for toxicological studies.

Recent observations have shown that cell lines derived from Reuber hepatoma are capable of hydroxylating bile acids (9) and of activating

0006-291X/80/100466-07$01.00/0
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aflatoxin B_1 to cytotoxic species when pretreated with a glucocorticoid (10). These two functions may be related to cytochrome P-450-dependent monooxygenases (11,12).

The studies described in the following were aimed at characterizing the monooxygenase(s) in these hepatoma cell lines by their substrate, inhibitor and inducer specificities. The results indicate that the cell lines may contain both cytochrome P-448- and cytochrome P-450-dependent monooxygenases.

MATERIALS AND METHODS

Cell cultures: The cell clones under study, FAZA 967, FaO, H5, HF1 and HF1-4, were kindly provided by Dr. M.C. Weiss, CNRS, Gif-sur-Yvette. They are known to express to different degrees liver specific functions, such as secretion of albumin or the activities of liver specific isozymes of alcohol dehydrogenase and aldolase (13,14). The "differentiated" clone FAZA 967 and its subclone FaO as well as the "undifferentiated" clone H5 are derived from FU-5 cells, descendents of the H-4-II-EC3 line (13). A hybrid cell clone, HF1, formed from FaO and H5, does not express the liver specific enzymes; however, a subclone, HF1-4, obtained by selection in glucose free medium, reexpresses the range of functions observed in the differentiated parental cell (14).

The cells were grown as monolayers in 100 mm dishes at 37°C in an atmosphere of 95% air/5% CO_2. The growth medium consisted of equal volumes of modified Ham's F12 and NCT 109, 7.5% fetal calf serum, 1 g bovine serum albumin, 7.1 mg taurine per liter, 100 units penicillin and 50 µg streptomycin per ml. The growth medium for the lines HF1 and HF1-4 was fortified by 15.6 mg hypoxanthine and 44.4 mg thymidine per liter.

Enzyme assays: For the determination of *in vitro* monooxygenase activities monolayers were washed with phosphate buffered solution, and the cells were collected and stored at -80°C as described previously (4). The hydroxylation of benzo(a)pyrene was determined by a modification (4) of the method of Nebert and Gelboin (15); epoxidation of aldrin was measured according to Wolff *et al.* (16).

Protein determination: Cellular protein was determined following the method of Lowry *et al.* (17) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

To analyze for the presence of various monooxygenase forms in the hepatoma cells three substrates were employed: 1) benzo(a)pyrene, which is preferentially metabolized by cytochrome P-448-dependent monooxygenases (=benzo(a)pyrene-hydroxylase; aryl hydrocarbon hydroxylase, AHH) (4,15,18); 2) aldrin, which is nearly selectively metabolized by cytochrome(s) P-450

(=aldrin epoxidase) (16,19); and 3) chenodeoxycholic acid which likewise is thought to be hydroxylated by cytochromes P-450 (11).

As shown in Table 1, there was a rough inverse correlation between AHH and aldrin epoxidase activities. The differentiated cell lines, FAZA 967 and FaO contained relatively high aldrin epoxidase activity and barely detectable AHH; the ratio of the 2 activities was the opposite in the undifferentiated cell lines, H5 and HF1. The hybrid cell line HF1-4, in contrast, expressed both enzymes at a high level of activity.

Exposure of the cells to dexamethasone enhanced the rate of oxidation of the 3 substrates to various degrees. The 6ß-hydroxylation of chenodeoxycholic acid was markedly increased in FAZA 967, FaO and in HF1-4 cells; aldrin epoxidase was induced in the FAZA 967 and HF1-4 cell lines but not in FaO cells; and AHH was found to be markedly induced only in HF1-4 cells. It is striking that induction of these enzyme activities occured only in the differentiated cell lines FAZA 967, FaO and HF1-4. Opposite results were obtained after exposure of cells to benz(a)anthracene, an inducer of cytochrome P-448-dependent reactions (15). Benz(a)anthracene treatment strongly induced the activity of AHH in the undifferentiated cell lines H5 and HF1, and had little effect on the differentiated cell lines except HF1-4 which was highly inducible to AHH. In contrast, benz(a)anthracene treatment did not induce aldrin epoxidase in any of the cell lines and did not elicit detectable levels of bile acid hydroxylation.

Monooxygenases are known to exhibit some degree of overlap in substrate specificities. This is true for aldrin epoxidation (16,19) and more so for AHH (7,18). The monooxygenase forms involved may be differentiated by their kinetic properties or their sensitivity to in vitro modifiers. For example, the substrate affinity for aldrin, determined by the apparent K_m, differed for the various cell lines: In the differentiated lines FAZA 967, FaO and HF1-4, the reaction exhibited a very low K_m of 0.1, 0.4 and 0.2 µM aldrin, respectively. In the undifferentiated lines H5 and HF1 the K_m was 3.7 and

Table 1. Oxidative metabolism of benzo(a)pyrene, aldrin and chenodeoxycholic acid in various hepatoma cell lines exposed to dexamethasone or benz(a)anthracene.

Cell Line	Benzo(a)pyrene-Hydroxylation (AHH) (pmol/min/mg prot.)			Aldrin-Epoxidation (pmol/min/mg prot.)			Chenodeoxycholic Acid Hydroxylation (nmol/24 hrs/mg prot.)		
	Control	DEX	BA	Control	DEX	BA	Control	DEX	BA
FAZA	0.06	0.24	0.15	14.1	22.6	13.3	0.2	9.4	0.2
FaO	0.19	0.94	0.14	23.0	17.5	18.3	0.2	10.2	0.2
H5	0.50	0.75	18.00	1.7	1.5	0.7	0.2	0.2	0.2
HF1	0.70	1.40	48.00	1.3	1.0	0.7	0.2	1.5	0.2
HF1-4	2.80	21.00	81.90	32.0	139.0	38.0	0.2	14.8	0.2

Cultures were exposed to fresh growth medium ("Control") or to medium containing either 1 μg/ml dexamethasone (DEX) or 5 μg/ml benz(a)anthracene (BA) for 18 hours. The experiment shown is one of 3 independent experiments yielding similar results. Duplicate determinations varied by less than 10% from the mean. For the determination of chenodeoxycholic acid hydroxylation, cultures were exposed to BA or DEX for 48 hrs. C^{14}-Chenodeoxycholate was added from the 24th to the 48th hr. The major product, α-muricholic acid, was extracted from the medium and separated by TLC as previously described (9).

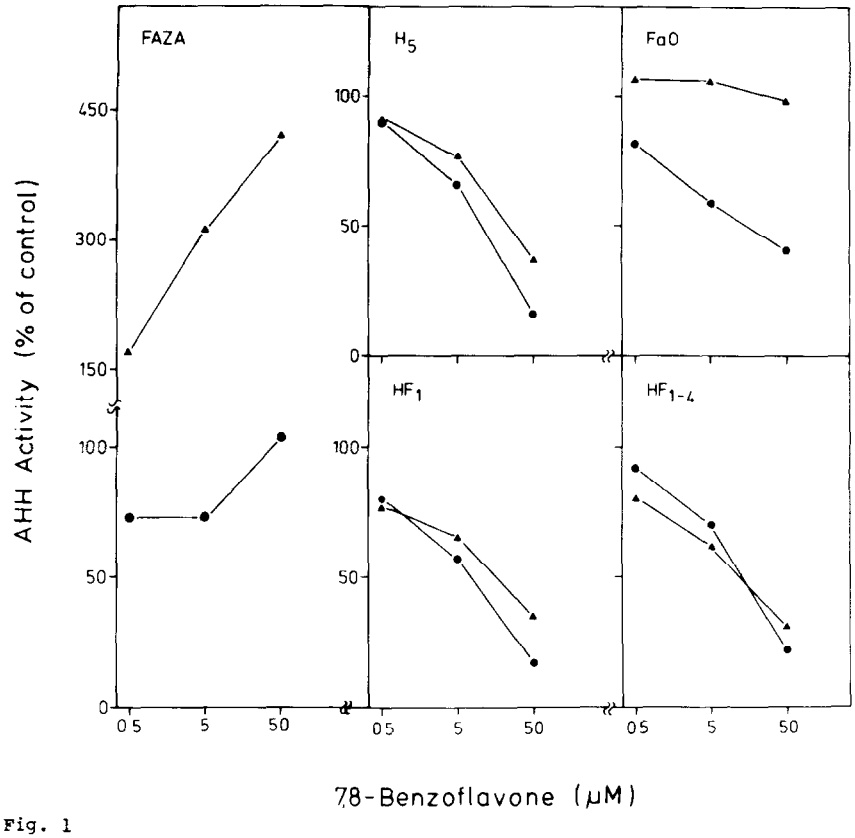

Fig. 1
In vitro effect of 7,8-benzoflavone on AHH activity in hepatoma cell lines. Cells were exposed to dexamethasone (▲—▲) or benz(a)anthracene (●—●) and assayed for in vitro AHH activity as described in Materials and Methods. 7,8-Benzoflavone was added at appropriate concentrations together with the substrate, benzo(a)pyrene, in 50 μl methanol/ml reaction mixture. Enzyme activities are expressed in % of control i.e., the activities in the absence of 7,8-benzoflavone (cf. Table 1).

4.3 μM aldrin, respectively i.e., about 10 times higher than in the differentiated lines. Previous observation showed that in a reconstituted system the K_m of cytochrome P-448 mediated aldrin epoxidation is considerably higher than that of the cytochrome P-450 mediated reaction (16). Treatment with the inducers dexamethasone or benz(a)anthracene did not significantly alter the apparent K_m. These findings suggest a) that the various cell lines contain at least two different forms of aldrin epoxidases and b) that the enzymes in untreated and inducer-treated cells are similar if not identical.

Different forms of AHH may be distinguished by their response to the synthetic flavonoid, 7,8-benzoflavone, that strongly inhibits cytochrome P-448-

dependent reactions and is without effect or stimulates cytochrome P-450-dependent forms (7,18). The effect of the flavone on AHH in various hepatoma cell lines is compatible with the presence of two monooxygenase forms. As shown in Fig. 1, 7,8-benzoflavone inhibited AHH activity in both untreated and benz(a)anthracene-treated cells of the undifferentiated lines, H5 and HF1. However, the flavone markedly increased AHH activity in the differentiated cell lines FAZA 967 and FaO, either after treatment with dexamethasone (Fig. 1) or without prior treatment (data not shown). In benz(a)anthracene-treated FaO and FAZA 967 cells, the enzyme activity was inhibited to a minor degree in keeping with the poor induction of AHH by the polycyclic hydrocarbon (Table 1).

In conclusion, the present results show that the differentiated hepatoma cell lines express monooxygenase functions which differ from those of the undifferentiated lines. According to the substrate specificity, the response to inducers and to *in vitro* modifiers, the monooxygenase form(s) found in the differentiated lines belongs to the cytochrome P-450-dependent enzymes, the form in the undifferentiated lines to the cytochrome P-448-dependent monooxygenases commonly observed in established cell cultures. The hybrid cell line HF1-4 appears to express both types of monooxygenases.

The availability of closely related cell lines exhibiting either cytochrome P-450- or P-448-dependent monooxygenase functions, or both, constitutes a major advance toward the development of cell culture systems that are suitable for analyzing the metabolic activation of foreign compounds and for screening of their cytotoxic, mutagenic or carcinogenic potential.

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