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TRANSFORMATION OF PRIMARY CULTURED AND CO-CULTURED ADULT RAT LIVER CELLS BY 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE

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Abstract. Under various conditions of culture and carcinogen treatment, the transformation of liver cells by 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) was studied. Primary liver cell (PLC) cultures from adult male rats and co-cultures with PLCs of ARL-D8 cells of a liver epithelial-like clear cell line from adult female rats were treated with 0.24 mM 3'-Me-DAB for 6 days. Four of 8 carcinogen-treated PLC cultures contained cells with marker chromosomes, and 3 of the 8 cultures contained gamma-glutamyltranspeptidase (GGT)-positive cells. Three of 5 carcinogen-treated co-cultures contained cells with marker chromosomes, and 2 of the 5 co-cultures contained GGT-positive cells. Pure cultures of ARL-D8 cells were treated for 6 or 12 days with 3'-Me-DAB (0.24 mM)-containing-medium perfused through the liver of adult male rats *in situ*. In the 6-day treatment, none of 5 carcinogen-treated cultures showed chromosomal abnormality or cytochemically exhibited GGT activity. However, in the 12-day treatment, 2 of the 5 carcinogen-treated cultures contained cells with marker chromosomes, and 2 of the 5 cultures contained GGT-positive cells. None of the control cultures exhibited chromosomal abnormality or GGT-positive cells. In summary, transformation markers increased in ARL-D8 cells when they were co-cultured with PLCs.

Key words : primary liver cell culture, epithelial-like clear cells, co-culture, 3'-methyl-4-dimethylaminoazobenzene, transformation.

Studies on *in vitro* malignant transformation of liver epithelial-like cells by aminoazo dyes have been attempted, but it is very difficult to demonstrate clearly *in vitro* malignant transformation by carcinogens because of frequent spontaneous-transformation of control cultures during experiments (1-4). Therefore, the development of a reliable *in vitro* carcinogenic system of liver cells using aminoazo dyes is urgently needed.

Aminoazo dyes are indirect carcinogens which require chemical alterations, mediated by drug-metabolizing enzymes, to react with intracellular targets (5). The main disadvantage of previous *in vitro* studies on aminoazo dye carcinogenesis is that the levels of drug-metabolizing enzymes are very low or absent in rat liver epithelial-like cells. Primary liver cell (PLC) cultures, in which liver-specific functions including drug-metabolizing enzyme activity can be maintained for about a

week, have been developed for such studies (6, 7).

In order to develop a reliable aminoazo dye carcinogenic system of liver cells *in vitro*, transformation of liver cells by 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) was studied using PLC cultures and a liver epithelial-like clear cell line, ARL-D8 (8), under various conditions of culture and treatment. Liver cell transformation was assessed in terms of chromosomal abnormality and gamma-glutamyl-transpeptidase (GGT) activity in the early stage of culture.

MATERIALS AND METHODS

PLC cultures. As reported previously (7), isolated liver cells, with an initial viability of greater than 80 % measured by trypan blue exclusion, were prepared by a collagenase-liver-perfusion technique from 3-month-old male Donryu rats inbred in this institute. The basal medium used was Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd., Japan) supplemented with bovine serum (20 %), penicillin (100 U/ml), streptomycin (100 μg/ml), kanamycin (60 μg/ml) and fungizone (1 μg/ml). The isolated liver cells were inoculated at a concentration of 3.5×10^5 cells/ml into 100 mm Falcon plastic dishes containing 10 ml of the basal medium supplemented with 10 μM dexamethasone-21-disodium phosphate (dexamethasone, Nippon Merck Banyu Co., Japan) and 10 μg/ml insulin (Sigma Chemical Co., USA), and cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 ℃.

Liver epithelial-like clear cell line, ARL-D8. A liver epithelial-like clear cell line, ARL-D8, was derived from cultures of liver cells which were isolated from adult female Donryu rats by a trypsin-liver-perfusion technique. The cells were consecutively treated with 7.7 μM dexamethasone for 8 days after initiation of primary culture (8). In this study, ARL-D8 cells were used, alone or co-cultured with PLCs prepared as above, as target cells for the assay of transformation by 3'-Me-DAB.

Co-culture of ARL-D8 cells with PLCs. For carcinogen treatment, 1.2×10^6 ARL-D8 cells and 3.6×10^6 PLCs were mixed and inoculated into 100 mm Falcon plastic dishes containing 10 ml of the basal medium supplemented with dexamethasone (10 μM) and insulin (10 μg/ml), and cultured under the same conditions as the PLC culture described above. The ARL-D8 cells were 39 days old at the time of initiation of co-culture.

Treatment of PLC cultures and co-cultures with 3'-Me-DAB. 3'-Me-DAB (Tokyo Kasei Co., Japan) dissolved in dimethylsulfoxide (DMSO, Sigma Chemical Co., USA) was added to the basal medium supplemented with dexamethasone (10 μM) and insulin (10 μg/ml) to give a final concentration of 0.24 mM 3'-Me-DAB (F) and 0.5 % DMSO (A) (9). Carcinogen-containing medium was added to PLC cultures and co-cultures by medium change following a 1-day attachment period, and thereafter the medium was renewed every 2 days. Control cultures were treated with 0.5 % DMSO in the same way. Following carcinogen treatment for 6 days, cultures were maintained in the basal medium. Cultures treated with 3'-Me-DAB were designated as 6F according to the treatment period and 3'-Me-DAB concentration (9). Similarly, control cultures were named 6A (9).

Preparation of 3'-Me-DAB-containing-medium perfused through the liver of adult male rats. Male Donryu rats (3 to 4 months old) inbred in this institute were used for preparation of 3'-Me-DAB-containing medium perfused through the liver *in situ*. To flush circulating blood from the liver completely, Hanks' balanced salt solution (100 ml) was perfused through the liver at a flow rate of 10 ml/min. Afterwards, 100 ml of the basal medium containing 3'-Me-DAB at 0.24 mM (F) or DMSO at 0.5 % (A) was recirculated at a flow rate of 5 ml/min through the

liver for 1 h while bubbling O_2 gas through the medium. After recirculation, perfused media was centrifuged at 2000 rpm for 10 min, and the supernatant was separated from the pellet. Perfused medium containing 3'-Me-DAB (PF) or DMSO (PA) was prepared freshly before use.

Treatment of ARL-D8 cells with perfused 3'-Me-DAB-containing-medium. ARL-D8 cells, cultured for 13 days, were inoculated at a concentration of 1.2×10^5 cells/ml into 100 mm Falcon plastic dishes containing 10 ml of the basal medium. Perfused 3'-Me-DAB-containing-medium (PF) was added to cultured ARL-D8 cells by medium change 1 day after inoculation, and thereafter the medium was renewed every 2 days. Control cultures were treated with perfused DMSO-containing-medium (PA) in the same way. Following treatment for 6 days, each culture was subcultured with 0.1 % trypsin (Difco, USA) and 0.02 % EDTA (ethylenediaminetetraacetic acid, Dotite, Japan) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline to prepare duplicate cultures. One of the duplicate cultures was treated for 6 more days with the perfused 3'-Me-DAB- or DMSO-containing-medium from day 1 after subculture. The other culture was maintained in the basal medium. Cultures treated with perfused 3'-Me-DAB-containing-medium were designated as 6PF and 12PF, and control cultures were named 6PA and 12PA.

Cytochemical assay of GGT. As reported previously (10), cytochemical assay of GGT in cultured liver cells fixed in cold acetone was carried out according to the method of Albert *et al.* (11). Nuclei of cultured liver cells were stained with hematoxylin. The percentage of GGT-positive cells in each liver cells culture was determined by scanning 1×10^4 cells.

Chromosome preparation. Chromosomes were prepared according to Rothfels and Siminovitch (12), and stained with 3 % Giemsa in Sörensen's phosphate-buffer.

RESULTS

PLC cultures. As reported previously (9), in 3'-Me-DAB-treated cultures, polygonal cells with granular cytoplasm, *i.e.*, mature hepatocytes, decreased rapidly in number due to the cytotoxic effect of the carcinogen, but epithelial-like cells with clear cytoplasm (epithelial-like clear cells) proliferated actively. In control cultures, on the other hand, mature hepatocytes survived well for 7 to 10 days after inoculation, although they gradually degenerated. Epithelial-like clear cells in control cultures appeared later than in carcinogen-treated cultures, and the growth of the cells was relatively slow in control cultures.

A series of 3'-Me-DAB-treated and control cultures were cytochemically tested for GGT activity 31 days after initiation of primary culture (Table 1). Three (6F-18, 21 and 23) of 8 carcinogen-treated cultures contained 0.01, 0.02 and 0.01 % GGT-positive cells, respectively, whereas none of 8 control cultures exhibited GGT-positive cells.

Chromosome analyses were carried out 31 days after initiating primary culture (Tables 1, 2). All 8 carcinogen-treated cultures and 7 of the 8 control cultures were diploid, *i.e.*, more than 80 % of the cell population was diploid. However, marker chromosomes were detected in 4 (6F-17, 18, 20 and 21) of the 8 carcinogen-treated cultures, but in none of the control cultures.

Co-cultures. ARL-D8 cells from adult female rats and PLCs from adult male rats were co-cultured for the assay of transformation by 3'-Me-DAB. ARL-D8 cells were well dispersed and in close contact with PLCs, especially primary mature

TABLE 1. NUMBERS OF MARKER-CHROMOSOME-POSITIVE AND GGT-POSITIVE LIVER CELL CULTURES INDUCED BY 3'-ME-DAB TREATMENT UNDER VARIOUS CONDITIONS

Liver cell culture[a]	3'-Me-DAB treatment period (day)	Culture age[b] (day)	Number of marker-chromosome-positive cultures[c]	Culture age[d] (day)	Number of GGT-positive cultures[c,e]
I. PLC culture:					
6F (8)	6	31	4 (8)	35	3 (8)
6A (8)	0	31	0 (8)	35	0 (8)
II. Co-culture:					
PLC-ARL-D8-6F (5)	6	20[f]	3 (5)	74[f]	2 (5)
PLC-ARL-D8-6A (5)	0	20[f]	0 (5)	74[f]	0 (5)
III. Cell line:					
ARL-D8-6PF (5)	6	17[g]	0 (5)	21[g]	0 (5)
ARL-D8-12PF (5)	12	24[g]	2 (5)	26[g]	2 (5)
ARL-D8-6PA (5)	0	16[g]	0 (5)	21[g]	0 (5)
ARL-D8-12PA (5)	0	24[g]	0 (5)	29[g]	0 (5)

[a]: Numbers in parentheses are the number of cultures initially started. [b]: When used for chromosome preparation. [c]: Numbers in parentheses are the number of cultures tested. [d]: When tested for GGT. [e]: Cultures containing 0.01 to 0.1 % GGT-positive cells. [f]: After initiation of co-culture. [g]: After initiation of 3'-Me-DAB treatment.

TABLE 2. CHROMOSOME DISTRIBUTIONS OF PLC CULTURES TREATED WITH 3'-ME-DAB

Liver cell culture	3'-Me-DAB treatment period (day)	Metaphase number	Modal chromosome number	Number of diploid cells	Number of cells with marker chromosomes
6F-17	6	50	42 (88)	43 (86)	1 (2)
6F-18	6	50	42 (90)	44 (88)	2 (4)
6F-19	6	50	42 (92)	46 (92)	0 (0)
6F-20	6	50	42 (96)	48 (96)	1 (2)
6F-21	6	50	42 (88)	43 (86)	1 (2)
6F-22	6	50	42 (94)	47 (94)	0 (0)
6F-23	6	50	42 (92)	46 (92)	0 (0)
6F-24	6	50	42 (96)	48 (96)	0 (0)
6A-17	0	34	42 (88)	30 (88)	0 (0)
6A-18	0	27	42 (100)	27 (100)	0 (0)
6A-19	0	50	42 (90)	45 (90)	0 (0)
6A-20	0	31	42 (84)	26 (84)	0 (0)
6A-21	0	50	42 (80)	40 (80)	0 (0)
6A-22	0	50	42 (92)	46 (92)	0 (0)
6A-23	0	50	42 (94)	47 (94)	0 (0)
6A-24	0	50	42 (72)	35 (70)	0 (0)

Chromosome analyses were carried out 31 days after initiation of primary culture. Numbers in parentheses show percentages.

TABLE 3. CHROMOSOME DISTRIBUTIONS OF 3'-ME-DAB-TREATED ARL-D8 CELLS CO-CULTIVATED WITH PLCs

Liver cell culture	3'-Me-DAB treatment period (day)	Modal chromosome number	Number of diploid cells	Number of cells with marker chromosomes
PLC-ARL-D8-6F-1	6	42 (94)	47 (94)	0 (0)
PLC-ARL-D8-6F-2	6	42 (86)	43 (86)	0 (0)
PLC-ARL-D8-6F-3	6	42 (90)	44 (88)	1 (2)
PLC-ARL-D8-6F-4	6	42 (88)	42 (84)	3 (6)
PLC-ARL-D8-6F-5	6	42 (88)	44 (88)	1 (2)
PLC-ARL-D8-6A-1	0	42 (94)	47 (94)	0 (0)
PLC-ARL-D8-6A-2	0	42 (96)	48 (96)	0 (0)
PLC-ARL-D8-6A-3	0	42 (88)	44 (88)	0 (0)
PLC-ARL-D8-6A-4	0	42 (92)	46 (92)	0 (0)
PLC-ARL-D8-6A-5	0	42 (84)	42 (84)	0 (0)

The culture age of ARL-D8 cells derived from adult female rats when used for co-cultivation with PLCs was 39 days. Chromosome analyses were carried out 20 days after initiation of co-cultivation. Fifty metaphase-spreads were counted in each chromosome preparation. Numbers in parentheses show percentages.

hepatocytes, 24 h after inoculation. Both in carcinogen-treated and control cultures, epithelial-like clear cells, mainly ARL-D8 cells, proliferated well.

10 cultures (5 carcinogen-treated and 5 control) were tested cytochemically for GGT activity 74 days after initiation of co-culture (Table 1). Two (PLC-ARL-D8-6F-2 and 4) of the 5 carcinogen-treated co-cultures contained 0.02 and 0.08 % GGT-positive cells, respectively, whereas none of the 5 control cultures contained GGT.

Chromosome analyses focusing on ARL-D8 cells were carried out 20 days after initiating co-culture (Tables 1, 3). ARL-D8 cells and PLCs were distinguishable from each other because of their different sexes. The 5 carcinogen-treated and 5 control cultures were diploid. However, 3 (PLC-ARL-D8-6F-3, 4 and 5) of the 5 carcinogen-treated co-cultures contained cells with marker chromosomes at frequencies of 2, 6 and 2 %, respectively, whereas no marker chromosomes were found in control co-cultures.

ARL-D8 cells. ARL-D8 cells (epithelial-like clear cells) were treated for either 6 or 12 days with 3'-Me-DAB- or DMSO-containing-medium which was perfused through adult male rat liver. ARL-D8 cells proliferated well both in carcinogen-treated and control cultures.

In the 6-day-treatment group, 5 carcinogen-treated and 5 control cultures were cytochemically negative for GGT activity (Table 1). In the 12-day-treatment group, 2 (ARL-D8-12PF-4 and 5) of 5 carcinogen-treated cultures exhibited 0.01 % GGT-positive cells, whereas none of the 5 control cultures were positive for GGT (Table 1).

TABLE 4. CHROMOSOME DISTRIBUTIONS OF ARL-D8 CELLS TREATED WITH 3'-ME-DAB-CONTAINING-MEDIUM PERFUSED THROUGH THE LIVER OF ADULT MALE RATS

Liver cell culture	Days in culture[a]	3'-Me-DAB treatment period (day)	Modal chromosome number	Number of diploid cells	Number of cells with marker chromosomes
ARL-D8-6PF-1	17	6	42 (84)	42 (84)	0 (0)
ARL-D8-6PF-2	17	6	42 (98)	49 (98)	0 (0)
ARL-D8-6PF-3	17	6	42 (86)	43 (86)	0 (0)
ARL-D8-6PF-4	17	6	42 (80)	40 (80)	0 (0)
ARL-D8-6PF-5	17	6	42 (88)	44 (88)	0 (0)
ARL-D8-12PF-1	24	12	42 (88)	44 (88)	0 (0)
ARL-D8-12PF-2	24	12	42 (80)	40 (80)	1 (2)
ARL-D8-12PF-3	24	12	42 (88)	42 (84)	1 (2)
ARL-D8-12PF-4	24	12	42 (94)	47 (94)	0 (0)
ARL-D8-12PF-5	24	12	42 (82)	41 (82)	0 (0)
ARL-D8-6PA-1	16	0	42 (92)	46 (92)	0 (0)
ARL-D8-6PA-2	16	0	42 (98)	49 (98)	0 (0)
ARL-D8-6PA-3	16	0	42 (90)	45 (90)	0 (0)
ARL-D8-6PA-4	16	0	42 (92)	46 (92)	0 (0)
ARL-D8-6PA-5	16	0	42 (94)	47 (94)	0 (0)
ARL-D8-12PA-1	24	0	42 (90)	45 (90)	0 (0)
ARL-D8-12PA-2	24	0	42 (96)	48 (96)	0 (0)
ARL-D8-12PA-3	24	0	42 (90)	45 (90)	0 (0)
ARL-D8-12PA-4	24	0	42 (90)	45 (90)	0 (0)
ARL-D8-12PA-5	24	0	42 (84)	42 (84)	0 (0)

The Culture age of ARL-D8 cells when used for this experiment was 13 days. Fifty metaphase-spreads were counted in each chromosome preparation. Numbers in parentheses show percentages.
[a]: After the initiation of 3'-Me-DAB treatment.

Chromosome analyses were carried out 16 and 24 days after initiating carcinogen treatment (Tables 1, 4). In the 6-day-treatment group, 5 carcinogen-treated and 5 control cultures were diploid and contained no cells with marker chromosomes. In the 12-day-treatment group, the 5 carcinogen-treated and 5 control cultures were also diploid, but marker chromosomes were detected in 2 (ARL-D8-12PF-2 and 3) of the 5 carcinogen-treated cultures. No marker chromosomes were detected in control cultures.

DISCUSSION

Since the indirect carcinogen, 3'-Me-DAB, induces unscheduled DNA synthesis in primary-cultured mature hepatocytes, it is obvious that 3'-Me-DAB is a genotoxic carcinogen which damages cellular DNA (13). In the primary culture of adult rat liver cells, 3'-Me-DAB may also damage cellular DNA of proliferating epithelial-

like clear cells. We believe it possible that epithelial-like clear cells are transformed by an active metabolite of 3'-Me-DAB produced in primary cultured mature hepatocytes. In the present experiments, chromosomal abnormalities were detected in proliferating epithelial-like clear cells in 4 of 8 carcinogen-treated PLC cultures, in 3 of 5 carcinogen-treated co-cultures and in 2 of 5 carcinogen-treated ARL-D8 cell cultures. These chromosomal abnormalities can be considered to be due to the effect of the carcinogen, since such chromosomal abnormalities were not detected in control cultures.

Although chromosomal abnormalities were detected in 3'-Me-DAB-treated PLC cultures, the percentage of cells with chromosomal abnormality in each culture was very low, only 2 to 4 %. That there are few epithelial-like clear cells in the starting materials for primary culture may be the reason for the low incidence of chromosomal abnormality (7). PLCs were co-cultured with ARL-D8 cells in the present study to make the starting materials rich in epithelial-like clear cells. ARL-D8 cells were well dispersed and in close contact with PLCs, especially mature hepatocytes. As a result, the percentage of cultures showing chromosomal abnormalities became slightly higher in carcinogen-treated co-cultures (60 %) than in carcinogen-treated PLC cultures (50 %). However, the percentage of cells with chromosomal abnormality in individual culture hardly changed.

Although pure cultures of ARL-D8 cells are richest in target cells among the 3 culture systems tested in this study, ARL-D8 cells are poor in liver-specific functions (8). Therefore, ARL-D8 cells may not be able to activate 3'-Me-DAB. To obtain an active metabolite of 3'-Me-DAB, carcinogen-containing-medium was perfused through adult male rat liver. Twelve-day treatment of ARL-D8 cells with perfused 3'-Me-DAB-containing-medium caused chromosomal abnormalities, but 6-day treatment did not. The efficiency of perfused 3'-Me-DAB-containing-medium in transforming epithelial-like clear cells was very low. Therefore, the active metabolite of 3'-Me-DAB may be unstable even if it is present in perfused medium. Furthermore, cell-to-cell contact may be necessary for cross-feeding of an active metabolite of 3'-Me-DAB.

GGT is an important marker of preneoplastic and neoplastic changes of liver cells, although not all preneoplastic and neoplastic lesions are positive for this enzyme (14, 15). In this study, GGT activity was detected cytochemically in proliferating epithelial-like clear cells in 3'-Me-DAB-treated cultures but not in control cultures. Therefore, the acquisition of GGT activity seems to be mainly associated with exposure to 3'-Me-DAB. The percentage of GGT-positive cultures was slightly higher in carcinogen-treated co-cultures (40 %) than in carcinogen-treated PLC cultures (37.5 %). However, GGT activity was not always detected in cultures showing chromosomal abnormalities.

The transformation markers increased in ARL-D8 cells when they were co-cultured with PLCs. Therefore, co-culture of ARL-D8 cells with PLCs as well

as PLC culture are suitable for studying the transformation of liver cells by 3'-Me-DAB *in vitro*.

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