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Note

Detection of the changes in serum proteins during chemical hepatocarcinogenesis by two-dimensional electrophoresis under non-denaturing conditions

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Administration of chemical carcinogens such as aminoazo dyes or 2-acetylaminofluorene to rats for about twelve weeks induces hyperplastic nodules and later hepatocellular carcinomas in the livers [1–6]. These livers have been extensively investigated as a model system to understand the mechanism of carcinogenesis and many biological changes; metabolism [7–9], enzymes [7–12], glycolipids [13,14], "specific proteins" such as α-fetoprotein or antigens [15–17] and cellular polypeptides [18,19] have been demonstrated. However, the changes in serum proteins after administration of chemical carcinogens have remained unknown because of the insufficient resolution of the analytical techniques used.

Manabe et al. [20] described a two-dimensional electrophoretic technique under non-denaturing conditions. As this technique does not employ denaturing agents such as sodium dodecyl sulphate (SDS) or urea during the run, it is possible to detect the proteins based on their biological activities [21,22]. With this electrophoretic technique, changes in serum proteins after experimental diseases and partial hepatectomy have been demonstrated [23–25]. In this paper, we describe studies of the changes in rat serum proteins after chronic administration of a chemical hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), using this two-dimensional electrophoretic technique.

EXPERIMENTAL

Reagents

Ampholines (pH range 3.5–10 and 4–6.5) were obtained from LKB (Bromma, Sweden). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′N′-tetramethyl-

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ethylenediamine (all of special grade for electrophoresis), Tris, glycine, sucrose, hydrochloric acid and ammonium persulphate (all of analytical-reagent grade) were purchased from Wako (Osaka, Japan). Coomassie Brilliant Blue R-250 was obtained from Sigma (St. Louis, MO, U.S.A.) and 3′-methyl-4-dimethylaminoazobenzene from Tokyo Kasei Kogyo (Tokyo, Japan). Peroxidase-conjugated goat anti-horse immunoglobulin G (IgG) and anti-rabbit IgG were purchased from Zymed Labs. (San Francisco, CA, U.S.A.).

Treatment of animals and serum samples

Male rats (Donryu strain) weighing approximately 130 g were used for all experiments. Rats were maintained for 0–4 months on an Oriental MF diet (Oriental Yeast, Tokyo, Japan) containing 0.06% of 3′-Me-DAB and water ad libitum. Control rats were maintained on the same diet without 3′-Me-DAB under the same conditions. At months 0, 1, 2, 3 and 4 after 3′-Me-DAB administration, blood was taken from the descending aorta of each rat under light ether anaesthesia. The blood was left to stand at 4 °C for 2 h, then centrifuged at 3000 *g* for 10 min. Sucrose was added to the serum to give a concentration of 10%, then the serum samples were stored at −20 °C until used.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed as described previously [24] with some modifications. Isoelectric focusing in the first dimension was performed on 4% cylindrical polyacrylamide gel (0.2% bisacrylamide, 14.5×0.5 cm) containing 2% Ampholine (mixture of pH range 3.5–10 and 4–6.5 in the ratio 9:1). The cathode electrode solution was 0.1 *M* sodium hydroxide and the anode solution was 0.01 *M* phosphoric acid. Electrophoresis was run at a constant current of 2 mA per gel for about 40 min (until the voltage reached 460 V), then at a constant voltage of 460 V for 20 h at 4 °C.

Polyacrylamide gel electrophoresis (PAGE) in the second dimension was performed on a 4–21% linear gradient (containing a 0–10% sucrose linear gradient and 0.2% bisacrylamide) slab gel of 14 cm long, 16 cm wide and 0.4 cm thick. Electrophoresis was run at a constant current of 36 mA at 4 °C in 0.05 *M* Tris–0.38 *M* glycine buffer (pH 8.3).

Staining and destaining

Gels were stained overnight in 0.05% Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (50:7:43, v/v/v). Destaining was carried out in methanol–acetic acid–water (30:7:63, v/v/v).

Determination of pH gradient and molecular mass

The pH gradient was determined as follows. The first-dimension isoelectric focusing gel was duplicated for each sample. One gel was cut into 10-mm sections and these sections were placed in individual vials containing 2 ml of distilled water. These vials were allowed to stand for 2 h at room temperature, then the pH was measured on a pH meter. The molecular mass under non-denaturing conditions was calculated using a molecular mass calibration graph [20]. The

molecular mass under denaturing conditions was determined with SDS-PAGE using cytochrome C (12 400), carbonic anhydrase (30 000), ovalbumin (45 000), bovine serum albumin (68 000), phospholylase a (94 000) and rabbit muscle myosin (220 000) as standard proteins.

Electroblotting and immunochemical staining

After two-dimensional electrophoresis, the gradient slab gel was swollen in 0.7% acetic acid for 6 h at 4°C, then placed with a nitrocellulose membrane in an electroblotting apparatus (Toyo Kagaku Sangyo, Tokyo, Japan). Electroblotting was performed at a constant voltage of 8 V/cm in 0.7% acetic acid for 1 h. The nitrocellulose membrane was soaked in phosphate-buffered saline (PBS) (pH 7.2) containing 3% bovine serum albumin (BSA) overnight at room temperature. Horse antiserum against rat α-fetoprotein (or other primary antiserum, 1:1000 dilution) was added to the PBS–BSA solution and kept at 37°C for 30 min. After thorough washing with PBS containing 0.05% Tween 20, the nitrocellulose membrane was again soaked in PBS–BSA solution, peroxidase-conjugated anti-horse IgG (or other secondary peroxidase-conjugated anti-IgG, 1:1000 dilution) was added, kept at 37°C for 30 min and then thoroughly washed with PBS containing 0.05% Tween 20. Staining was carried out in 50 m*M* Tris–HCl buffer (pH 7.6) containing 0.025% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide for 10 min.

RESULTS

Before examination of serum proteins after 3′-Me-DAB administration, we determined them in the serum of eight Donryu rats (130 g body weight) by two-dimensional electrophoresis, which confirmed that there was no significant difference in the two-dimensional electrophoretic patterns among the rats. An example of the two-dimensional electrophoretic pattern of normal rat serum is shown in Fig. 1a. Major serum proteins were located on the gel by comparing the patterns with those of human plasma proteins [26]; some of these are shown in Fig. 1a.

The changes in serum proteins after 3′-Me-DAB administration were then examined. Fig. 1b shows a two-dimensional electrophoretic pattern of serum proteins obtained at month 3 after 3′-Me-DAB administration. Considerable changes were observed for more than ten proteins; these are shown by arrows in Fig. 1b (proteins in area C are shown in Fig. 2C) and some of them are named P1–P6 for convenience. These changes in the two-dimensional electrophoretic patterns were not observed with those of control rats. The increase in IgG was considered to be an age-related change, as a similar increase was observed in control rats. The p*I* values and the molecular masses of P1–P6 were calculated as follows (expressed as p*I*/MW): 4.9/900 000 for P1, 4.3/80 000 for P2, 4.4/79 000 for P3, 4.6/80 000 for P4, 4.8/70 000 for P5 and 4.9/71 000 for P6.

Time-dependent changes in the two-dimensional electrophoretic patterns of serum proteins after 3′-Me-DAB administration were examined. Serum samples obtained at months 1, 2, 3 and 4 after 3′-Me-DAB administration were analysed

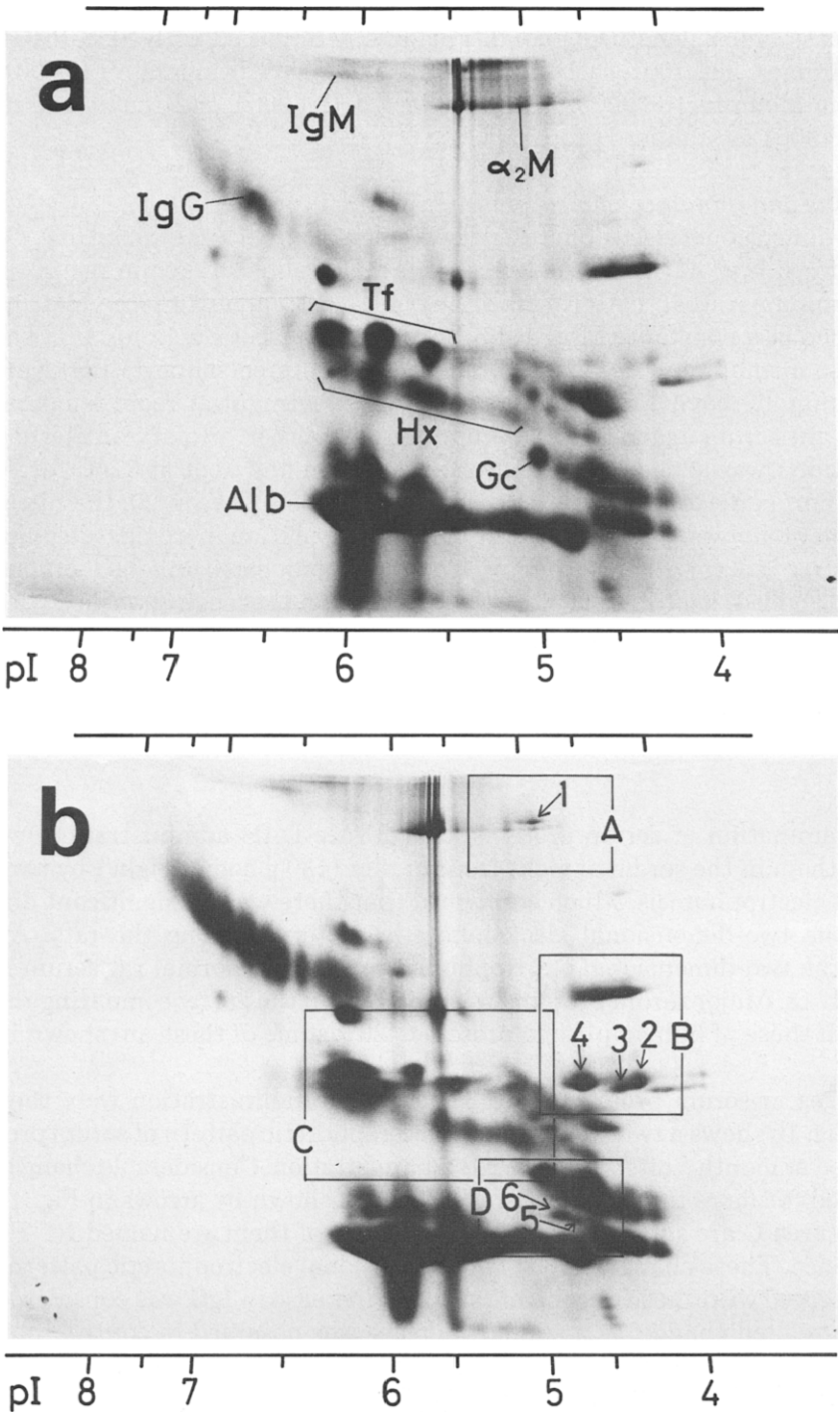

Fig. 1. Two-dimensional electrophoretic pattern of rat serum proteins. (a) Before experiment, (b) three months after 3′-Me-DAB administration. The positions of major serum proteins were tentatively located on the gel by comparing the two-dimensional pattern of rat serum proteins with those of human plasma proteins [26]. Abbreviations: IgM = immunoglobulin M; IgG = immunoglobulin G; α_2M = α_2-macroglobulin; Tf = transferrin; Hx = hemopexin; Gc = Gc-globulin; Alb = albumin.

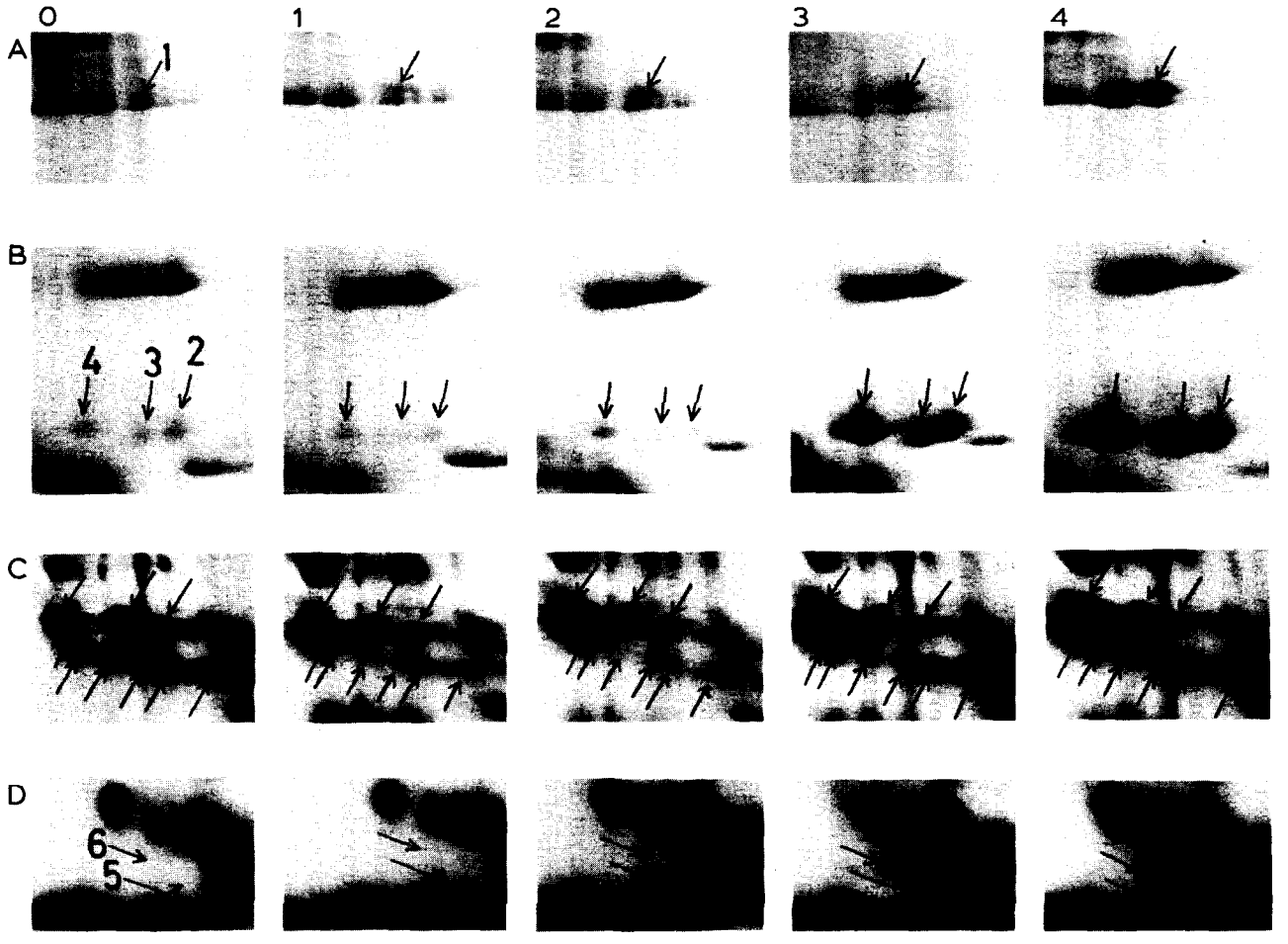

Fig. 2. Time courses of the changes in the two-dimensional electrophoretic pattern of rat serum proteins after 3′-Me-DAB administration. Serum samples taken 0, 1, 2, 3, and 4 months after 3′-Me-DAB administration were analysed by two-dimensional electrophoresis. Time-dependent changes on the gel sections in areas A, B, C and D (indicated in Fig. 1b) are shown. Rapidly increasing or decreasing proteins after 3′-Me-DAB administration are shown by arrows.

and the protein distributions were compared with those of the normal pattern. In Fig. 2, the time courses of the markedly changed proteins on the two-dimensional slab gel are shown. Fig. 2A is the time course of P1. This protein occurred slightly in normal rat serum, gradually increased during months 1–2 after 3′-Me-DAB administration and then increased dramatically during months 3–4. Fig. 2B shows the time courses of P2, P3 and P4. These proteins were also found in normal rat serum. However, their amounts decreased during months 1–2 after 3′-Me-DAB administration (P2 and P3 almost disappeared), and then increased dramatically during months 3–4. Fig. 2C shows the time courses of transferrin and hemopexin. These two proteins were tentatively identified by comparing the two-dimensional patterns with those of human plasma proteins [24]. The upper arrows show transferrin and the lower arrows hemopexin. Transferrin was observed as some spots over a wide p*I* range, but after 3′-Me-DAB administration the distribution of its basic side increased dramatically and that of its acidic side decreased. Hemopexin was also observed as several spots over a wide p*I* range, but the distribution of its most basic side gradually increased during months 1–2 and then increased dramatically during months 3–4. In contrast, the other distribution of hemopexin decreased during months 1–2 and then recovered to the original level during months 3–4. Fig. 2D shows the time courses of P5 and P6. These proteins could not be detected in serum during 0–2 months after 3′-Me-DAB administration, but a dramatic increase was observed during months 3–4.

It is known that the amounts of α-fetoprotein and α_2-acute phase macroglobulin in rat serum increase rapidly in serum after administration of carcinogens [16,27]. Therefore, we examined their locations on the two-dimensional gel by the immunochemical procedure as described under Experimental. Horse antiserum against rat α-fetoprotein was kindly provided by Dr. Nam-ho Choi, School of Pharmaceutical Sciences, Showa University. Rabbit antiserum against rat α_2-acute phase macroglobulin was prepared in our laboratory [28]. P1 was identified to be α_2-acute phase macroglobulin and both P5 and P6 were identified to be α-fetoprotein.

P2, P3 and P4 were further characterized by SDS-PAGE. Stained proteins on the two-dimensional gel were individually punched out, homogenized with 0.1 *M* sodium hydroxide solution containing 2% β-thiodiglycol and then centrifuged. The supernatant was dialysed against distilled water, lyophilised and then analysed by SDS-PAGE in the presence of 2-mercaptoethanol. The results indicated that these proteins each formed a single band at a position corresponding to a molecular mass of approximately 73 000.

DISCUSSION

In order to define the mechanism of carcinogenesis, biological and morphological changes after administration of chemical carcinogens have been extensively investigated [7–19]. Few studies have been made of the changes in serum proteins, however, because of the insufficient resolution of the analytical techniques available. As shown in Figs. 1 and 2, the two-dimensional electrophoretic technique could clearly detect changes in more than ten proteins after 3′-Me-

DAB administration. As our two-dimensional electrophoretic technique does not employ denaturing agents such as SDS or urea during the run, it is possible to determine both the p*I* and molecular mass of proteins under native conditions. Further, as our technique does not require equilibration of the first-dimension gel, there is no loss of the proteins during the course of the run, which makes it possible to measure accurately the time-dependent changes in the amounts of proteins on two-dimensional gels.

The changes of serum proteins after 3′-Me-DAB administration can be divided into four types: type I includes proteins that newly appeared or increased dramatically in amount during months 3–4 after 3′-Me-DAB administration (e.g., P1, P5, P6 and transferrin of the basic side), type II includes those which decreased in amount during months 1–2 and then increased dramatically during months 3–4 (e.g. P2, P3 and P4), type III includes those which decreased in amount during months 1–2 and then gradually recovered to their original levels (e.g., hemopexin in area C) and type IV includes those which did not change. We suggest that the proteins of types I, II and III (especially types I and II) have a close relationship with hepatocarcinogenesis after 3′-Me-DAB administration. The quantification of these proteins may be useful in determining the degree of hepatocarcinogenesis.

Both P5 and P6 were identified to be α-fetoprotein by two-dimensional electrophoresis followed by the immunochemical procedure. As for the heterogeneity of rat α-fetoprotein, similar observations were made by both disc electrophoresis and isoelectric focusing [29]. Recently, Hirai and Taga [30] demonstrated further heterogeneity of rat α-fetoprotein using lectin crossed immuno-affino-electrophoresis.

P2, P3 and P4 were proteins that increased dramatically during months 3–4 after 3′-Me-DAB administration. The molecular masses of these proteins were calculated to be 80 000 for P2, 79 000 for P3 and 80 000 for P4 on two-dimensional gels under non-denaturing conditions. As these proteins showed almost the same molecular masses (approximately 73 000) on SDS-PAGE in the presence of 2-mercaptoethanol, these proteins are considered to be in the monomeric state.

Similar changes in P2, P3 and P4 could also be observed in AH-66 or AH-130 tumour-bearing rat sera. Therefore, we suggest that these proteins have a close relationship with carcinomas. However, the function and role of these proteins are still unclear. The relationship between these proteins and carcinogenesis is under investigation in our laboratory.

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