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DETECTION OF THE CHANGES IN CELLULAR PROTEINS IN REGENERATING RAT LIVER BY HIGH-RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS

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SUMMARY

The changes in cellular proteins in regenerating rat liver after partial hepatectomy were examined by high-resolution two-dimensional electrophoresis. The cellular proteins in regenerating rat livers were separated into two fractions (soluble and insoluble protein fractions) and the proteins in each fraction were analysed by means of two-dimensional electrophoresis. A rapid increase in three proteins and a rapid decrease in two proteins were detected after partial hepatectomy. The changes in these proteins were in parallel with the regeneration rate of liver, suggesting a close relationship with the proliferation of liver after partial hepatectomy.

INTRODUCTION

Since the first report of Higgins and Anderson [1] on the quantitative assessment of liver regeneration after partial hepatectomy, many studies on the regenerating liver have been reported. However, what controls the proliferation after partial hepatectomy and how the rapid regenerative growth is stimulated are not yet fully clear. Many investigators have suggested that the proliferation of regenerating liver after partial hepatectomy is controlled by a humoral and a specific factor(s), but such factors have not been identified. On the other hand, it has been also considered that metabolic changes after partial hepatectomy contribute to the stimulation of such factors. Based on this question, the changes in various metabolites after partial hepatectomy have been investigated [2—6]. Until recently, however, the changes in proteins after partial hepatectomy have remained unknown because of the insufficient resolution of the analytical techniques used.

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In previous papers [7—9], we reported that two-dimensional electrophoresis could be used to detect the changes in serum proteins assumed to be closely related to the proliferation of liver after partial hepatectomy. In this paper, we describe studies of the changes in cellular proteins in regenerating rat liver after partial hepatectomy using two-dimensional electrophoresis.

EXPERIMENTAL

Reagents

Ampholines (pH range 3.5—10 and 5–8) were obtained from LKB (Bromma, Sweden), urea (ultra-pure grade) from Schwarz/Mann (New York, NY, U.S.A.), Nonidet P-40 from Iwai Kagaku Yakuhin (Tokyo, Japan), Agar Noble from Difco (Detroit, MI, U.S.A.) and Coomassie Brilliant Blue R-250 from Sigma (St. Louis, MO, U.S.A.). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (all of special grade for electrophoresis), sodium dodecyl sulphate, 2-mercaptoethanol (both of biochemical grade), Tris base, glycine, hydrochloric acid and ammonium persulphate (all of analytical-reagent grade) were purchased from Wako (Osaka, Japan).

Animals and partial hepatectomy

Male rats (Wistar strain) weighing approximately 150 g were used in all experiments. Partial hepatectomy was performed under light ether anaesthesia according to the method of Higgins and Anderson [1]. After partial hepatectomy, the rats were maintained for 1—17 days with food and water ad libitum. Sham operation was carried out in an identical manner to partial hepatectomy.

Preparation of cellular proteins

Under light ether anaesthesia, livers were perfused thoroughly with 0.9% sodium chloride, removed, homogenized with an equal volume of homogenization buffer (10 m*M* Tris—HCl buffer containing 5 m*M* magnesium chloride, pH 7.4) and then centrifuged at 105 000 *g* for 1 h at 0°C. The supernatants obtained were diluted with homogenization buffer to give a protein concentration of 50 mg/ml, designated the "soluble protein fraction". The precipitates were washed twice by rehomogenizing with ten volumes of homogenization buffer and centrifuging at 105 000 *g* for 1 h at 0°C. The final precipitates were suspended in homogenization buffer to give a protein concentration of 50 mg/ml, designed the "insoluble protein fraction". Each protein sample was placed in an individual vial and an equal volume of sample buffer (0.1 *M* Tris—HCl buffer containing 6.5% SDS and 10% 2-mercaptoethanol, pH 7.4) was added. Under these condition, the SDS:protein ratio is 1.3. These vials were capped and kept in a boiling water-bath for 3 min. After cooling, Ampholine and urea were added to the samples to give concentrations of 2% and 9 *M*, respectively. These samples were centrifuged at 3000 *g* for 10 min and the supernatants (500 μg of the protein) were applied to two-dimensional electrophoresis.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out according to the modified

method of O'Farrell [10]. Isoelectric focusing in the first dimension was performed on a 4% polyacrylamide gel (14.5 × 0.2 cm) containing 2% Ampholine, 2% Nonidet P-40 and 9 *M* urea. The Ampholine used was a mixture of pH ranges 3.5—10 and 5—8 in the ratio 4:1. The cathode solution was 0.1 *M* sodium hydroxide and the anode solution 0.01 *M* phosphoric acid. Electrophoresis was run at 200 V for 30 min, 300 V for 30 min, 400 V for 13 h and then 800 V for 1 h at room temperature.

Polyacrylamide gel electrophoresis in the second dimension was performed on a 10—20% linear gradient slab gel of 14 cm long, 16 cm wide and 0.2 cm thick. The acrylamide solutions were prepared as follows: for high concentration, 20% acrylamide (0.5% N,N'-methylenebisacrylamide), 0.1% SDS, 0.07% TEMED and 0.05% ammonium persulphate in 0.375 *M* Tris—HCl buffer (pH 8.8); for low concentration, 10% acrylamide (0.25% N,N'-methylenebisacrylamide), others as for high concentration. The gradient gel was prepared at 3 ml/min and polymerized for 2 h in a water-bath at 33°C. After the polymerization, the first-dimension gel was placed on the slab gel without equilibration with 1% agar—0.125 *M* Tris—HCl buffer (pH 6.8, 60°C) containing 0.1% SDS and 5% 2-mercaptoethanol. Electrophoresis was run at a constant current of 20 mA for 6—7 h in 0.025 *M* Tris—0.192 *M* glycine buffer (pH 8.4) containing 0.1% SDS until the bromophenol blue dye reached the bottom of the gel.

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). Destaining was carried out in 7% acetic acid at 80°C for 15 min and then in methanol—acetic acid—water (30:7:63, v/v) at room temperature.

Determination of pH gradient and molecular weight

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 the sections were placed in individual vials containing 1 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 weight was determined 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.

RESULTS

Before the determination of cellular proteins in regenerating rat liver, we determined them in the livers of eight Wistar rats (150 g body weight) by two-dimensional electrophoresis. After separation into two fractions (soluble and insoluble protein fractions), two-dimensional electrophoresis was carried out, which confirmed that there was no significant difference in the two-dimensional electrophoretic patterns among the rats. Examples of the two-dimensional electrophoretic patterns of soluble and insoluble proteins in normal rat liver are shown in Fig. 1a and b, respectively.

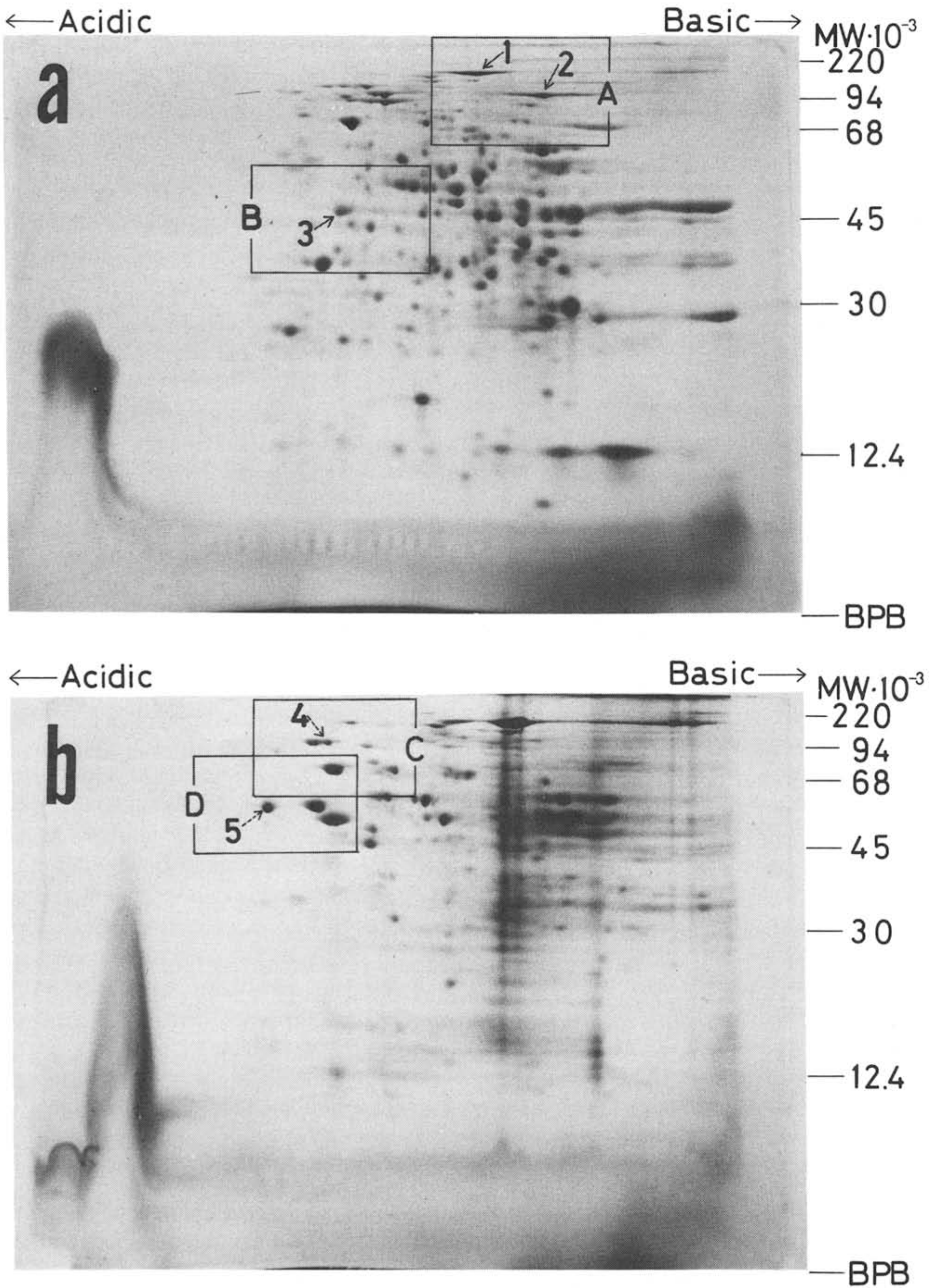

Fig. 1. Two-dimensional electrophoretic patterns of cellular proteins in normal rat liver. Cellular proteins were separated into two fractions (soluble and insoluble protein fractions), treated as described under Experimental and subjected to two-dimensional electrophoresis. Solid arrows show the increased proteins after partial hepatectomy and dotted arrows show the decreased proteins. (a) Soluble protein (500 μg); (b) insoluble protein (500 μg).

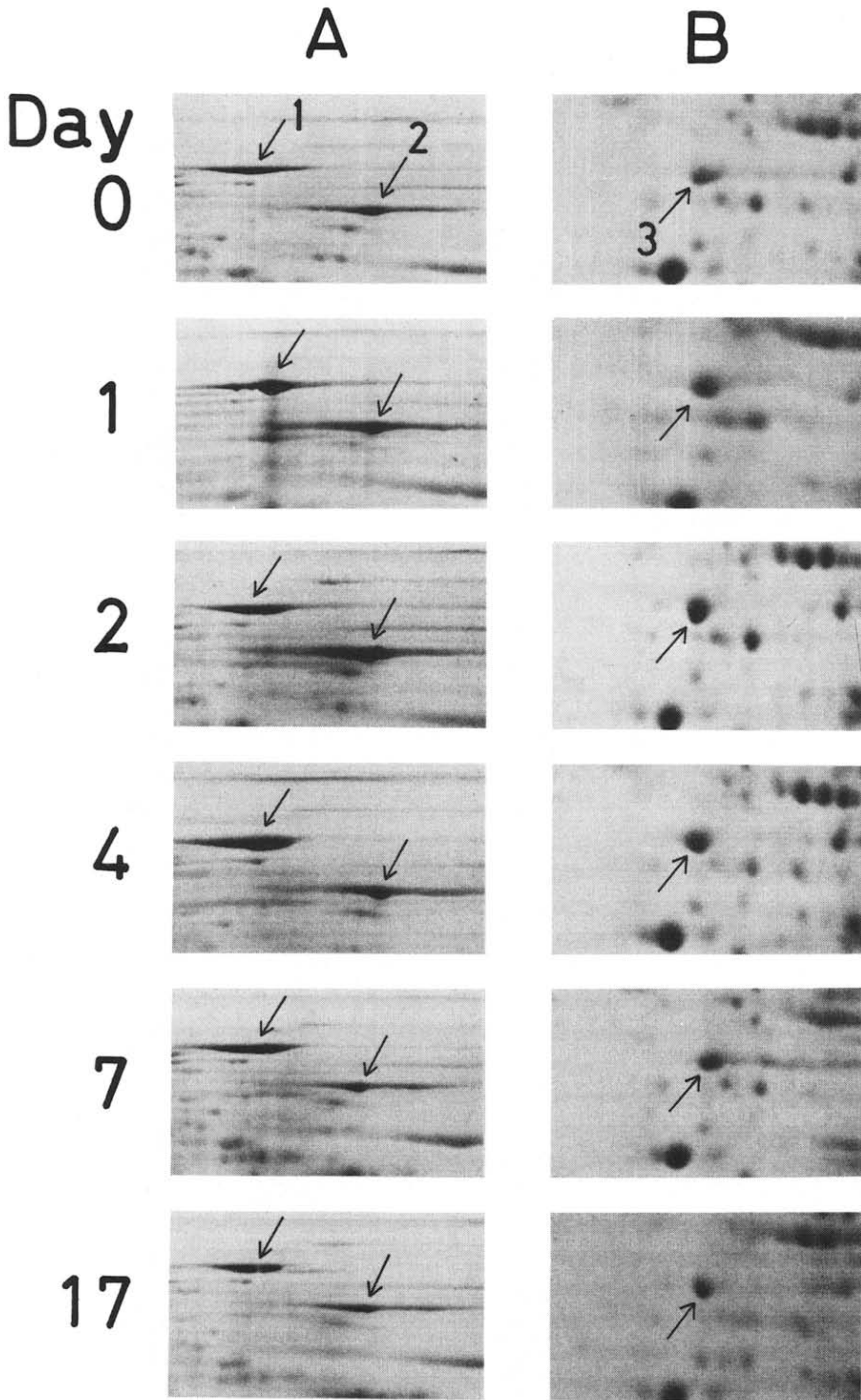

Fig. 2. Time courses of the changes in increased cellular proteins after partial hepatectomy in the two-dimensional electrophoretic pattern. Rat livers taken on days 0, 1, 2, 4, 7 and 17 after partial hepatectomy were treated as described under Experimental and 500 μg of the protein samples were subjected to two-dimensional electrophoresis. Time-dependent changes on the gel sections in areas A and B (indicated in Fig. 1a) are shown. Rapidly increasing proteins after partial hepatectomy are shown by arrows.

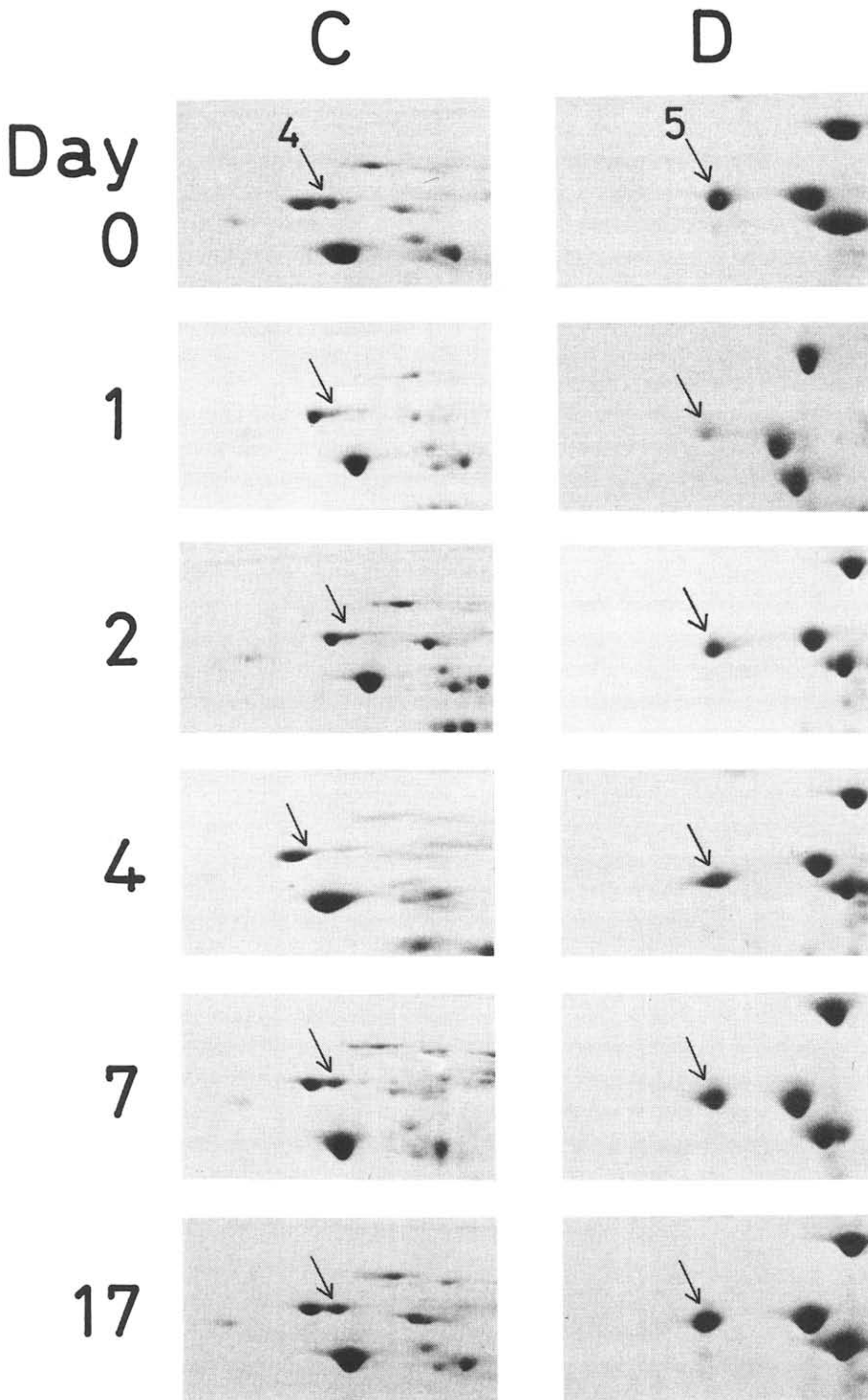

Fig. 3. Time courses of the changes in decreased cellular proteins after partial hepatectomy in the two-dimensional electrophoretic pattern. Rat livers taken on days 0, 1, 2, 4, 7 and 17 after partial hepatectomy were treated as described under Experimental and 500 μg of the protein samples were subjected to two-dimensional electrophoresis. Time-dependent changes on the gel sections in areas C and D (indicated in Fig. 1b) are shown. Rapidly decreasing proteins after partial hepatectomy are indicated by arrows.

The changes in cellular proteins in regenerating rat livers were then examined. Soluble and insoluble proteins in regenerating rat livers obtained on days 1, 2, 3, 4, 7, 10 and 17 after partial hepatectomy were prepared in the same manner as normal liver, subjected to two-dimensional electrophoresis, and the protein distributions were compared with those of the normal patterns. A rapid increase in three proteins was observed in the soluble fraction (the positions of these proteins in the two-dimensional electrophoretic pattern are shown by solid arrows in Fig. 1a and these are named proteins 1, 2 and 3 for convenience) and a rapid decrease in two proteins was observed in the insoluble fraction (the positions of these proteins are shown by dotted arrows in Fig. 1b and these are named proteins 4 and 5). Under the experimental conditions used, these proteins may deviate from the native p*I* values because of SDS—protein complex formation, but the p*I* values and the molecular weights were calculated approximately estimated as follows (expressed as p*I*/MW); 6.3/140 000 for protein 1, 6.6/90 000 for protein 2, 5.3/45 000 for protein 3, 5.0/100 000 for protein 4 and 4.5/53 000 for protein 5.

Figs. 2 and 3 show the time courses of the rapid increase and decrease in proteins, after partial hepatectomy, on polyacrylamide slab gels. The positions and areas of the two-dimensional electrophoretic patterns are shown in Fig. 1.

Fig. 2 shows the time courses of the rapidly increasing proteins. Protein 1 increased rapidly after partial hepatectomy, was at a maximum on day 4, then gradually decreased and returned almost to its original level on day 17. Proteins 2 and 3 also increased rapidly after partial hepatectomy, but the maximal changes were observed on day 2. Fig. 3 shows the time courses of the rapidly decreasing proteins. Proteins 4 and 5 decreased rapidly after partial hepatectomy, were at a minimum on day 1, gradually increased and recovered almost to their original levels on day 17.

DISCUSSION

To investigate liver regeneration, the changes in various metabolites after partial hepatectomy have been studied [2—6]. However, the changes in proteins after partial hepatectomy are not fully known because of the poor analytical techniques used. Until recently, changes in proteins after partial hepatectomy had been studied by one-dimensional electrophoretic techniques such as cellulose acetate membrane electrophoresis [4] and polyacrylamide gel electrophoresis [6]. However, these one-dimensional electrophoretic techniques could provide little information on the changes in proteins supposed to be closely related to the proliferation of liver cells after partial hepatectomy. As shown in Figs. 1—3, the two-dimensional electrophoretic technique described in this paper could clearly detect the changes in several cellular proteins in regenerating rat liver. As our two-dimensional electrophoretic technique does not require equilibration of the first-dimension gel, there is no loss of the proteins, which makes it possible to measure accurately the time-dependent changes in the amounts of proteins on polyacrylamide slab gels.

It is also possible to assume that the changes in cellular proteins described above are due to operation wounds. Therefore, we determined the cellular

proteins in sham-operated rat liver in the same manner and compared the protein distributions with those after partial hepatectomy. However, no changes were observed.

As shown in Figs. 2 and 3, rapid changes after partial hepatectomy were observed within the first 4 days, and these changes were in parallel with the regeneration rate [11, 12] of liver cells. Therefore, we conclude that these changes contribute to the proliferation of liver cells after partial hepatectomy. However, the function and role of these proteins are still unclear. The relationship between these cellular proteins and the regeneration of liver cells is under investigation in our laboratory.

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