Identification and Purification of a Normal Rat Liver Plasma Membrane Surface Protein which Disappears after Chemical Carcinogenesis

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Abstract: The electrophoretic patterns of plasma membrane surface proteins of normal rat liver cells and rat hepatomas were compared in 10% non-denaturing and 7~15% gradient non-denaturing gel. Chemical carcinogens, 2-Me DAB (2-methyl-4-dimethylaminoazobenzene) and DENA (diethylnitrosamine), were used to induce hepatoma in rats. One protein which disappeared in hepatoma was identified in normal rat liver by non-denaturing gel electrophoresis. Rabbit antisera were raised against this specific protein, and the protein was purified by Sephacryl S-200 column and immunoaffinity chromatography using the purified antibody. The purified protein showed two bands of molecular weights approximately 50 kD_a and 52 kD_a by SDS-polyacrylamide gel electrophoresis, which reacted specifically with the antibody. However only one band was observed in non-denaturing gel and also in isoelectric focusing with a pI value of 6.6. This study showed the existence of an unique protein on the plasma membrane surface of normal rat liver cells which disappeared in rat hepatomas induced by chemical carcinogens.

Key words: chemical carcinogens, disappearing protein, hepatoma.

Chemical carcinogens disrupt hepatocyte physiology in a way that may contribute to carcinogenesis. Chemical carcinogens induce cell injury and proliferation in the pathogenesis of hepatocellular carcinoma. Almost all chemicals implicated as carcinogens can cause cellular mutations resulting in changes of components and structures of cell surface or inside the cell.

Particular attention has been focused on tumor specific antigens that appear on the cell surface during malignant transformation. Baldwin *et al.* (1971, 1973) reported on a tumor specific antigen (MW 55 kD_a) from the plasma membrane of 4-dimethylaminoazobenzene-induced rat hepatoma. Meltzer *et al.* (1971) reported tumor specific antigens (MW 75 kD_a and 150 kD_a) extracted with 3 M KCl from guinea pig hepatoma tissues induced by DENA. The studies on human specific antigens on the plasma membrane surface have been mainly performed with adenosarcoma cell lines and monoclonal antibodies (Metzgar *et al.*, 1984; Varki *et al.*, 1984; Starling and Wright, 1985).

Lee *et al.* (1988) investigated tumor specific antigens on the plasma membrane surface of rat hepatoma induced by 2-Me DAB and DENA and showed the presence of proteins (MW 65 kD_a and 55 kD_a) on the human liver cell cross-reacting with the antibody against rat hepatoma associated antigens.

This study shows the identification and purification of a new protein that was present only in normal rat liver cells and disappeared from rat hepatoma induced by the chemical carcinogens, 2-MeDAB and DENA.

Materials and Methods

Materials

Rats used for the study were male Sprague-Dawleys obtained from the animal breeding facility of the Bio-Potency Evaluation program, Korea Research Institute of Bioscience and Biotechnology, and the rabbits used were New Zealand white rabbits. 2-Me DAB was from Janssen (Belgium) and DENA from Sigma (St. Louis, USA). Electrophoresis chemicals were purchased from Bio-Rad (Richmond, USA) and Sigma CNBr-activated Sepharose 4B was purchased from Pharmacia-LKB. All chemicals were of the highest purity commercially available.

Induction of rat hepatoma

Hepatocellular carcinoma was induced in male Sprague-Dawley rats weighing 80~100 g by the method

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of Lee *et al.* (1988).

Extraction of plasma membrane surface proteins

Extraction of surface proteins from the plasma membrane of normal rat liver and rat hepatoma was carried out using 3 M KCl according to the modified method of Lim *et al.* (1982). Livers were perfused with phosphate buffered saline (PBS, pH 7.4), minced and washed five times with PBS by centrifugation at 5,000×*g* for 30 min. The plasma membrane surface proteins were extracted with 3 M KCl in PBS, pH 7.4 (2 ml per g of tissue) for 18 h at 4℃ with constant stirring. The mixture was centrifuged at 20,000×*g* for 30 min and the supernatant was dialyzed against PBS, pH 7.4 followed by 1 mM EDTA-water, pH 8.0. The dialysate was centrifuged at 110,000×*g* for 60 min at 4℃ and the clear supernatant was sterilized by passing it through a 0.45 μm membrane filter (Gelman). The solution was divided into aliquots and stored frozen at −70℃. Protein concentrations were determined by the modified micromethod of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE)

Discontinuous non-denaturing PAGE and SDS-PAGE were run according to the procedure of Laemmli (1970), with or without SDS using a vertical slab-cell (Protean II cell or Bio-Rad or Mighty small, Hoefer). Homogeneous (10%) and gradient (7∼15%) polyacrylamide gels were used as separating gels in non-denaturing PAGE, and homogeneous (10%) polyacrylamide gel was used in SDS-PAGE. The stacking gel was 4% in both cases.

Preparation of antiserum

New Zealand White rabbits were immunized with an antigen subcutaneously into several sites on the back. The antigen was prepared according to the method of Tjian *et al.* (1975) by cutting stained protein band from 10% non-denaturing polyacrylamide gel, lyophilizing the crushed gel and emulsifying with PBS (pH 7.4) and Freund's complete adjuvant. After 3 weeks, a booster injection was administered with the same antigen in Freund's incomplete adjuvant. Blood was collected one week later and the second booster was carried out after one week.

Preparation of immunoaffinity column

Rabbit immunoglobulin G (IgG) was purified from rabbit antiserum by the method of James and Stanworth (1964) using 40% saturated ammonium sulfate solution (pH 7.4). The precipitate was dissolved in the dialysis buffer (0.15 M NaCl in 50 mM Tris, pH 8.6) and dialyzed against the dialysis buffer. A Protein A-Sepharose 4B column was equilibrated with the same dialysis buffer and the dialyzed fraction was applied to the column. IgGs were eluted by 0.15 M NaCl in acetate buffer (pH 4.3) followed by 0.15 M NaCl in 0.5 M glycine-HCl buffer (pH 2.3). The purified IgGs were dialyzed against 0.5 M NaCl in 0.1 M sodium bicarbonate buffer (pH 8.5) and attached to CNBr-activated Sepharose 4B as described by Choo *et al.* (1981).

Purification of the normal rat liver specific protein

Twelve mg of the normal rat liver plasma membrane proteins was applied to a Sephacryl-S 200 column and eluted with 30 mM Tris-HCl (pH 7.4). The fractions containing the specific protein were pooled and applied to an immunoaffinity column. The immunoaffinity column was washed with 0.1 M Tris-HCl buffer (pH 8.3) containing 0.5 M NaCl to remove any non-specific bindings. The bound protein was eluted with 50 mM triethylamine solution (pH 11.5) and equilibrated with PBS by ultrafiltration.

Westernblot analysis

Electrophoretic transfer of proteins from a polyacrylamide gel to a nitrocellulose membrane was performed by the modified method of Towbin *et al.* (1979). The second antibody used was the goat anti-rabbit IgG conjugated with horse-radish peroxidase.

Isoelectric focusing

The isoelectric focusing was carried out in 5% non-denaturing polyacrylamide gel containing 2% pharmalytes (pH 3.5∼10) using a vertical slab gel system (Mighty Small, Hoefer).

Amino acid sequence analysis

Automated amino acid sequence analysis was performed with an Applied Biosystems protein sequencer (Model 470). PTH derivatives of amino acids were identified by HPLC.

Results

Identification of a normal rat liver specific protein

The electrophoretic patterns of plasma membrane surface proteins from normal rat liver and hepatoma induced by 2-MeDAB and DENA were compared using 10% non-denaturing PAGE (Fig. 1). Since a protein migrates according to the size and charge in non-denaturing gel electrophoresis a 7∼15% gradient non-denaturing gel electrophoresis was carried out to remove the charge effect and the same result was obtained

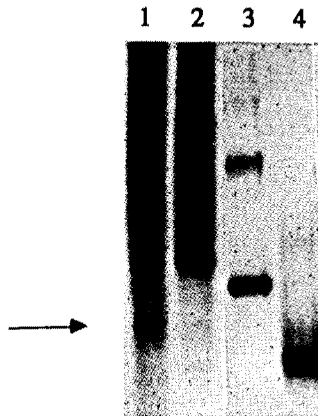

Fig. 1. 10% non-denaturing gel electrophoresis of plasma membrane surface proteins isolated from normal rat liver (lane 1) or rat hepatoma (lane 2). Lane 3 shows albumin, bovine and lane 4 shows albumin, egg. An arrow indicates the protein band which is apparent only in normal rat liver and disappeared from hepatoma.

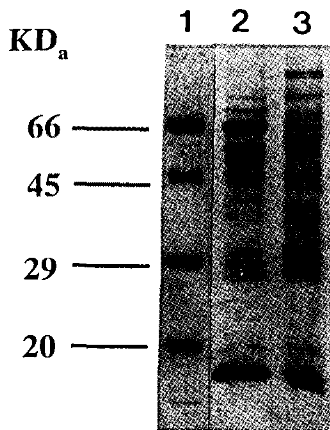

Fig. 2. 10% SDS-polyacrylamide gel electrophoresis. Lane 1 shows molecular weight marker proteins. Lane 2 shows plasma membrane surface proteins isolated from rat hepatoma and lane 3 shows proteins from normal rat liver.

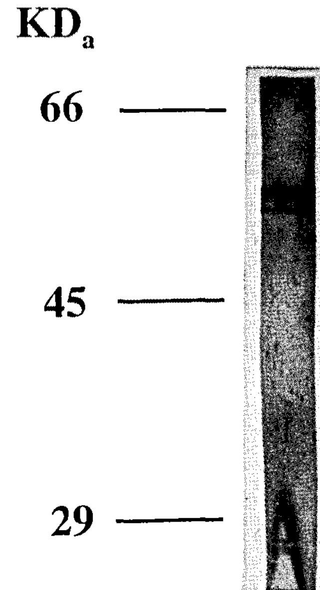

Fig. 3. Western immunostaining of the proteins of normal rat liver.

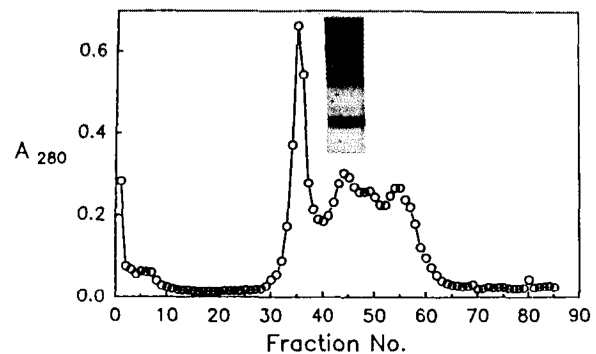

Fig. 4. Elution profile of Sephacryl S-200 chromatography.

(data not shown). In 10% or gradient non-denaturing PAGE, one protein band which disappeared from hepatoma was identified in normal rat liver. This specific protein band was sliced from the gel and used to immunize rabbits. The antisera obtained from the rabbits were used for western immunostaining and immunoaffinity chromatography.

SDS-PAGE and western immunostaining

10% SDS-polyacrylamide gel electrophoresis was run with plasma membrane surface proteins from rat hepatoma and normal liver to see the difference in the electrophoretic patterns (Fig. 2). These bands were electrophoretically transferred to nitrocellulose membrane and immunostaining was performed with rabbit antisera raised against the normal rat specific protein. As shown in Fig. 3 the antisera reacted specifically with the two proteins of normal liver and did not react with any other proteins of hepatoma.

Purification and characterization of the normal rat liver specific protein

As shown in Fig. 4, three peaks were separated by Sephacryl S-200 column chromatography and the second peak contained the protein of interest obtained by 10% non-denaturing gel electrophoresis. The protein was further purified with the immunoaffinity column. After removing the nonspecific binding proteins from the column, the protein to be purified was eluted with 50 mM triethanolamine (pH 11.5) (Fig. 5). In 10% non-denaturing gel, only one protein band which disappeared on the hepatoma cell surface was detected (Fig. 6).

The purity and molecular weight of the purified protein was determined by 10% SDS-polyacrylamide gel electrophoresis. Fig. 7 shows that two protein bands

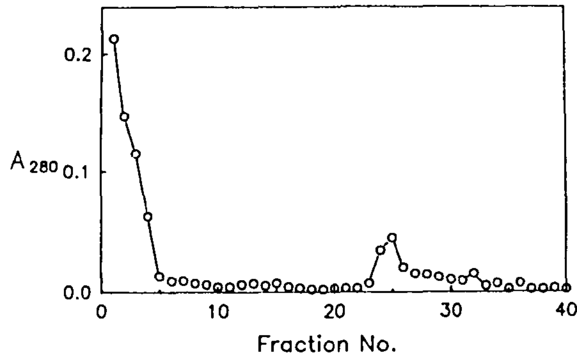

Fig. 5. Elution profile of IgG-Sepharose chromatography. Pooled Sephacryl S-200 chromatography fractions (No. 41~51) were applied to the column.

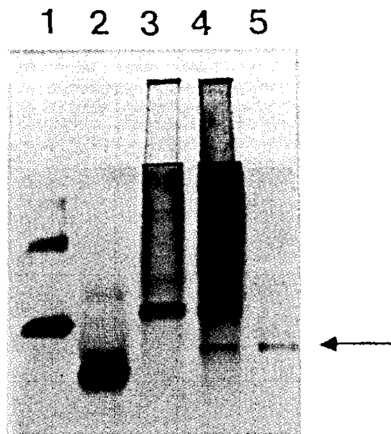

Fig. 6. 10% non-denaturing gel electrophoresis of the protein purified by immunoaffinity chromatography (lane 5). Lane 1 is albumin, bovine, and lane 2 is albumin, egg. Lane 3 and 4 are plasma surface proteins from rat hepatoma (lane 3) or from normal liver (lane 4).

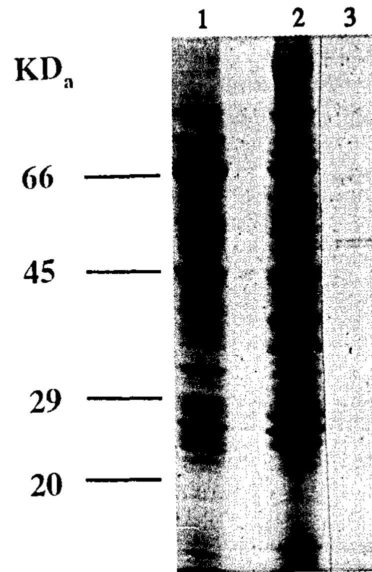

Fig. 7. 10% SDS-polyacrylamide gel electrophoresis of the protein purified by immunoaffinity chromatography (lane 3). Lane 1 and 2 are plasma surface proteins from rat hepatoma (lane 1) or from normal liver (lane 2).

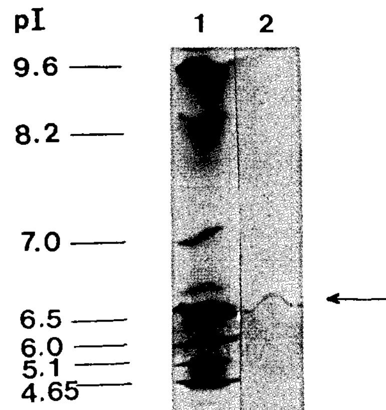

Fig. 8. Isoelectric focusing of the protein purified by immunoaffinity chromatography (lane 2). Vertical isoelectric focusing was performed and Bio-Rad isoelectric focusing standards were used to indicate the pH gradient (lane 1).

were stained in the gel. The molecular weights were estimated to be 50 and 52 kD_a from the standard curve. This result coincided with the western immunoassay, suggesting that the one protein band in non-denaturing gel may exist as two isoform proteins or this protein may have different glycosylation.

In the isoelectric focusing of the purified protein only one band, with a pI value of 6.6 was observed (Fig. 8).

Amino acid sequence analysis

The N-terminal amino acid sequence of the purified protein was analysed by automated amino acid sequencer (ABI) and its N-terminal was found to be blocked.

Discussion

There is convincing evidence that the site of action of most carcinogenic agents is the genetic material in cells; many known and suspect chemical carcinogens cause mutations. Chemical mutagenesis could be an important mechanism for inactivation of tumor suppressor gene or activation of DNA repair gene.

Recent studies show that expression of tumor suppressor genes, such as the p53 gene, is frequently lost or altered in human hepatocellular carcinoma cell lines (Bressac *et al.*, 1990). In more than 70% of colorectal cancers and in 50% of larger adenomas, allelic loss at chromosome 18q is very common. Using restriction site polymorphisms, these allelic losses were mapped to chromosome band 18q21, and the gene was identified by a molecular cloning technique and was called DCC for deleted in colorectal cancer (Fearon, 1990). The DCC mRNA is reduced or absent in more than 85% of the colorectal tumor cell lines examined (Fea-

ron, 1992). The DCC protein has a trans-membrane spanning domain with extracellular domains. Since the protein disappearing after chemical carcinogenesis is also a membrane protein, this type of structure may suggest a role for this protein in adhesion or metastatic invasion of cancer cells. Although the roles of the appearing or disappearing proteins are not clear, they could be used as useful tools in tumor diagnosis. In many studies (Meltzer *et al.*, 1971; Baldwin *et al.*, 1973; Hahm *et al.*, 1984; Kim *et al.*, 1985), the proteins appearing during carcinogenesis are extensively studied, but the disappearing proteins are poorly studied. In our previous study (Lee *et al.*, 1988) two disappearing proteins (MW 52 kD_a and 28 kD_a) were found. In this study we found a protein which disappeared in rat hepatoma. Since this protein specifically appears in normal rat liver, the disappearance of the protein may indicate malignant transformation. Therefore the antibody raised against the disappearing protein may be an useful tool for hepatoma diagnosis in rat. Further studies should be carried out to identify this protein which disappears after chemical carcinogenesis.

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References

Baldwin, R. W., Barker, C. R., Embleton, M. J., Glaves, D., Moore, M. and Pimm, M. V. (1971) *Ann. N. Y. Acad. Sci.* **177**, 268.

Baldwin, R. W., Harris, J. R. and Price, M. R. (1973) *Int. J. Cancer* **11**, 385.

Bressac, B., Galvin, K. M., Liang, T. J., Isselbacher, K. J., Wands, J. R. and Ozturk, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1973.

Choo, K. H., Jennings, I. G. and Cotton, G. H. (1981) *Biochem. J.* **199**, 527.

Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E. and Simons, J. W. (1990) *Science* **247**, 49.

Fearon, E. R. (1992) *Cancer Surveys* **12**, 119.

Hahm, K-S., Lee, N. J. and Kim, Y. S. (1984) Abstract Presented at the 36th Annual Meeting of Korean Biochemical Society.

James, K. and Stanworth, D. R. (1964) *J. Chromatography* **15**, 324.

Kim, K. S., Hahm, K-S. and Kim, Y. S. (1985) Abstract Presented at the 37th Annual Meeting of Korean Biochemical Society.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Lee, M. K., Han, M. H. and Hahm, K-S. (1988) *Korean Biochem. J.* **21**, 402.

Lowry, O. H., Rosebrough, N. A., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 256.

Lim, I. K., Hwang, D. S. and Kim, Y. S. (1982) *Yonsei J. Med. Sci.* **15**, 108.

Meltzer, M. S., Leonard, E. J., Rapp, H. J. and Borsos, T. (1971) *J. Natl. Cancer Inst.* **47**, 703.

Metzgar, R. S., Rodriguez, N., Finn, O. J., Lan, M. S., Daasch, V. N., Fernsten, P. D., Meyers, W. C., Sindelar, W. F., Sandler, R. S. and Seigler, H. F. (1984) *Proc. Natl. Acad. Sci.* USA **81**, 5242.

Kim, Y. S., Hahm, K-S., Kim, K. S. and Lee, N. J. (1988) *Yonsei Med. J.* **29**, 17.

Starling, J. J. and Wright, Jr. G. L. (1985) *Cancer Res.* **45**, 804.

Tjian, R., Stinchcomb, D. and Losick, R. (1975) *J. Biol. Chem.* **250**, 8824.

Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci.* USA **76**, 4350.

Varki, N. M., Reisfeld, R. A. and Walker, L. E. (1984) *Cancer Res.* **44**, 681.