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Monoclonal Antibody Against Placental Alkaline Phosphatase

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SUMMARY

Several monoclonal antibodies against rat placental alkaline phosphatase (PLAP) were produced by the mouse hybridoma technique. An immunohistochemical staining was performed using the monoclonal antibody against PLAP in various tissues such as placenta, fetal liver, and chemically induced hepatoma. It is revealed that the hepatoma cells as well as fetal hepatocytes were positive for the PLAP by the immunohistochemical staining. An electrophoretogram of the n-butanol extract of the hepatoma tissue on the cellulose acetate membrane also showed the existence of the similar band to that of placenta. The data obtained indicate that the trophoblastic gene product, PLAP, is expressed in chemically induced hepatoma and in fetal hepatocytes as well.

Key words: Monoclonal antibody, Placental alkaline phosphatase, Hepatoma

INTRODUCTION

We have reported the existence of the intestinal type of tumor cells in hepatoma tissue induced by 3'-methyl-4-dimethyl-aminoazobenzene (3'-Me-DAB) in rat liver which was associated with the appearance of the intestinal isoenzyme in the cellulose acetate membrane zymogram (Yoshida *et al.*, 1978). The phenomenon was designated as "Intestinalization" which seems to be a similar event usually seen in human stomach as intestinal metaplasia.

In human, the PLAP is produced by several malignancies such as lung cancer, ovarial cancer and seminoma (Fishman *et al.*, 1976). Therefore it is of interest to determine whether the hepatoma cells possess the trophoblastic gene product, PLAP, or not. In the present study, the monoclonal antibody aganinst PLAP was

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produced by the mouse hybridoma technique, and various tissues including fetal liver and hepatoma induced by 3'-Me-DAB were stained by an immunohistochemical method.

MATERIALS AND METHODS

PLAP purification

PLAP was purified from full-term placentas of pregnant rats of Wistar strain by the method of Sugiura *et al.* (1975) with slight modification. To determine the alkaline phosphatase activity, two methods were used.

Method A (Felix and Fleisch, 1974): Reaction mixture contained 0.05 M 2-amino-2-methyl-1, 3-propandiol-HCl (pH 10.3), 35 mM Na_2CO_3, 1 mM $MgCl_2$ and 3.8 mM p-nitrophenol phosphate as a substrate. After enzyme solution was added, the incubation was made at 37℃ for 10 min. The reaction was stopped with 0.1 M NaOH and released p-nitrophenol was read at 405 nm.

Method B (Kaneko, 1968): The activity of alkaline phosphatase was assayed in a medium containing 0.01 M veronal buffer (pH 9.3) and 0.01 M β-glycerophosphate as a substrate with addition of 5 mM $MgCl_2$. The reaction mixture was incubated at 37℃ for 30 min. and released Pi was determined. The protein content was determined according to Lowry *et al.* (1961) using bovine serum albumin as a standard.

Electrophoresis of alkaline phosphatase

After a portion of hepatoma tissue which has been induced by 3'-Me-DAB was removed for morphological examination, the remainder and the various tissue samples were subjected to electrophoresis on the cellulose acetate membrane as described (Yoshida *et al.*, 1978).

Production of monoclonal antibodies

By using purified PLAP as antigen, hybridomas were selected from fusions between spleen cells of sensitized BALB/c mice and NS-1 myeloma cell line (Slaughter *et al.*, 1980). The culture media were tested for antibodies to PLAP by enzyme linked immunosorbent assay.

Detection of monoclonal antibody by enzyme linked immunosorbent assay

The bottom of 96 wells of polyvinyl microtiter plate was coated with 5 μg/m*l* of purified antigen. After incubation with the media of cloned hybridomas, horse raddish peroxidase-conjugated antimurine immunoglobulin antibody was added. After the incubation with substrate containing 0.4 mg/m*l* o-phenylene diamine, 0.01 % H_2O_2 in phosphate-citrate buffer pH 5.0, horse raddish peroxidase activity was determined with a microplate spectrophotometer at 610 nm. Several positive hybridomas were obtained, of which 3A2 is used for immunohistochemical staining of various tissues.

Immunohistochemical staining

Placenta, fetal liver, hepatoma induced by 3'-Me-DAB was fixed with cold acetone and embedded into paraffin of low melting point. 2 μm thin sections were cut and stained with the monoclonal antibody, 3A2, aganinst PLAP by indirect immunoperoxidase method (Nakane, 1970). Serial section was stained with hematoxylin and eosin to confirm the hitology of each tissue. As the control for the specific staining, non-immune mouse serum and phosphate-buffered saline without the monoclonal antibody were used instead of the first antibody.

Enzyme activity inhibition test by monoclonal antibody

An aliquot of the monoclonal antibody was added into the enzyme solution and incubated overnight at 4℃. After the centrifugation at 10,000 ×g for 10 min, the remaining activities in the supernatants were measured.

Determination of immunoglobulin subclass of monoclonal antibody

Ouchterlony immunodiffusion method was done to determine the subclass of immunoglobulin of the monoclonal antibodies by using specific antibody against IgM, IgG_1, IgG_{2a} (Miles Laboratory, Kenkakee, Illinois).

RESULTS

Purification of PLAP

The purification procedures are summarized in Table 1. The alkaline phosphatase from rat placenta was purified up to about 850-fold from the enzyme level in the placenta homogenate. As shown in Fig. 1, the purified enzyme gave a single band of protein by polyacrylamide disc electrophoresis.

Electrophoresis of alkaline phosphatase

The alkaline phosphatase electrophoretogram of the hepatoma tissue and other comparative tissues including early and late hyperplastic nodules (Onoé *et al.* 1976) showed that hepatoma possessed the placental type of alkaline phosphatase as well

Table 1 *Purification of rat placental alkaline phosphatase*

Purification step	Total Protein (mg.)	Total activity* (units)	Specific activity (units/mg. protein)
1 Placenta homogenate	5,010	4,425	0.88
2 Butanol extract	735	7,300	9.93
3 DEAE-cellulose	246	6,216	25.27
4 Sephacryl S-300	30	4,059	135.30
5 CM-cellulose	2.3	1,774	771.30

* The activity was measured by the method A.
The unit of the enzyme is defined as that amount which liberates 1 μmole of p-nitrophenol/10 min./m*l*.

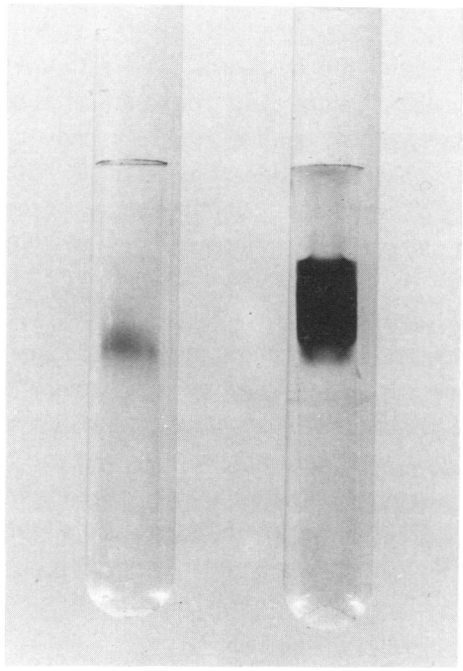

Fig. 1 Disc gel electrophoresis of the purified enzyme. Left tube stained with Coomassie Brilliant Blue ; right stained for alkaline phosphatase activity.

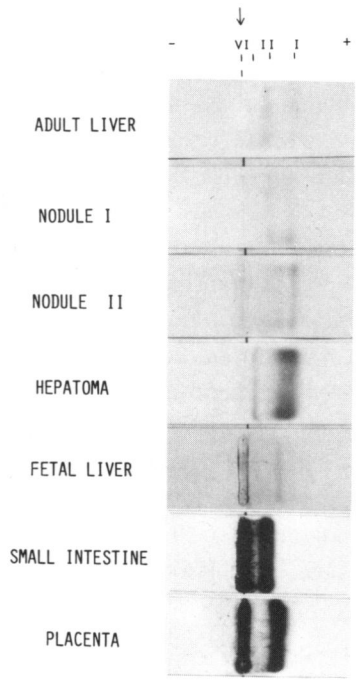

Fig. 2 Zymograms of alkaline phosphatase of comparative tissues. Hepatoma contains placental-type isozymes in addition to fetal liver types. Each 16 μl of tissue extract were applied on the strip, except 2 μl for intestinal mucosa.

as the fetal liver type (Fig. 2).

Determination of immunoglobulin subclass of monoclonal antibody

The monoclonal antibody used for immunohistochemical staining, 3A2, in the present study was revealed IgM by Ouchterlony immunodiffusion method (Fig. 3).

Enzyme activity inhibition test

The remaining enzyme activity after the incubation with specific monoclonal antibody is shown in Fig. 4. The activity was inhibited with the increase of the antibody. However the remaining activity was still detected at about 20% of the original activity.

Immunohistochemical staining

Acetone fixed sections were stained with the monoclonal antibody against PLAP. In placenta both cytoplasm and cell membrane of the trophoblasts covering

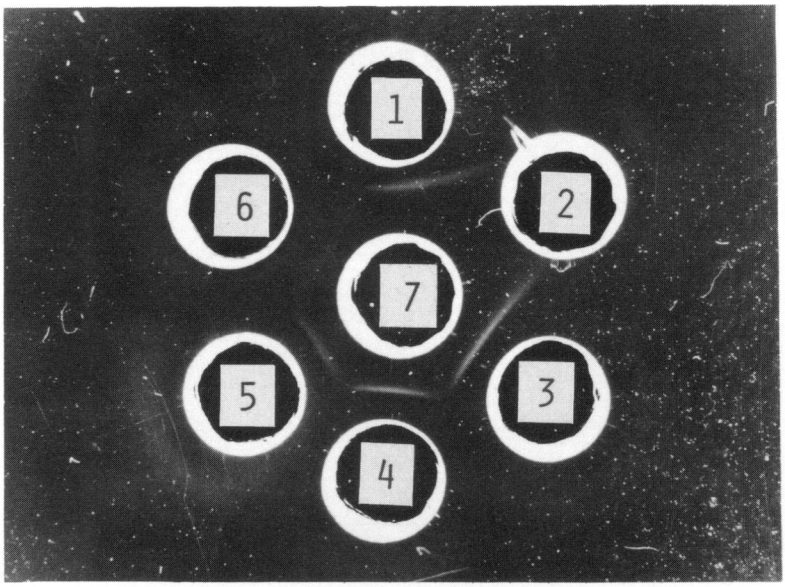

Fig. 3 Immunological determination of immunoglobulin subclass of the several monoclonal antibodies. 1, monoclonal antibody 4C1 ; 2, 9C5 ; 3, 3A2-B6 ; 5, 6, normal mouse serum ; 7, anti murine IgM antibody.

capillary lumen were positive (Fig. 5). In fetal liver, hepatocytes among extramedullary hematopoietic cells were strongly stained (Fig. 6). Chemically induced heptoma tissues, both trabecular type (Fig. 7) and adenocarcinomatous type (Fig. 8) were also positive. The sections stained with nonimmune mouse serum or phosphatebuffered saline without the monoclonal antibody were not stained.

DISCUSSION

It was clearly demonstrated, from the zymograms of alkaline phosphatase in chemically induced hepatoma tissue and in placenta, that the hepatoma possessed the PLAP (Fig. 2). Therefore an immunohistochemical investigation on the study whether the PLAP is expressed in the hepatoma or not was performed. The monoclonal antibody against PLAP was produced to give the specificity to the staining by the mouse hybridoma technique (Köhler and Milstein, 1975). One of the PLAP specific antibodies, 3A2 was used for a immunohistochemistry. In term placenta, both plasma membrane and cytoplasm of the trophoblasts lining many capillary lumens were positive for the peroxidase activity indicating the presence of the PLAP (Fig. 5). This localization of the PLAP was same as that of the enzyme activity stained by histochemical method of Burstone (1961) in which naphthol-AS--phosphate was used as a substrate (Yoshida, unpublished data). On the other

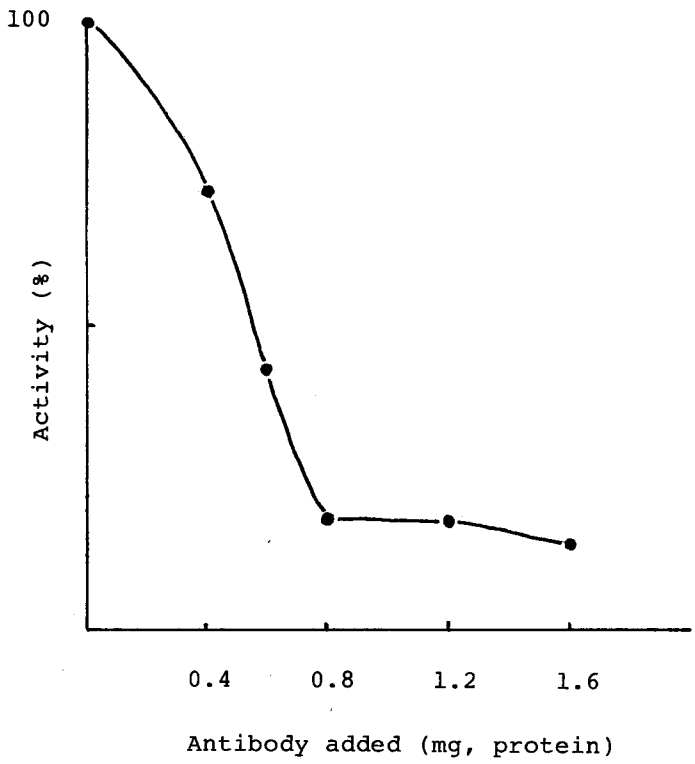

Fig. 4 The activity inhibition test by anti-PLAP monoclonal antibody was performed as follows. The various volume of crude monoclonal antibody solution (4 mg protein/m*l*) was added to 0.5 m*l* of PLAP (100 μg protein), and to final volume was made to 1.0 m*l* with PBS solution. The antibody itself had no alkaline phosphatase activity. After the mixtures were left overnight at 4℃ and then centrifuged at 10,000×g for 10 min. The activity of the supernatants were assayed. The initial activity of PLAP was 2.24 μmol Pi/30 min/m*l* with β-glycerophosphate as substrate.

hand, in fetal liver tissue, the PLAP was strongly positive in cytoplasm of the fetal hepatocytes which distributed among many extramedullary hematopoietic cells (Fig. 6). Proteins produced by the trophoblasts such as human chorionic gonadotropin have not been found in another organ during embryogenesis. Therefore it is of interest that the PLAP was expressed in the fetal hepatocytes. As shown in Fig. 2 the zymograms of alkaline phosphatase indicate that the band of the fetal liver seems very similar to that of the placenta. Moreover the cytochemical staining of alkaline phosphatase in the fetal liver showed canalicular pattern (data not shown)

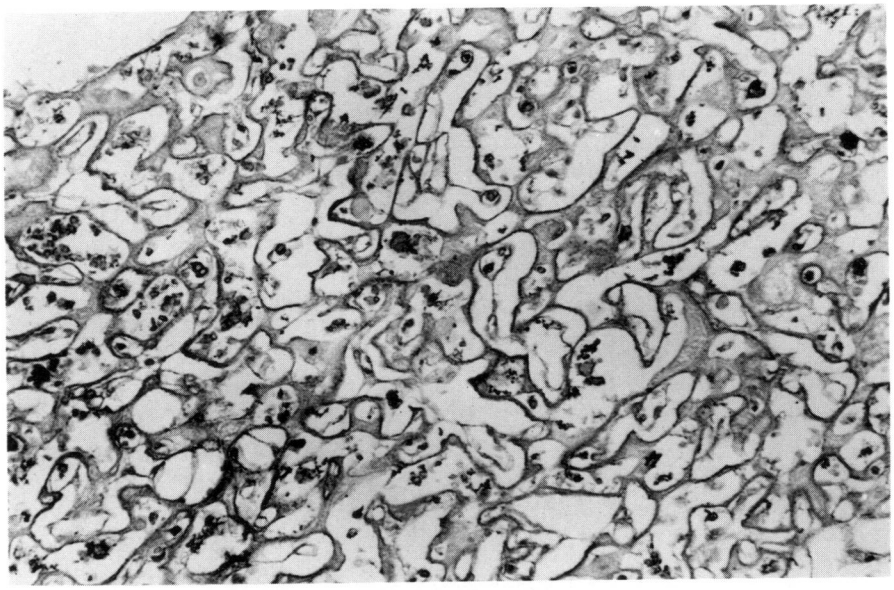

Fig. 5 Immunohistochemical staining of placenta by the monoclonal antibody against PLAP. Trophoblasts covering capillary lumen are positive. ×100.

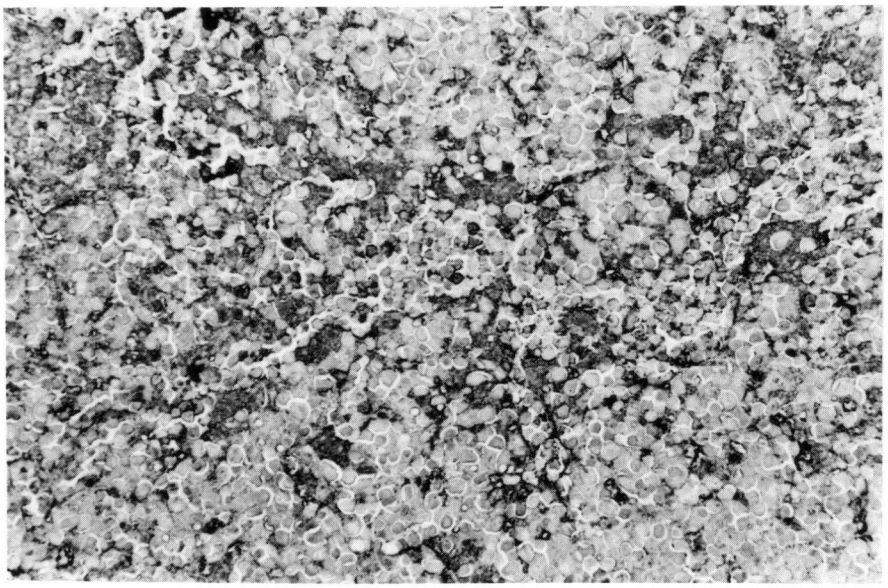

Fig. 6 Immunohistochemical staining of fetal liver by the monoclonal antibody. The cytoplasm of fetal hepatocytes are strongly stained. ×100.

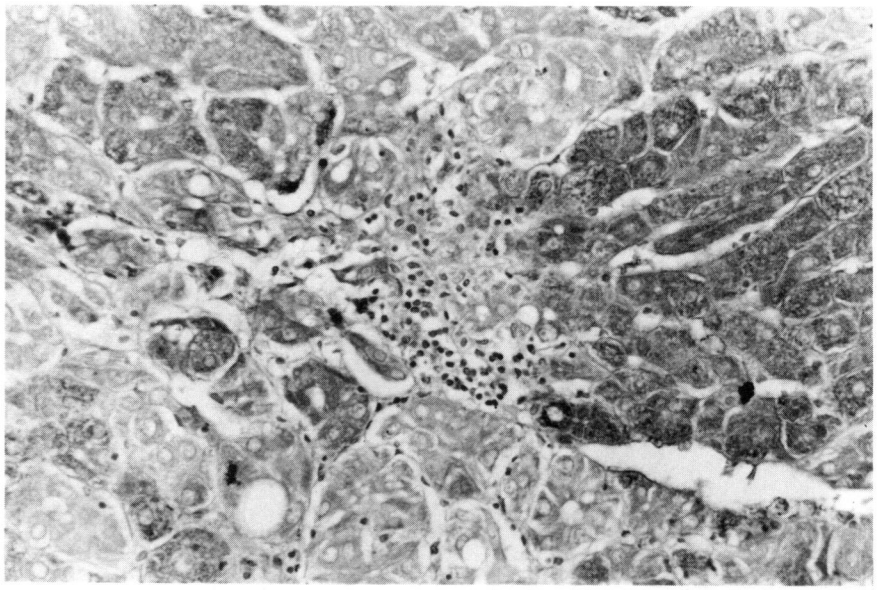

Fig. 7 Immunohistochemical staining of the trabecular type of hepatoma induced by 3'-Me-DAB. The cytoplasm of the hepatoma cells are positive. ×270.

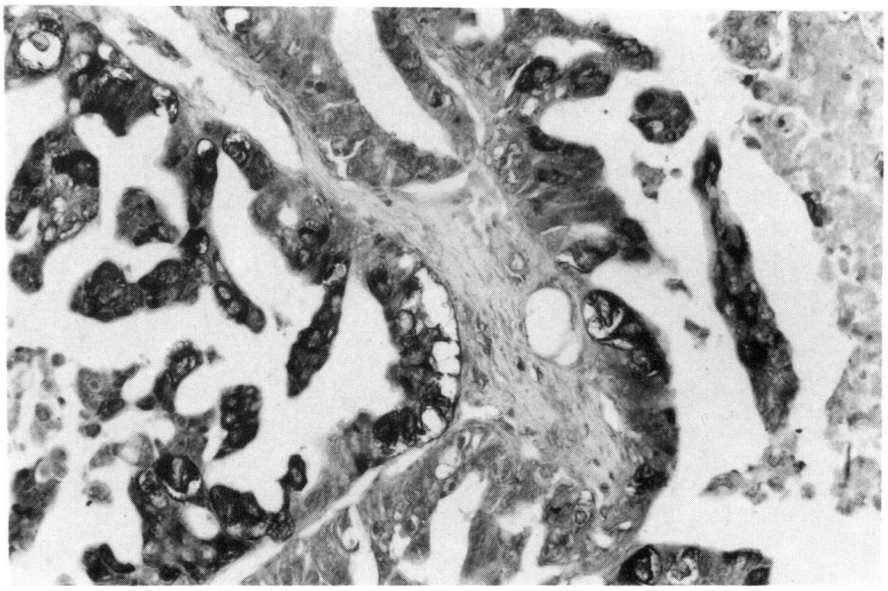

Fig. 8 Immunohistochemical staining of the adenocarcinomatous type of hepatoma by 3'-Me-DAB. The tumor cells proliferating papillary are positively stained. ×330.

among the neighboring hepatocytes, while the immunohistochemistry by the monoclonal antibody revealed that the PLAP was detected in the cytoplasm of the fetal hepatocytes homogeneously. It is not clear why this difference between the enzyme activity and the antigenecity occurred. However it might be possible to state that at least the substance antigenic to the PLAP existed in the fetal hepatocytes. The difference might also show that the enzymatically active site and the antigenic determinant in a molecule may not be associated each other. The result of the enzyme activity inhibition test by adding the monoclonal antibody into the enzyme solution in which the activity was not inhibited completely might be related to this (Fig. 4).

The PLAP was demonstrated in the hepatoma tissue induced by 3'-Me-DAB immunohistochemically. The presence of the PLAP within the hepatoma cells had no correlation with the histological pattern of hepatoma, whatever it may be.

The authors reported the appearance of the intestinal isoenzyme in the undifferentiated type of hepatoma (Yoshida *et al.*, 1978). The evidence indicates that the hepatoma cells could undergo intestinalization. This might also be interpreted that the tumor cells showed disdifferentiation (Sugimura *et al.*, 1972). However since liver, stomach and small intestine originate from common ancestry, the foregut, the intestinalization of the hepatoma cells should be undertaken as an event during the course of normal ontogenetic process of the liver, that is retrodifferentiation (Uriel, 1976). Same fact was found in the case of the expression of aldolase isozyme in hepatoma (Schapira *et al.*, 1971).

Among the placental gene products isoferritin possessing the subunit of lower pI value was reported to be found in fetal liver and hepatoma (Arosio *et al.*, 1976; Niitsu *et al.*, 1976). The trophoblastic gene products were also found in other cancer tissue rather than in trophoblastic malignancy, choriocarcinoma (Fishman *et al.*, 1976; Ibsen and Fishman, 1979). Why and how these gene products are expressed by the cancer cells remains unanswered. The monoclonal antibody to these might be a clue for the question.

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