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Relationship Between Appearance of AFP-Producing Cells and Serum AFP Levels in Chemically Induced Mouse Hepatoma

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Summary (Report II)

The relationship between the productivity of AFP, the serum AFP level by single assay and the pattern of periodic assay of AFP was studied using chemically induced hepatoma in mice. The model animal used in this experiment was the mouse with hepatoma induced by Direct Deep Black-Extra (DDB-EX), one of the derivatives of benzidine. The productivity of AFP in the tumor tissue is demonstrated as a density of AFP-positive cell by immunofluorescent technique. In the particular group of tumors with high level of serum AFP determined by a single assay, it may be assumed that the productivity of AFP in the tumor tissue is high and the size of the tumor has a positive correlation with the titer of serum AFP level. But the single-assay serum AFP level does not always reflect the productivity of AFP in the tumor tissue. The pattern of serial change of serum AFP level, however, tends to have a high correlation to the degree of productivity of AFP in the tumor tissue. The pattern of serial change of AFP provides a more sensitive indicator for the activity of the tumor tissue than the single assay serum AFP level.

Introduction

Since the first report by ABELEV et al.,[1] it has become apparent that alphafetoprotein (AFP) is synthesized in hepatocellular carcinoma and released into the blood. The reappearance of this embryospecific protein in a patient with hepatoma is a very interesting subject to investigate. Although there are exceptional hepatomas that do not produce AFP, serum AFP can be detected by agar-gel precipitation method in more than 70% of the patients[7]. Furthermore, by means of the recently developed more sensitive radioimmunoassay technique, clinical diagnosis can be made in 90% of the patients with hepatoma[5].

Serial assay of serum AFP levels in a patient with hepatoma may also be helpful in obtaining other information about the disease. In clinical practice we have noticed a good correlation of

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serial change of serum AFP levels with the prognosis of hepatoma patients and with histological grading of the tumor tissue. Hepatoma patients were put into three groups based on serial changes of serum AFP levels[8]: (1) the high level group, (2) the moderate level group and (3) the low level group. We pointed out the important differences among these groups, such as survival time in clinical course or histological appearance, especially in the differentiation of tumor cells. In our previous paper, we reported induction of hepatic tumors in mice using Direct Deep Black-Extra (DDB-EX), one of the benzidine dyes, and the resemblance of the tumor to the human hepatoma in pathological findings and AFP production. In this paper, we will discuss the characteristic feature of the changing pattern of serial AFP levels in DDB-EX hepatoma.

The relationship between the AFP level in the tumor tissue to the AFP level in the serum was determined by studying the distribution of AFP-producing cells by immunofluorescent technique.

Materials and methods

Chemically induced hepatoma

Seventy ICR (SLC) male mice were obtained from Shizuoka Laboratory Animal Co. (Shizuoka, Japan), and 0.3% solution of Direct Deep Black-Extra (DDB-EX. Kyoei Chemical Ind. Co., Kyoto, Japan) was given as drinking water for 50 weeks according to the method described in the previous report[3].

Experimental series

Group I

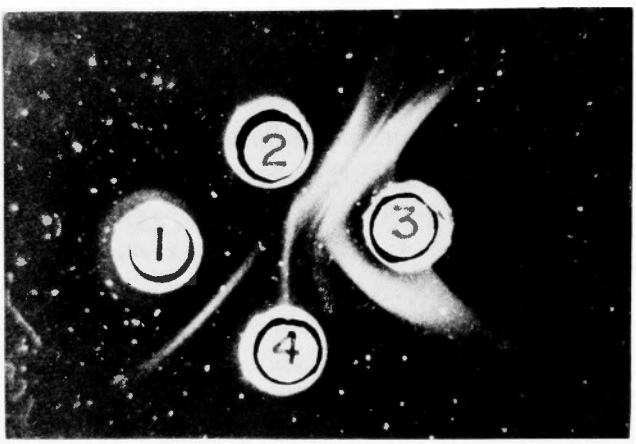
This group consisted of 50 mice, which were sacrificed at the 60th week from the beginning of the study. Histological examination of hepatic tumor was performed by immunofluorescent technique. Serum AFP concentrations were determined by single radial immunodiffusion (SRID) method and latex fixation (LF) method.

Group II

This group consisted of 20 mice whose serum AFP levels were assayed serially every two or four weeks from the 16th to the 60th week. Blood samples for determination of AFP were obtained by puncture of the orbital space of the mouse using a capillary tube. The experimental animals were sacrificed at the 60th week by exsanguination through the exposed abdominal aorta and offered as specimens for immunofluorescent technique.

Antiserum

From the pooled serum of mice with DDB-EX hepatoma, a crude antigen containing mouse AFP was prepared by preparative polyacrylamide gel electrophoresis. Immunization of rabbits was performed four times at weekly intervals with single dosages of 4 mg of protein. Two weeks after the last injection, rabbit serum was collected from the ear vein. Albumin fraction was removed with saturated ammonium sulfate. Immunoabsorption of crude antiserum was done



Results of agar-gel precipitation method.
Well 1; absorbed pure antibody
Well 2; normal mouse serum
Well 3; crude antibody
Well 4; mouse serum containing AFP

Fig. 1. The monospecific precipitation line can be seen between the absorved antiserum (Well 1) and mouse AFP (Well 4) in agar-gel plate. No line appears between Well 1 and Well 2.

with pooled normal adult mouse serum. Figure 1 shows the monospecific precipitation line between absorbed antiserum and mouse AFP in agar gel plate.

FITC conjugate

The antiserum was dialysed overnight with phosphate buffer solution at pH 9.1. Fluorescein isothiocyanate (FITC) in the ratio of 1/50 of the protein content was mixed with the antiserum by magnetic stirrer for 6 hours at 4°C. Unconjugated dye was removed by filtration through Sephadex G-25 column. DEAE-cellulose column was used for purification of FITC conjugate by elution with 0.65 M phosphate buffer solution (pH 6.4). The purified antiserum was condensed through a diaflomembrane and absorbed with normal mouse liver powder. It was employed in direct immunofluorescent technique.

FITC-labelled goat antibodies monospecific to rabbit γ-globulin used for indirect staining were obtained from Dr. TAKAHASHI (Second Department of Pathology, Kyoto University, Faculty of Medicine).

Quantification of serum AFP

Serum AFP level was routinely assayed by single radial immunodiffusion (SRID) method. A small amount of serum AFP undetectable by SRID method was determined by the more sensitive latex fixation (LF) method described by Singer[12]. The lowest level detectable by this method is 250 ng/ml[2].

Specimens for immunofluorescent technique

Specimens of hepatic tumor were cut into small blocks and frozen in cold 95% ethanol/acetic

acid placed in dry ice/acetone at −70°C. A minimum of five blocks were cut from different places in each tumor mass. ENGELHARD[4] emphasized that the best demonstration of both mouse and human AFP could be obtained by the paraffin embedding technique of SAINTE-MARIE[11] and the ethanol/acetic acid fixation according to HAMASHIMA et al.[6] The present experiment was conducted using both methods.

Immunofluorescent staining

Sections were deparaffinized, hydrated and washed in several changes of cold PBS, pH 7.2, and subjected to immunofluorescent staining by either direct or indirect method.

A. Direct method

One drop of FITC-labelled antiserum was put on each tissue section. After incubation for one hour at 37°C, the sections were washed with several changes of staining buffer solution and then examined by fluoromicroscope.

B. Indirect method

Indirect immunofluorescent staining was done using FITC-labelled goat antiserum to rabbit γ-globulin. Tissue sections were incubated with antiserum to mouse AFP for 30 minutes at 37°C and washed well with PBS. Thereafter they were stained with FITC-labelled antiserum at room temperature. After an overnight incubation, tissue specimens were washed again in PBS, mounted in buffered glycerol and sealed with paraffin wax. Serial sections were simultaneously stained with hematoxylin-eosin for identification of structural localization.

C. Blocking test

Control staining was carried out for comparative study on specificity of fluorescence. Non-labelled antiserum was dropped on the tissue sections and incubated for 6 hours or more in a moist chamber at 37°C. The tissue sections were washed well with cold staining buffer and then stained.

Results

A. *Localization of fluorescence in each tumor cell*

Specimens were made from five different places in each hepatoma and all-round observation was made to give a strict evaluation. In most cases specific fluorescences with diffuse or fine granular appearance were observed in the cytoplasm of the tumor cell but not in the nuclei. In a few cases fluorescences were found in the cytoplasmic membrane or the perinuclear zone, as other authors have reported also[10]. The fluorescence intensity of the cytoplasm was found to be much stronger than that of the cytoplasmic membrane or the perinuclear zone.

B. *Relationship between the density of AFP-producing cells and serum AFP level*

Hepatic tumor was observed in 45 out of 50 mice sacrificed at the 60th week (Group I). Serum AFP was detected in 32 mice by SRID, in eight by LF method. In the remaining five mice no serum AFP could be detected by either method.

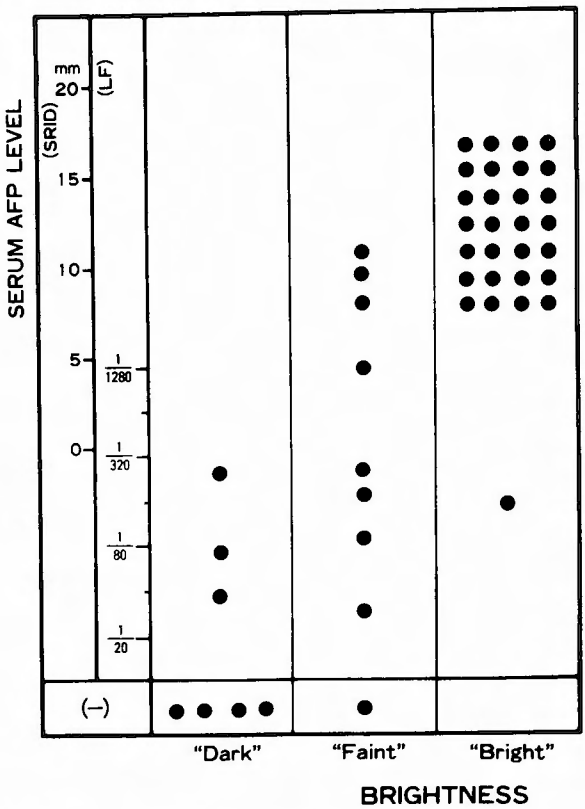


Fig. 2. Relationship between the density of AFP-producing cells and serum AFP level.

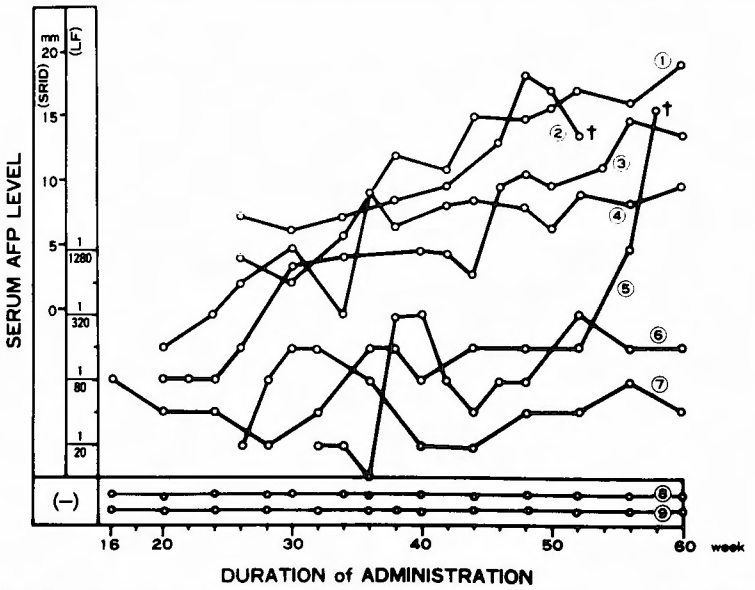


Fig. 3. Serial changes of serum AFP level. Lines 1, 2, 3, 4 represent the high level group; 5, 6, 7 the moderate level group; 8, 9 the low level group.

The results are demonstrated in Figure 2. Serum AFP levels of 32 mice were found to be high by SRID method. Most of the tumor tissues showed "bright" visual field because of the high density of AFP positive cells, but a few tissues showed "faint". There were eight mice with moderate level of AFP detectable only by LF method. The degree of brightness in these mice spread over a wide range. The five mice whose serum AFP levels were negative by both SRID and LF method showed "dark" or "faint" visual fields with scattered fluorescence.

The relationship between the cell differentiation and the density of AFP-positive cells was studied. Poorly differentiated hepatoma tended to show "bright" visual field, moderately differentiated tissue "faint", and well differentiated tumor tissue "dark".

C. *Serial changes of serum AFP level*

Although Group II consisted of 20 mice, in only nine of them could satisfactory blood samples be collected successively throughout the experiment. Figure 3 shows the changing pattern of periodically tested AFP levels in the nine. In four mice (Nos. 1, 2, 3, 4) the AFP levels showed a pattern of rapid and continuous elevation throughout the experiment, high enough to be detected even by SRID method (high level group). In three mice (Nos. 5, 6, 7), serum AFP was first detected at the 16th–20th week by LF method. Though a temporary decrease was observed, the overall level gradually increased. The AFP value remained, however, within a moderate level range detectable only by LF method. One of these animals (No. 5) showed a rapid elevation of serum AFP level after the 50th week and died soon after (moderate level group). However, in two of the remaining animals (Nos. 8, 9), serum AFP was continuously negative by LF method throughout the whole period. Autopsy revealed the presence of rather large hepatic tumors (low level group).

D. *Relationship between the pattern of change of AFP level and the density of AFP-positive cells*

In tissue containing a large number of AFP-positive cells, the visual field appeared "bright" because of the large amount of fluorescence. We classified the density of AFP-positive cells

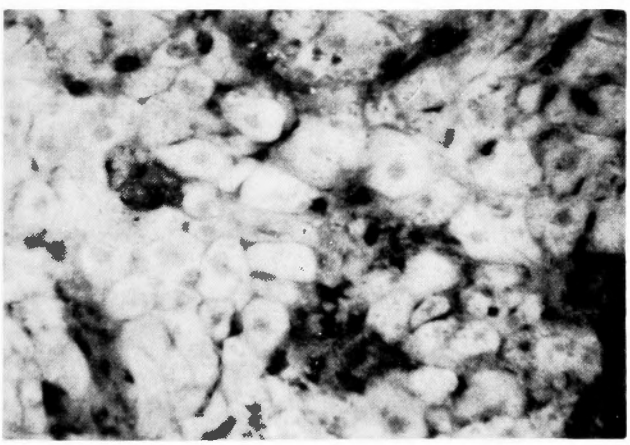


Fig. 4. Immunofluoresence technique. High density of AFP-positive cells makes "bright" classification Animal in high level group.

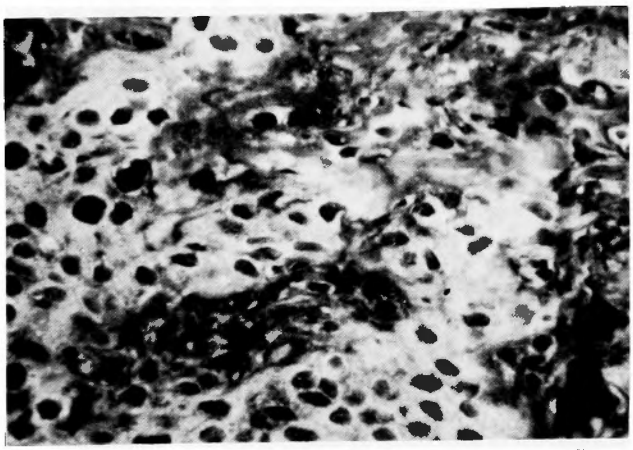


Fig. 5. Immunofluorescence technique. Moderate level group. Fewer AFP-positive cells make "faint" classification.

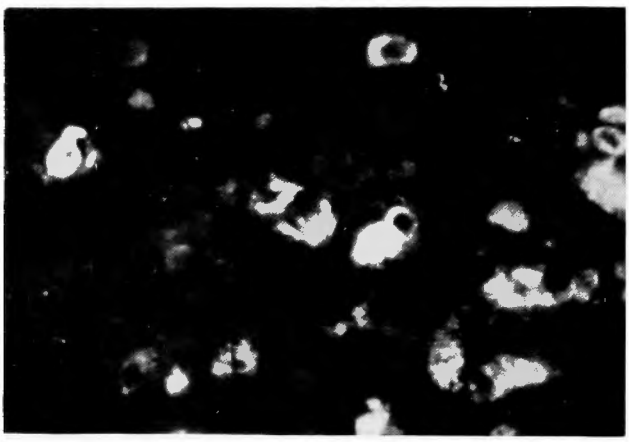


Fig. 6. Immunofluorescence technique. Sparse fluorescence. "Dark" classification.

Table 1. Correlation between the degree of brightness and the pattern of change of AFP level in the nine mice whose serum AFP levels were serially assayed.

No. of MOUSE	CHANGES of AFP LEVEL	BRIGHTNESS
1		
2	HIGH LEVEL GROUP	BRIGHT
3		
4		
5		
6	MODERATE LEVEL GROUP	FAINT
7		
8	LOW LEVEL GROUP	DARK
9		

into three grades, "bright" (Fig. 4), "faint" (Fig. 5) and "dark" (Fig. 6), according to the brightness of the visual field.

Table 1 shows a correlation between the degree of brightness and the pattern of change of AFP level in the nine mice whose serum AFP levels were serially assayed. Most of the tumor tissue of the high level group (Nos. 1–4) exhibited diffuse, dense fluorescence (grading "bright") with a histology of poorly differentiated hepatocellular carcinoma. Each of Nos. 5–7 mice belonging to the moderate level group had a solitary large tumor with moderate cell differentiation. The density of fluorescence in the tumor tissue was "faint" with sparse distribution of AFP-positive cells (grading "faint"). Mice Nos. 8 and 9 (low level group) had each a solitary large tumor of the liver with histology of well differentiation. Fluoromicroscopic examination showed more scattered fluorescent cells. The intensity of the visual field was graded "faint" for No. 8 and "dark" for No. 9.

Discussion

It is well known that most human hepatomas produce AFP, although some hepatomas lack this productivity.

In our previous report[3] on chemically induced hepatoma, it was observed that tumor size tended to have a positive correlation to the titer of the serum AFP in a group of mice with hepatoma in which AFP levels were high enough to be detected by SRID method. The larger the occupancy of the tumor in proportion to the entire liver, the higher the serum AFP level was found to be. These tumors showed a histological appearance of poor differentiation. However, there were many mice with large tumors in which serum AFP could not be detected by either SRID method or LF method. These tumors showed a histological appearance of moderate or well differentiation. Similarly some other authors[13] reported that clinically as well as experimentally serum AFP level does not always correlate with the size of a tumor.

McIntire[9] pointed out the importance of dynamic aspects on a serial change of serum AFP level, which is the most sensitive indicator of tumor activity, but the level did not appear to correlate with tumor size.

In our previous report[8], from a quantitative analysis of serum AFP in 106 patients, the relationship between the degree of malignancy of the tumor and the pattern of change of serum AFP level in human hepatoma was determined. Based on the change of serial levels of AFP three main groups (high, moderate and low level group) were delineated, a classification which appeared to be a good indicator of survival time of the patient and histological grading of the tumor tissue.

Three factors are considered significant in the regulation of serum AFP level: (1) the total content of AFP produced in the tumor tissue, (2) the release of AFP from the tumor into the blood stream and (3) the disappearance of AFP from the blood stream. In examining the disappearance, the half-life of this protein was reported to be constant for 3.5–4 days. Because of this constant rate of excretion, the serum AFP level is considered to be regulated by the total content of AFP being produced in a mass and the release of AFP into blood circulation. Generally, the

total content of a protein produced in a mass is represented as a product of the volume of protein synthesized in the mass multiplied by the size of the tumor. Thereafter, besides the release of AFP from the tumor tissue into blood circulation, serum AFP level is considered to be reflected by the productivity of AFP and/or the size of the tumor. Similarly, the pattern of serial change of serum AFP level may be also reflected by the productivity of AFP and/or the growth rate of tumor size, because the pattern consists of successive serum AFP levels. Thus, both the single assay level and serial assay levels of serum AFP might be regulated by the productivity of AFP in the tumor tissue. It is important to clarify the relationship between the productivity of AFP, the serum AFP level by single assay and the pattern of periodic assay of AFP.

In the present examination a new type of experimental hepatoma was used which was induced by Direct Deep Black-Extra (DDB-EX), one of the derivatives of benzidine. One characteristic of this chemically induced hepatoma is its high degree of resemblance to human hepatoma, especially in histological appearance. This experimental hepatoma was used to study the relationship described above. This is the first report on such a relationship using chemically induced hepatoma in miec.

The productivity of AFP in the tumor tissue is demonstrated as a density of AFP-positive cells by immunofluorescent technique. In the particular group of tumors with a high level of serum AFP, the tumor tissue tends to show high productivity of AFP. However, no such tendency is found between the productivity of AFP and the serum AFP level in the group of tumors with lower concentrations of serum AFP. In the previous paper, it was reported that the size of tumor has a positive correlation to the titer of serum AFP in the group of mice with high concentration of AFP detectable by SRID method. However, the correlation was not observed in the mice whose AFP was undetectable by SRID method. From these findings, in the group of tumors with high level of serum AFP determined by a single assay, it may be assumed that the productivity of AFP in the tumor tissue is high and the size of the tumor has a positive correlation with the titer of serum AFP level. However, the single-assay serum AFP level does not always reflect the productivity of AFP.

In nine mice serial assay of AFP was made every two or four weeks. From the analysis of serial assay of serum AFP, three groups of mice with clearly delineated serum AFP levels was noticed, namely, high, moderate and low. By histological observation with immunofluorescent technique, the density of AFP-positive cells in the tumor tissue of these three groups was compared. It was observed that tumors in the high level group tend to have a high productivity of AFP. In the moderate level group, fluorescence was sparsely scattered showing a "faint" or "dark" visual field. Some cases whose serum AFP levels on serial assay were undetectable by either SRID or LF method showed "dark" appearance because of the low density of AFP-positive cells. These findings show that the pattern of serial change of serum AFP level has a high correlation with the degree of productivity of AFP in the tumor tissue.

In the high level group the serial change of AFP level tended to increase lineally with a steep gradient, whereas the moderate and low level groups showed an irregular pattern without a constant gradient. An occasional decrease of AFP level was observed in the latter groups.

The gradient of the pattern of serial change is considered to be regulated by the degree of productivity of AFP and/or the growth rate of the tumor. In the previous paper, in the particular group of mice whose high serum AFP level was detectable by SRID method, it was observed that the tumor size has a correlation with the single-assay level of serum AFP. From these findings, it is speculated that the productivity of AFP is constant and corresponds to the gradient of the pattern, and the size of the tumor mainly influences the change of serum AFP level in proportion to the stage of development.

In short, a single assay of serum AFP level can reveal the total content of AFP in a tumor mass, but not the productivity of AFP in the tumor tissue. The pattern of change of serum AFP levels determined by periodical assay, high, moderate or low, correlates with the productivity of AFP in the tumor mass. Accordingly, a hepatoma showing a pattern of high level of serum AFP indicates a hepatoma with a high productivity of AFP and a hepatoma showing a moderate or low level of serum AFP produces the AFP in moderate or low amount.

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和文抄録

マウス肝癌における AFP 陽性細胞の出現と血中 AFP 値の関係

京都大学医学部外科学教室第1講座

麻田 勇，松本 由朗，戸部 隆吉

肝癌組織の AFP 産生能と血中 AFP 値及びその経時的変動パターンとの関係についてマウス実験肝癌を用いて検討を行った．

Direct Deep Black EX (DDB-EX) で発癌した肝癌マウスを使用した．螢光抗体性で染色した AFP 陽性細胞の組織内密度により，組織内の AFP 産生能を表した．血中 AFP 値の高い動物では組織内 AFP 産生能は高く，又，腫瘍の大きさと血中 AFP 濃度にも相関関係がみられた．しかし AFP の1回測定値だけでは必ずしも組織内 AFP 産生能とは組関がみとめられなかった．これに対し AFP の経時的変動パターンは AFP の産生能をよく反映していることが知られた．