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Intracellular Synthesis of α-Fetoprotein and Fibrinogen without Secretion by Zajdela Rat Ascites Hepatoma Cells[1]

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SUMMARY

Transplantable Zajdela rat ascites hepatoma cells, previously considered "nonproducers," synthesize detectable amounts of intracellular α-fetoprotein (AFP) and fibrinogen, but fail to secrete or release these serum proteins. Evidence for defective secretory mechanisms for serum proteins in these hepatoma cells (*a*) explains the failure to detect AFP in either the serum or ascitic fluid of rats bearing this hepatoma, (*b*) indicates that some hepatoma cells should be classified as "nonsecretors," rather than nonproducers of AFP, and (*c*) suggests that failure to detect AFP in some human and animal hepatomas *in vivo* and *in vitro* may also reflect failure of secretion rather than failure of intracellular synthesis.

INTRODUCTION

Although AFP[2] synthesis by hepatoma tissues and cells is well documented (1, 5, 25), the following information is consistent with the conclusion that not all hepatomas produce this fetoprotein. (*a*) Approximately 90% of patients with histologically proven primary liver cancer are now found to have elevated serum AFP levels measured by radioimmunoassay; however, the remaining 10% have serum AFP levels within the normal accepted range (8, 12, 13, 15, 17, 18). (*b*) Reportedly, numerous carcinogen-induced and transplantable hepatomas and isolated clones of hepatoma cell lines fail to produce detectable amounts of AFP in the serum or ascitic fluid of these hepatoma-bearing animals *in vivo* (2, 22, 26, 27) or in the culture fluid *in vitro* (6, 7, 25). (*c*) Immunofluorescence studies revealed both AFP-positive and AFP-negative tumor cells in hepatoma tissue (4, 16, 24). Taken together, these observations have been interpreted to indicate that hepatomas consist of mixed populations of AFP-producing and -nonproducing tumor cells.

In a previous study (20), transplantable Zajdela rat ascites hepatoma cells were shown to synthesize intracellular α-macrofetoprotein (AMFP) and albumin but not to secrete these serum proteins *in vitro*. Failure to detect AFP in the serum or ascitic fluid obtained from such tumor-bearing animals *in vivo* or in cell culture fluid *in vitro* suggests that these hepatoma cells either are a typical nonproducer strain and have lost their capacity to produce AFP without this loss affecting their malignant properties, or they synthesize intracellular AFP but fail to secrete or release it. This study was undertaken to differentiate between these alternatives.

MATERIALS AND METHODS

Hepatoma Cell Protein Synthesis *in Vitro*. Zajdela ascites hepatoma cells (C strain) (28) were transplanted i.p. into adult male Charles River CD rats, and cells were harvested 6 to 7 days later. Hepatoma cells were separated from the ascitic fluid by centrifugation at 50 × *g* for 15 min at 5°, then were washed 3 times with 0.9% NaCl solution. For the short-term synthesis studies, hepatoma cells (13×10^7) were incubated in 3.5 ml of RPMI Medium 1640 with 10% fetal calf serum plus antibiotics containing 3 mCi L-[*U*-^{14}C]leucine (New England Nuclear, Boston, Mass.) at 37° with shaking in air. Puromycin (Calbiochem, San Diego, Calif.) was added to some flasks at zero time minus 15 min to a final concentration of 100 μg/ml. After incubation for 1 to 3 hr, the total hepatoma cell-incubation medium was frozen and thawed 3 times, brought to a final concentration of 1% sodium deoxycholate to dissolve cell membranes, then dialyzed against frequent changes of 0.9% NaCl solution containing 0.1% unlabeled L-leucine at 4° to remove free radioactive leucine.

The dialyzed samples were centrifuged and the clear supernatants were reconstituted to their original 3.5 ml volume by the addition of 0.9% NaCl solution. Then either 0.5 ml of pooled rat amniotic fluid was added to provide sufficient carrier AFP for immunoprecipitation or 0.5 ml of normal adult rat plasma was added to provide carrier fibrinogen. Aliquots of these samples were used for isolation of AFP, fibrinogen, and total cell protein.

Isolation of AFP, Fibrinogen, and Total Cellular Protein. AFP was isolated immunochemically from a 1.5-ml aliquot of dialyzed total hepatoma cell-incubation medium to which 0.5 ml of pooled rat amniotic fluid had been added, by the addition of 2.4 ml of unabsorbed monospecific rabbit antiserum to rat AFP. This amount rabbit antiserum to rat AFP was shown to yield maximum precipitation of AFP in preliminary titration and resulted in precipitation of 98 to 100% of the total AFP present in these samples, based on observations that (*a*) a 2nd addition of antiserum did not result in visible immune precipitation, and (*b*) a 2nd addition of rat amniotic fluid, followed by rabbit antiserum, to these samples did not result in the appearance of additional radioactivity in immune precipitates.

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[2] The abbreviations used are: AFP, α-fetoprotein; RPMI, Roswell Park Memorial Institute.

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Rabbit antiserum to rat AFP was prepared by injecting 0.2 mg purified rat AFP emulsified in Freund's complete adjuvant at 10-day intervals for a total of 4 injections. Purified rat AFP was isolated from amniotic fluid by disassociation of specific immune precipitates by the Nishi method (14). The monospecificity of the rabbit antisera was checked by immunoelectrophoresis of rat amniotic fluid against unabsorbed rabbit antisera and against antisera absorbed with pooled normal adult rat sera. Both antisera revealed a single precipitation band in the α_1-globulin region.

To reduce nonspecific absorption of radioactivity to a minimum, collected immune precipitates were washed twice with cold 0.9% NaCl solution, disassociated by the addition of 5.0 ml 0.1 M glycine-HCl buffer, pH 1.8, allowed to stand for 30 min at 37°, and centrifuged. The antigen-antibody complex was reprecipitated by adjustment of the clear supernatants to pH 7.0 by the addition of 0.4 M sodium phosphate (monobasic) and then was allowed to stand first at 37° for 2 hr, then overnight at 4°. Immune precipitates were collected by centrifugation, washed twice with 0.9% NaCl solution, dissolved in 2.0 ml of 0.1 N NaOH, then precipitated with 2.0 ml of cold 10% trichloroacetic acid. The acid-precipitated protein was washed twice with cold 5% trichloroacetic acid and twice with 0.9% NaCl solution.

Fibrinogen was isolated from a 1.5-ml aliquot of dialyzed total hepatoma cell-incubation medium extract, to which 0.5 ml of normal rat plasma had been added, by the addition of bovine thrombin as described previously (19).

Total hepatoma cell protein was obtained by precipitation with trichloroacetic acid. A 0.5-ml aliquot of dialyzed total hepatoma cell-incubation medium was diluted 10-fold with 0.9% NaCl solution; then, an equal volume of cold 10% trichloroacetic acid was added. The samples were kept at 5° for 2 hr, centrifuged, and the collected precipitates were washed twice with cold 5% trichloroacetic acid and twice with cold 0.9% NaCl solution.

Confirmation of AFP Synthesis by Radioimmunoelectrophoresis. Zajdela ascites hepatoma cells (13×10^7) were incubated in 3.5 ml of RPMI Medium 1640 with 10% fetal calf serum plus antibiotics for 24 hr at 37° with shaking in air. L-[^{14}C]leucine (3.5 μCi) was added at 0, 4, 8, and 20 hr during the incubation. After 24 hr, the total hepatoma cell-incubation medium was frozen and thawed, treated with sodium deoxycholate, and dialyzed as described above. After dialysis, the samples were centrifuged and the clear supernatants were concentrated 10-fold with a Minicon-B15 (Amicon Corp. Lexington, Mass.), and then were used for immunoelectrophoresis and radioautography. Microimmunoelectrophoresis was performed with 1% agarose and barbital buffer, pH 8.6, then diffused against monospecific rabbit antiserum to rat AFP. After immunoelectrophoresis, the slides were washed for 24 hr in repeated changes of 0.9% NaCl solution and, finally, in distilled water. Next they were dried and placed against Kodak no-screen industrial-type X-ray film and exposed for 3 months to obtain positive radioautographs.

Hepatoma Cell Protein Secretion *in Vitro*. To determine whether Zajdela ascites hepatoma cells secrete AFP and fibrinogen *in vitro*, these hepatoma cells (13×10^7) were incubated for 24 hr in 3.5 ml of RPMI Medium 1640 with 10% fetal calf serum plus antibiotics at 37° with shaking in air. A single amount of 3.0 μCi of L-[^{14}C]leucine was added at zero time. Hepatoma cell viability was monitored throughout the 24-hr incubation period microscopically by the trypan blue exclusion method. At intervals during 24 hr, hepatoma cells were separated from the incubation medium and washed twice with 0.9% NaCl solution, and then the cells were resuspended in 3.5 ml of fresh RPMI Medium 1640. AFP, fibrinogen, and total protein were isolated from both the separated hepatoma cells and the original incubation medium by methods described above.

RESULTS

Incubation of ascites hepatoma cells with L-[^{14}C]leucine resulted in rapid and continuous incorporation of radioactivity into AFP, fibrinogen, and total cell protein during 3 hr (Chart 1). In contrast, the addition of puromycin, a known inhibitor of cellular protein synthesis, markedly inhibited incorporation of radioactivity into these same proteins. The inhibition produced by puromycin supports the conclusion that incorporation of radioactivity observed in its absence reflects *de novo* protein synthesis rather than nonspecific absorption of free L-[^{14}C]leucine.

Synthesis of AFP by Zajdela ascites hepatoma cells was confirmed by radioimmunoelectrophoresis. Concentrated dialyzed extracts obtained from hepatoma cells incubated with L-[^{14}C]leucine to which carrier rat amniotic fluid had been added resulted in a single precipitin arc for AFP in the appropriate α_1 region, and produced a positive radioautography in duplicate experiments (Fig. 1). This demonstrates

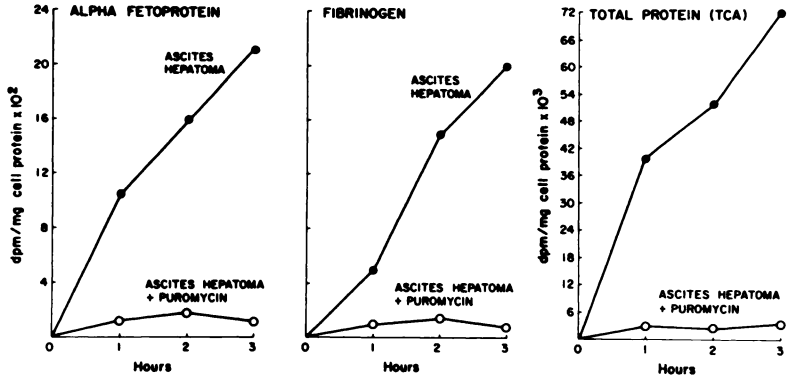

Chart 1. Time course of incorporation of L-[^{14}C]leucine into AFP, fibrinogen, and total protein during incubation of Zajdela rat ascites hepatoma cells. L-[^{14}C]leucine was added to zero time, and puromycin (at a final concentration of 100 μg/ml) was added at zero time minus 15 min. AFP was isolated immunochemically; fibrinogen, by addition of thrombin; and total protein, by trichloroacetic acid (*TCA*) precipitation. Each *point* is the mean of 3 incubation flasks.

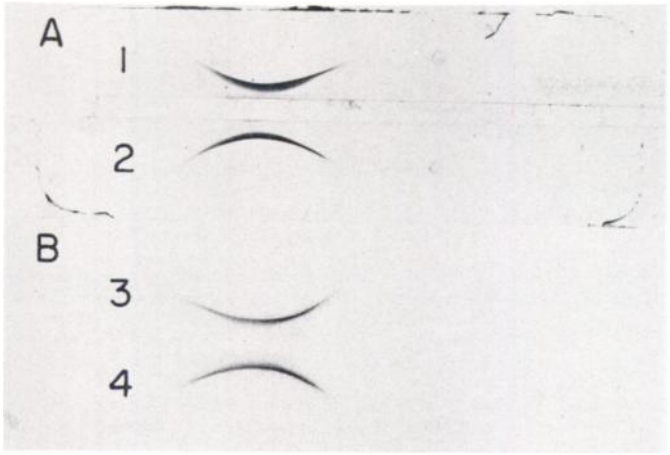

Fig. 1. Immunoelectrophoresis patterns (*A, 1* and *2*) and their radioautographs bottom (*B, 3* and *4*) obtained after incubating Zajdela rat ascites hepatoma cells with L-[^{14}C]leucine. The anode was to the *left*, and precipitin arcs were developed with the use of rabbit monospecific antiserum to rat AFP.

the monospecificity of the rabbit antiserum used and confirms *de novo* intracellular synthesis of AFP by these hepatoma cells.

To determine whether hepatoma cells secrete these serum proteins, cells were separated from the incubation medium at intervals during incubation with radioactive precursor. Chart 2 demonstrates that incubation of Zajdela rat ascites hepatoma cells for 24 hr resulted in rapidly increasing incorporation of leucine radioactivity into intracellular AFP, fibrinogen, and total cell protein, which reached a maximum at 16 hr, then declined thereafter. On the other hand, no radiolabeled AFP, fibrinogen, or total protein was detected in the incubation medium from 0 to 8 hr; only trace amounts were found at 16 hr, followed by the appearance of rapidly increasing amounts of these labeled proteins from 16 to 24 hr. On the other hand, it is of interest that the AH-66 strain of rat ascites hepatoma cells secretes or releases newly synthesized AFP and albumin within 0 to 60 min of their synthesis (10). Hepatoma cell viability monitored during these 24-hr incubations did not change appreciably from 0 to 8 hr (94 to 93%), decreased slightly at 16 hr to 90%, and then decreased markedly at 20 and 24 hr to 72 and 65%, respectively. The close correlation found at 16 to 24 hr between decreasing hepatoma cell viability, decreasing intracellular protein synthesis, and the appearance of rapidly increasing amounts of radio-labeled proteins in the incubation medium strongly suggests that the appearance of these radiolabeled proteins in the incubation medium resulted from cell death rather than from secretion. These data are consistent with the conclusion that healthy viable Zajdela rat ascites hepatoma cells do not secrete newly synthesized AFP or fibrinogen.

DISCUSSION

This study demonstrates that Zajdela rat ascites hepatoma cells, previously considered nonproducers, synthesize intracellular AFP and fibrinogen but fail to secrete or release appreciable amounts of these serum proteins. The evidence for synthesis of both AFP and fibrinogen in this study, and for synthesis of α-macrofetoprotein and albumin in a previous study (20), confirms the liver parenchymal cell origin of this strain of hepatoma cells and indicates that they retain synthesis of at least 2 fetospecific and 2 liver-specific antigens. The presence of undefined embryospecific liver antigens in Zajdela rat ascites hepatoma cells was reported earlier (9).

Failure of Zajdela ascites hepatoma cells to secrete rather than to synthesize serum proteins satisfactorily explains the failure to detect AFP in the serum or ascitic fluid of animals bearing this transplantable hepatoma and is consistent with the conclusion that secretory mechanisms for serum proteins are defective in this strain of hepatoma cells. Others have also reported evidence for defective serum protein secretion by hepatomas (3, 21).

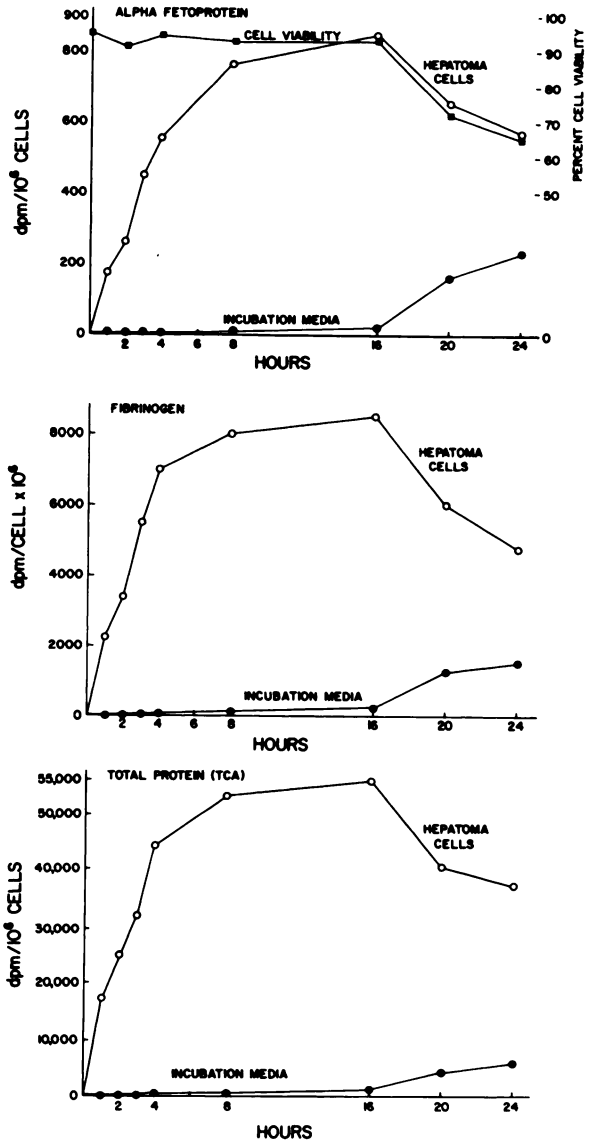

Chart 2. Time course of incorporation of L-[^{14}C]leucine added at zero time into AFP, fibrinogen, and total protein present in intracellular Zajdela rat ascites hepatoma cells and in the incubation medium. AFP was isolated immunochemically; fibrinogen, by the addition of thrombin; and total protein by trichloroacetic acid (*TCA*) precipitin. Each *point* is the mean of 3 incubation flasks.

Hepatomas are currently classified as either producers or nonproducers of AFP, based on its positive or negative detection in the serum or ascitic fluid of animals with carcinogen-induced and transplantable hepatomas *in vivo* (2, 22, 26, 27) or in the culture fluid of hepatoma cells *in vitro* (6, 7, 25). On the other hand, the present evidence that Zajdela ascites hepatoma cells synthesize detectable amounts of intracellular AFP without secretion indicates that some hepatomas should be classified as nonsecretors rather than nonproducers of this fetoprotein. It is of interest to speculate that failure to detect AFP in some human and animal hepatomas *in vivo* and *in vitro* may also reflect failure of secretion rather than failure of intracellular synthesis.

The unanswered question whether all hepatomas should be classified as secretors and nonsecretors rather than as producers and nonproducers of AFP has an important bearing on its possible biological role. The association of AFP production with fetal liver, regenerating adult liver, and hepatomas (23) suggests a causal relationship between AFP production and hepatocellular growth and proliferation. However, the generally accepted view that many, but not all, hepatoma cells produce AFP appears to indicate that AFP is not required for hepatoma cell growth, and would, therefore, preclude such a causal relationship. On the other hand, information indicating that all hepatoma cells synthesize detectable amounts of intracellular AFP, but some fail to secrete it, would be consistent with the conclusion that AFP synthesis is necessary for continued growth of hepatoma cells. Such information would serve to refocus attention on the possible relationship between AFP production and hepatocellular proliferation. It is apparent that an answer to this question can only be obtained by simultaneously measuring both intracellular synthesis and secretion of AFP by hepatoma cells, rather than relying exclusively on its appearance in the serum or culture fluid, which is the current practice.

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