Nucleic Acid and Phospholipid Synthesis in the Regenerating Liver of Tumor-bearing Mice

ATHANASIOS THEOLOGIDES AND B. J. KENNEDY

Department of Medicine, University of Minnesota Medical Center, Minneapolis, Minnesota 55455

SUMMARY

The metabolic interrelationships between the growth of a rapidly proliferating tissue and of a transplanted tumor were investigated in the same animal. Liver regeneration and tumor growth were studied in C3H mice bearing a transplanted mammary carcinoma.

The presence of the tumor did not affect significantly the rate of DNA synthesis in the regenerating liver from 24 to 36 hours after partial hepatectomy. It had no effect on the liver phospholipid and RNA turnover rates. The tumor itself did not show any variation in the rate of incorporation in phospholipids and RNA throughout the 48-hour study period, but demonstrated an early fall and a subsequent rise in DNA synthesis.

INTRODUCTION

The presence of a malignant growth elsewhere in the body alters markedly the metabolic pattern of the liver of the host. Although this has been substantiated by numerous studies, it is not clear whether it is due to a purely metabolic effect caused by the increased demands on the organism for the new tissue formation, or whether hormonal factors liberated by the tumor cause modifications in the metabolic processes of distant tissues. The fact that a malignant tumor continues to grow in the presence of body wasting and cachexia suggests that the autonomous growth of the neoplasm might have a priority for essential metabolites at the expense of the host tissue.

Any competition for essential metabolites or any alteration in the metabolic pattern of the host or of the tumor might be detected by investigating the metabolism of a rapidly proliferating tissue and a malignant neoplasm in the same animal. An excellent experimental model of such a rapidly proliferating tissue and anabolic focus is the regenerating liver following partial hepatectomy. By studying the regeneration in a tumor-bearing animal, any metabolic interrelations and mutual influences between regeneration and malignant growth would be uncovered.

For this purpose, DNA synthesis and RNA and phospholipid rates of turnover in the regenerating liver were compared in tumor-bearing and control mice.

MATERIALS AND METHODS

C3H (Bittner Z) mice of both sexes, 6-10 weeks old, were used. Their weight varied from 18-24 gm, but within a given experiment, mice of a similar age and similar weight were used. The animals were fed Purina laboratory chow and water ad libitum, and they were kept under standardized environmental conditions (lights on at 6 A.M., off at 6 P.M., and temperature 78 ± 1°F).

A spontaneous mammary carcinoma that arose in a C3H female mouse was used. Following successive subcutaneous transplantations of a small piece of tumor on the back of C3H mice, after several passages, the tumor appears to attain a relatively uniform rate of growth (7). This rate of growth was maintained throughout the study period and was such that tumors of 1.5-2 cm in diameter were recovered 5 weeks after inoculation, at the time of the experiments. In the control mice, a piece of muscle from the thigh of the tumor donor was transplanted subcutaneously. Ether anesthesia was used in these procedures.

All partial hepatectomies were performed between 8 and 10 A.M. under Nembutal anesthesia according to a modification (53) of the Higgins and Anderson technic (47) used on rats. The average amount of the median and left lateral lobes of the liver that is removed is 65.5% of the total liver (53). Mortality after surgery was less than 10%. After the operation, the animals were allowed Purina laboratory chow and water ad libitum.

Two hours prior to sacrifice of each group of partially hepatectomized control and tumor-bearing animals, the radioisotope, HP (carrier-free), as orthophosphate, was injected intraperitoneally. Using a 400-microcurie/ml solution of HP in normal saline, 4 microcuries/gm of body weight were injected. Following injection, the animals were housed in separate jars until the time of sacrifice.

The animals were sacrificed at 6, 12, 18, 24, 30, 36, 42, and 48 hours after partial hepatectomy. These time points represent the time of sacrifice of the first mouse in each experiment. The center of the sacrificing period was 20-40 minutes later depending on the number of animals at each experiment. This number varied from 6 to 10 in each control and in each tumor-bearing experiment.
group. The remaining lobes of the liver and the tumor in the tumor-bearing mice were removed.

The method for isolation of DNA and RNA used in this work is a modification and adaptation to radiochemical experiments of the methods of Schmidt and Thannhauser (86), Schneider (87), and Davidson and Smellie (24). This modified method has evolved in Barnum's laboratory (6, 8) and has been reviewed and evaluated recently for mouse liver by Hammer (44).

Each animal was analyzed individually. The liver and tumor were homogenized with ice-cold 5% trichloracetic acid in a Potter-Elvehjem homogenizer (81). The homogenate was transferred to a heavy-duty centrifuge tube, and after centrifugation, an aliquot of the supernatant was filtered into ashing tubes for acid-soluble phosphorus specific activity determination. The remainder of the supernatant was discarded. The pellet was resuspended and washed twice with 5% trichloracetic acid to remove any remaining acid-soluble phosphorus. To protect the nucleic acid from hydrolysis by the acid, the samples were kept cold in an ice bath during the two acid washes and the first wash with alcohol.

Following the two acid washes, the precipitate was extracted twice with 95% ethanol and once with Bloor's alcohol:ether (3:1) reagent with boiling for one minute. The sediment was then suspended in ethanol. An aliquot of the combined alcohol and Bloor's extracts was filtered into ashing tubes for phospholipid phosphorus specific activity determination.

The isolation of DNA and RNA was subsequently accomplished by extraction of the nucleic acids from the acid-insoluble lipid-free tissue residue in hot, concentrated salt solution. For this purpose, 10% NaCl buffered with 0.4 M tris(hydroxymethyl)-amino- methane (Tris) (0.4 M Tris-10% NaCl) at a pH of 7.8 was used. The tissue residue was heated for 30 minutes at 90-95°C with 3 ml and again for 15 minutes with 2 ml of 0.4 M Tris-10% NaCl. The nucleic acids were precipitated from the combined extracts with ethanol. The supernatant was discarded and the precipitate was washed with a 2:1 mixture of 95% ethyl alcohol and 0.4 M Tris-10% NaCl (alcohol-saline). For further removal of any protein contaminant, the two salt extraction steps, alcohol precipitation and alcohol-saline wash, were repeated once. Subsequently, to separate the RNA from the DNA, the RNA was hydrolyzed with hot alkali followed by precipitation of the DNA by cold acid. For the RNA hydrolysis, 0.1 N NaOH was used at 80-85°C for 30 minutes. The DNA was then precipitated with 2 N HCl and 10% trichloracetic acid. The supernate was filtered into ashing tubes for RNA-phosphorus specific activity determination. The precipitated DNA was washed twice with ice-cold 5% trichloracetic acid. The steps of the alkali hydrolysis, the acid precipitation, and the trichloracetic acid washes of the DNA were repeated once to remove any remaining RNA.

The purified DNA was then hydrolyzed with 5% trichloracetic acid for 30 minutes at 80-85°C. The hydrolysate was subsequently filtered into the DNA ashing tube for DNA-phosphorus specific activity determination.

For the wet ashing of acid-soluble phosphorus, phospholipid, RNA, and DNA, after the addition of 6 N H2SO4 for a final concentration of 0.6 N, the samples were heated first in an oven at about 90°C and finally in an ashing block at about 240°C. The oxidation and the clearing of the sample was completed by dropwise addition of HNO3. After clearing, any pyrophosphates formed were hydrolyzed by adding H2O and boiling for 15 minutes. Then the acid-soluble phosphorus, phospholipid, and RNA were diluted to a volume of 10 ml and the DNA to 5 ml.

The samples were counted at infinite thickness in plastic cups fitted with Mylar bottoms using an end-window Geiger tube, and the counts per minute of each sample were corrected for counter loss, background, and decay. Phosphorus content was determined by the Fiske and SubbaRow method (31). The specific activity of the sample was calculated as counts per minute per microgram of phosphorus. The relative specific...
activity is an expression of the specific activity of DNA, RNA, and phospholipid phosphorus as a percent of that of acid-soluble phosphorus. The use of the acid-soluble specific activity to calculate the relative specific activity tends to compensate for any error of injection of the isotope, although there was uniformity within a given experiment in the amount of \(^{32}\)P injected and no significant variation in that amount was observed from experiment to experiment.

With the extensive sequence of purification steps in this method, highly purified DNA and RNA are obtained (44).

RESULTS

The presence of the tumor did not alter measurably the usual time of onset of liver regeneration following partial hepatectomy (Chart 1). Initiation of marked DNA synthesis in tumor-bearing and control animals was between 24 and 30 hours.

The rate of DNA synthesis was higher in the regenerating liver of tumor-bearing mice at 24 to 36 hours \( (P\) value at 24 hours, \( P < 0.05\), at 30 hours, \( P < 0.001\), and at 36 hours, \( P < 0.05\) ). At 42 hours, the rate of DNA synthesis was similar in both groups of animals. At 48 hours, the rate of DNA synthesis was significantly higher \( (P = 0.05)\) in the control than in the tumor-bearing mice.

There was no difference in the rates of \(^{32}\)P incorporation in RNA in the two groups (Chart 2). No difference was observed either in phospholipid turnover rates in the two groups (Chart 3).

In the tumor (Chart 4), the phospholipid and RNA turnover rates remained the same throughout the 48-hour study period. An interesting finding in the tumor metabolism was a significant fall in the rate of DNA synthesis at 18 hours and a marked increase at 30 hours posthepatectomy.

DISCUSSION

It has been observed that the average weight of the tumor-free livers of rats and mice with spontaneous or transplanted malignant tumors elsewhere in the body is greater than that of tumor-free comparable animals (4, 70, 99, 100).

Quantitative studies of DNA, RNA, purines, pyrimidines, methionine, arginine, histidine, threonine, glycine, and cystine content of the liver in transplanted tumor-bearing animals showed a marked increase of these components per gm of dry tissue (19-21, 63, 82). A similar rise in the concentration of nucleic acids was observed in mouse liver during gestation (83).

Work from another laboratory (55, 56, 77-79) showed that there was a significant increase in the DNA turnover rates in...
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Liver phospholipid specific activity as percent of acid-soluble phosphorus specific activity at various times after partial hepatectomy. $^{32}P$ was injected intraperitoneally two hours before sacrifice. Computations were done on a log scale and antilogs were taken.

The regenerating liver provides an excellent experimental model for a rapidly proliferating tissue. Following partial hepatectomy in rats and mice, restoration of the liver weight occurs rapidly. Although nothing is known regarding the mechanism by which this process of regeneration is initiated, controlled, and terminated, extensive work has been done regarding the rate and the extent of this restoration (8, 9, 12, 13, 45-47, 52, 54, 61, 94, 96). An attractive hypothesis for the cybernetics and the mechanism of restoration of lost liver tissue has been that of humoral control factors. According to this theory, following the partial hepatectomy, changes take place in the blood constituents capable of initiating and controlling the process of regeneration.

Akamatsu (2) observed that tissue cultures of rabbit liver grew better in plasma from partially hepatectomized animals than in normal plasma. Christensen and Jacobsen (23), Bucher et al. (14), Wenneker and Sussman (97), and Hurowitz and Studer (49) and their co-workers demonstrated that partial hepatectomy on one parabiotic partner resulted in increased cellular proliferation and growth not only in the liver of the hepatectomized animal, but also of the intact liver of the parabiont. But other investigators (3, 51, 85) failed to elicit any statistically significant response in hepatic mitotic index or DNA synthesis in the nonhepatectomized parabiotic partner.

Glinos and co-workers (37-41) found that blood serum from partially hepatectomized animals enhanced the growth of various tissue cultures, but he also observed similar enhancing effect by diluting the normal serum or by decreasing the concentration of serum constituents in vitro through plasmapheresis. On the other hand, a high concentration of normal serum...
demonstrated inhibitory effects. Friedrich-Freksa and Zaki (33, 101) observed that the injection of serum from rats subjected previously to partial hepatectomy increased the mitotic rate in the liver of normal rats. Similar results were obtained also by Stich and Florian (93), Smythe and Moore (92), Adibi et al. (1), Hughes (48), and Survis et al. (95). Laquerrière and Laumonier (60), using a similar procedure, have reported an increase in the DNA content of the liver nuclei studied histophotometrically. Other workers (29, 57, 64, 72, 80) failed to show any significant increase in liver mitosis or DNA-phosphorus specific activity following the injection of serum or plasma from partially hepatectomized donors into normal recipients.

A contradiction exists in the evidence for the reduction in frequency of mitosis in the regenerating liver following injection of serum or plasma from a normal animal (57, 64, 92, 93, 95). Equally conflicting are the data of enhancement of the normal posthepatectomy, regenerative activity by injection of serum from similar animals undergoing liver regeneration (57, 64, 72, 92, 95).

Additional evidence for the presence of humoral changes influencing growth was brought about by Paschkis and his colleagues (73, 76) who found a significant increase in the mitotic index of some other tissues in animals undergoing liver regeneration. Sigel et al. (91), using a technic of introducing liver autotransplants, observed that after the excision of most of the non-transplanted liver in the animal, the autotransplanted hepatic graft grew larger. Ballantine (5) noted excessive growth of fetal liver following maternal partial hepatectomy, and he considered this as an evidence of stimulatory factor passing through the placenta. Although Paschkis et al. (74) had reported previously that liver regeneration was identical in nonpregnant and pregnant animals, Gershbein (35) found later that pregnancy was causing a marked increase in extent of liver regeneration in partially hepatectomized rats.

Most of the reviewed evidence seems to indicate that following partial hepatectomy, a change in the blood constituents occurs, which affects liver growth and probably other tissue growth. Although the problem of a relationship between regeneration and tumor formation (88) is beyond the scope of the present discussion, pertinent at this point is the question of whether blood changes and humoral factors during regeneration might affect tumor formation and tumor growth, or whether any simultaneous malignant growth might affect liver restoration.

Glinos et al. (40) observed that liver regeneration was relatively effective in accelerating the rate of appearance of tumors resulting from exposure to a carcinogen. Cantarow, Paschkis, and their colleagues (15, 75) found that certain transplanted tumors grew more rapidly in the partially hepatectomized than in the intact control rats and also that liver regeneration was
not depressed in the presence of growing tumor. Fisher and Fisher (28) reported that hepatectomy immediately after intra-
portal tumor cell injection resulted in an increased incidence of hepatic metastases over control animals. DePeyster et al. (25) noted that partial hepatectomy resulted in augmentation of subcutaneous tumor takes and increased tumor growth in parabiotic animal experiments where one partner had a partial hepatectomy and the other received implantation of tumor cells subcutaneously. Analogous results were also obtained by Enchave Llanos and Saffle (27). Gersbein (36) reported that subcutaneous transplantation of various tumors did not alter the extent of liver regeneration.

A potential effect upon hepatic regeneration of serum or plasma from patients with cancer and from tumor-bearing animals has been investigated by several workers. The results remain contradictory as to whether normal serum inhibits liver regeneration and serum from patients with cancer produces a stimulation of regenerating liver (11, 30, 50, 67-69, 98).

Gel'stein (34) observed that the liver response to partial hepatectomy is markedly suppressed during the first state of carcinogenesis induced in the liver by orthoaminoazotoluene. Bolasny et al. (11) studied the effects of partial hepatectomy on mammary carcinoma in the rat induced by 3-methylcholanthrene. They concluded that partial hepatectomy inhibits the induction of mammary carcinoma.

The present work has demonstrated that the transplanted tumor had no measurable influence on the usual time of onset of DNA synthesis by the remaining liver after partial hepatectomy. This finding cannot be presented as evidence of absence of any growth-stimulatory factors liberated by the tumor, because a 24-hour period might be required for intracellular metabolic changes prior to initiation of DNA synthesis and mitosis.

An enhancement in the rate of DNA synthesis in the liver of tumor-bearing mice was demonstrated for the period 24 to 36 hours after partial hepatectomy. At 42 hours, there was no difference in the rate of DNA synthesis in the two groups. At 48 hours, the rate was significantly higher in the control animals.

These observations indicate that the presence of the tumor initially stimulated the rate of DNA synthesis of the rapidly proliferating normal tissue. But later, the presence of the tumor had the reverse effect.

This variation of the effect of the tumor on liver regeneration at various posthepatectomy periods explains the contradictory reports in the literature, where stimulation, inhibition, or no effect on liver restoration has been described. Obviously, the posthepatectomy period studied was different. The observations by Enchave Llanos (20) are in accord with this explanation. He found a variation in mitotic activity responsiveness of the regenerating liver of the recipient when homogenates of regenerating liver were injected at different times of the day.

The significant fall in tumor DNA synthesis at 18 hours and the rise at 30 hours remains unexplained.

It appears from the present study that the presence of tumor elsewhere in the animal affects the rate of DNA synthesis during restoration of liver after partial hepatectomy, causing an initial stimulation and a subsequent depression. The regenerating liver, on the other hand, affects also the rate of DNA synthesis of the tumor, causing an initial suppression and a subsequent stimulation.

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Athanasios Theologides and B. J. Kennedy


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