ABSTRACT

The microcirculation of nodules (0.5 to 10 mm in diameter) from diethylnitrosamine-treated rats was studied in perfused livers. Microlight guides were placed on nodules and surrounding tissue on the capsular surface of the liver to measure fluorescence due to fluorescein-dextran (12 μm), a dye confined to the vascular space, infused via the hepatic artery and portal vein separately or simultaneously. The fluorescence increase due to fluorescein-dextran infusion via the artery and vein simultaneously was used to compare vascular space in nodules with that of surrounding tissue. The vascular space of nodules less than 1 mm in diameter was only about one-half as large as that of surrounding tissue. In contrast, in nodules 1 to 2 mm in diameter, the vascular space was similar to values from surrounding tissue. This was largely due to an increase in the fluid entering via the artery. As nodules grew from 2 to 10 mm in diameter, the vascular space decreased as a function of nodule size to 40% of surrounding tissue. The sum of fluorescence increases due to fluorescein-dextran infused via the artery and vein separately always equaled values obtained from simultaneous infusions. From these measurements, the fraction of vascular fluid observed by the microlight guide that entered the liver via the artery was calculated. In tissue surrounding nodules, fluid entering from the artery was 19% of the total, a value approximating the fraction of fluid pumped into the liver via the artery (25%). The percentage of fluid in the nodule that entered the liver via the hepatic artery increased progressively to 100% of the total as nodules grew from 2 to 10 mm in diameter. Thus, nodules become increasingly dependent on the hepatic artery and less dependent on blood supply via the portal vein as they grow.

INTRODUCTION

Several carcinogen treatment regimens induce large numbers of histochemically defined, microscopic foci of altered hepatocytes in rodent livers; however, only a small fraction of these foci become macroscopic nodules, and relatively few emerge as hepatocellular cancers (15, 29, 30, 33). A variety of factors may influence the process of carcinogenesis after the initial target cell-carcinogen interaction including changes in oxygen and nutrient delivery and hormone concentrations. Prat et al. (31) observed that portal caval anatomosis performed on rats having carcinogen-induced hepatic foci and nodules increased the incidence and frequency of nodules and malignant tumors. However, Rothstein et al. (32) did not observe any effect of diversion of portal blood supply with ameroid constrictors on the number of nodules per volume of liver at latter time points. Thus, the effect of alterations of blood flow on the progression of foci to nodules and cancer in liver is complex and not understood completely.

Previous work has shown that metastatic (1-3, 5, 25) and primary liver carcinomas (27, 28, 37) in rodents have diminished blood flow from the portal vein and normal or increased arterial blood supply. In addition, the ability to induce capillary ingrowth is characteristic of several cancers (13, 38). However, few studies have addressed the vascular supply of small, putative preneoplastic lesions. In studies with radiolabeled microspheres, Solt et al. (35) observed that large (>4 mm in diameter) diethylnitrosamine-induced nodules generally have low blood flow via the portal vein, and few exhibit increased blood flow via the hepatic artery. Since microcirculatory changes may take place in much smaller lesions, we previously characterized the portal circulation of small nodules, 0.5 to 9.0 mm in diameter, by measuring intracellular pyridine nucleotide fluorescence and fluorescence due to infused dyes (9) with fiber-optic microlight guides placed on the surface of livers perfused via the portal vein. In surrounding tissue and nodules less than 2 mm in diameter, pyridine nucleotide fluorescence increased sharply when inflow perfusate oxygen tension was lowered due to reduction of pyridine nucleotides indicating previous normoxia; however, when perfusate oxygen tension was lowered in nodules greater than 2 mm in diameter, fluorescence did not increase. This demonstrated that NADH was reduced maximally previously; i.e., the nodules were anoxic under these experimental conditions. Anoxia in nodules greater than 2 mm in diameter was due to restricted portal circulation, since fluorescence due to infused 7-hydroxycoumarin and fluorescein could be detected from normal tissue and small nodules but not from nodules greater than 2 mm in diameter. It was concluded that nodules undergo a loss of portal circulation as they grow from 1 to 4 mm in diameter (9).

Methods to compare the vascular space in local areas on the liver surface and to quantitate the fraction of fluid entering the liver via the portal vein or hepatic artery based on local fluorescence of fluorescein-dextran, a dye confined primarily to the vascular space, have been developed (10). In the present study, the microcirculation of nodules from rats treated chronically with diethylnitrosamine for 3 to 4 months was studied using these new methods.

MATERIALS AND METHODS

Treatment of Rats. Male Fischer 344 rats [CDF(F344)/CrBr] weighing 130 to 150 g (Charles River Breeding Laboratories, Inc., Wilmington, MA) were given diethylnitrosamine (40 ppm) (Eastman Kodak, Rochester, NY) in the drinking water for 3 to 4 months. Rats (250 to 400 g) were then utilized for perfusion experiments 1 to 4 weeks after diethylnitrosamine treatment was administered.
Liver Perfusion. Perfusion fluid was Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with oxygen (95%) and carbon dioxide (5%). Rats were anesthetized with pentobarbital (80 mg/kg). The abdominal cavity was opened, ties were placed around the portal vein and common hepatic artery, and the branch of the common hepatic artery supplying the stomach was ligated. The portal vein was then cannulated and perfused at flow rates of 24 ml/min. Next, the common hepatic artery was cannulated with a 28-gauge needle, and flow was initiated at 8 ml/min. Effluent perfusate was collected via a cannula placed in the vena cava and directed past a Teflon-shielded, Clark-type oxygen electrode to assess tissue viability (34). Flow rates ranged from 2 to 3 ml/min/g.

Surface Fluorescence Measurements. Fluorescein-dextran (Sigma Chemical Co., St. Louis, MO) was dissolved directly in perfusate at a concentration of 12 mM. To measure fluorescein-dextran fluorescence, a dual-channel, microlight guide consisting of 2 optical fibers glued together to form a tip 170 μm in diameter (16, 18) was placed on the surface of nodules (gray-white color) or surrounding tissue (brown) using a micromanipulator. Light from a xenon lamp was passed through a glass filter (No. 5562; Swift Glass, Elmira, NY), and the resultant excitation light (400 to 460 nm) was transmitted to the tissue through one channel of the microlight guide. Through the parallel channel, emitted light greater than 515 nm was transmitted to a photomultiplier using a gelatin secondary filter (Wratten No. 16; Kodak). The signal was amplified and recorded as described elsewhere (16, 18). Microlight guides observe a volume extending about 300 μm deep into the perfused liver and about 100 μm wide. This volume includes about 300 hepatocytes (23).

Histology. At the end of each experiment, the nodule studied was marked by inserting thin glass fibers, frozen in liquid nitrogen and sectioned serially (6 μm) perpendicular to the capsular surface. Sections were stained for γ-glutamyltranspeptidase (24), glucose-6-phosphatase (40), and ATPase (41), and the maximum diameter of each nodule was measured using a calibrated eyepiece reticle in a Nikon Optiphot microscope.

RESULTS

Chart 1 illustrates typical surface fluorescence measurements from tissue surrounding a nodule and in a 1.5- and a 3-mm-diameter nodule. Microlight guides were placed on nodules and surrounding tissue on the surface of livers perfused simultaneously via the hepatic artery and portal vein at flow rates of 8 and 24 ml/min, respectively. After a stable fluorescence baseline was established, the perfusate entering via the portal vein was switched to buffer containing fluorescein-dextran (12 μM). After a short lag due to the dilution of dye (10), surface fluorescence from surrounding tissue and the 1.5-mm-diameter nodule increased to steady-state values of 78% and 69% over basal fluorescence, respectively, in about 15 s (Chart 1). At the termination of fluorescein-dextran infusion, fluorescence returned to baseline values in 15 to 20 s. In contrast, fluorescein-dextran infused via the portal vein did not increase the fluorescence of the nodule 3 mm in diameter. Fluorescein-dextran was then infused via the hepatic artery. In surrounding tissue and the 1.5-mm-diameter nodule, fluorescence increased 17% and 13% above basal values, respectively (Chart 1). The increase in fluorescence in the 3-mm-
diameter nodule was 4-fold greater than the increase from surrounding tissue or small nodules. When fluorescein-dextran was infused simultaneously via the vein and artery, the fluorescence increase closely approximated the sum of the increases due to infusion via the vein and artery separately (Chart 1). For example, in surrounding tissue, the sum of the increases due to infusion via the vein and artery (78 + 17 = 95%) equaled the increase due to infusion simultaneously via the artery and the vein (94%). Three to 7 fluorescence measurements were made from each nodule and surrounding tissue with the microlight guides reset each time in order to average nodule microheterogeneity and to determine the response of tissue on all sides of the nodule.

The average fluorescence increase due to fluorescein-dextran infusion via the vein or artery in 56 individual nodules is plotted as a function of nodule size in Chart 2. As nodules increase in size from 0.8 to 4 mm in diameter, the fluorescence increase due to fluorescein-dextran infusion via the portal vein decreased progressively (Chart 2A). Fluorescence due to infusion of fluorescein-dextran via the portal vein could not be detected in nodules greater than 5 mm in diameter. In contrast, when fluorescein-dextran was infused via the artery, most nodules less than 1.5 mm in diameter showed only a slight increase in fluorescence, while large fluorescence increases were observed in nodules greater than 1.5 mm in diameter (Chart 2B). There was no relationship between fluorescence increases detected in surrounding tissue and the size of nodule studied.

The increase in fluorescence due to fluorescein-dextran infusion via the vein and artery separately or simultaneously was expressed as a percentage of the increase detected in surrounding tissue. In the smallest nodules studied (0 to 1 mm in diameter), the fluorescence increase due to fluorescein-dextran infusion via the artery and vein simultaneously was about half that of surrounding tissue (Chart 3); however, the fluorescence increase equalled surrounding tissue in nodules 1 to 2 mm in diameter (Chart 3). In nodules larger than 2 to 4 mm in diameter, fluorescence increases were 40 to 70% of surrounding tissue (Chart 3).

When dye was infused exclusively via the portal vein, the increase in fluorescence was less than surrounding tissue in all size groups studied (Chart 4A). In nodules less than 2 mm in diameter, the increase in fluorescence was about 50 to 60% of surrounding tissue. Further, this value decreased progressively as a function of size as nodules grew greater than 2 mm in diameter (Chart 4A). In contrast, the relationship between fluorescence increases due to infusion of fluorescein-dextran exclusively via the hepatic artery and nodule size was complex (Chart 4B). In nodules less than 1 mm in diameter, the fluorescence increase was only 40% of that of surrounding tissue; however, this value increased progressively to readings 8-fold greater than that of surrounding tissue in nodules 5 mm in diameter. In the largest nodules studied (5- to 10-mm diameter), fluorescence increases due to infusion via the artery tended to be only slightly greater than surrounding tissue.

In livers perfused exclusively via the portal vein, fluorescence from the liver surface was directly proportional to the concentration of fluorescein-dextran infused (10). This relationship also held when the concentration of dye was altered by dilution in experiments where livers were perfused via the artery and vein (10). The sum of fluorescence increases due to dye infused via the artery and vein approximated the value obtained from simultaneous infusions closely (Chart 1); therefore, the fraction of vascular fluid derived from the hepatic artery could be calculated from the dilution of dye in the vascular space observed by light guides placed on livers perfused via the artery and vein. For example, fluorescein-dextran (12 μM) infused via the artery and vein simultaneously increased fluorescence 94% over basal, whereas fluorescein-dextran infused only via the artery increased fluorescence 17% over basal (normal tissue in Chart 1) due to dilution of dye by fluid entering the liver via the portal vein. From these data, the fraction of vascular fluid derived from the hepatic artery was calculated (e.g., 17%/100 = 18%). In tissue surrounding nodules, the average fluorescence increase due to
infusion via the artery was $19 \pm 2\%$ ($n = 56$) of the total, in fairly close agreement with the fraction of fluid pumped into the liver via the artery (25%). In nodules, however, the percentage of fluorescence increase due to dye infused via the artery was related strictly to nodule diameter. As nodules grew from 1 to 4 mm in diameter, the value increased from 20% to 83%. In large nodules (>4 mm), over 80% of the fluorescence arose from dye infused via the hepatic artery (Chart 5).

Tissues were stained for one positive marker for hyperplastic nodules (6, 12), $\gamma$-glutamyltranspeptidase, and 2 negative markers (11, 12, 14), glucose-6-phosphatase (40) and ATPase (41). Nodules were predominantly $\gamma$-glutamyltranspeptidase positive (83%) and ATPase (65%) and glucose-6-phosphatase (63%) negative, confirming earlier results (9). We could not detect any relationship between staining characteristics and microcirculation in nodules.

**DISCUSSION**

**A New Method for Studying Microcirculation of Hepatic Nodules.** The differential pigmentation of periportal (light) and pericentral (dark) regions of the liver lobule has been used to place microsight guides on sublobular areas of the perfused liver to monitor local biochemical processes noninvasively (4, 7, 8, 17–21). We have taken advantage of the color differences between hyperplastic nodules (white-gray) and surrounding tissue (brown) to place microsight guides on nodules induced with diethylnitrosamine (9). In our initial studies, perfusate entering via the portal vein did not penetrate nodules larger than 2 to 3 mm in diameter (9); however, the role of the hepatic artery was not evaluated.

Fluorescein-dextran fluorescence from the liver surface has been used to quantitate vascular space (10). This method is based on the observation that fluorescence due to fluorescein-dextran was directly proportional to vascular space when the vasoconstricting hormone, epinephrine, was infused into perfused livers from normal rats. Differences in vascular space between periportal and pericentral regions determined with the
MICROCIRCULATION OF HEPATIC NODULES

Chart 5. Percentage of vascular fluid in nodules derived from the hepatic artery. The average fluorescence increase (percentage over basal) due to fluorescein-dextran infusion via the artery divided by the sum of fluorescence increases due to infusion of dye via the artery and vein was calculated (see Chart 1 and "Results"). Error term is absent in nodules 5 to 10 mm in diameter because all dye entered the liver via the hepatic artery (Chart 4A). Other conditions as in Chart 4. Numbers in parentheses, number of nodules in each size group.

fluorescein-dextran method were validated with morphometric measurements of vascular space in sublobular areas (10). Thus, fluorescence of fluorescein-dextran infused simultaneously via the artery and vein can be used to quantitate vascular space in nodules and surrounding tissue. Furthermore, the percentage of fluid that entered the nodules via the artery was quantitated (e.g., fluorescence increases due to infusion via the artery divided by the increase due to infusions via the artery and the vein simultaneously). Moreover, since fluorescence signals are proportional to the concentration of dye observed by the light guide (10), nodules and surrounding tissue can be compared.

There are several advantages of this new methodology. (a) The fluid entering the vascular space via the portal vein and hepatic artery can be evaluated in individual nodules. (b) Nodules less than 1 mm in diameter can be studied, since the tip of the microlight guide is very small (170-µm diameter).

Changes in the Microcirculation of Nodules as a Function of Size. The vascular space in small nodules was decreased by about one-half (Chart 3). Restriction of portal and arterial circulation in small nodules is most likely due to the compression of tissue in the small nodules. This is consistent with twisted configurations of double and triple thick plates of hepatocytes often observed in nodules (12). In larger nodules (1 to 2 mm in diameter), the vascular space occupied by dye delivered via the artery increased to 400% of surrounding tissue, while the portal value remained constant at around 60% of surrounding tissue (Chart 4). These data are consistent with the hypothesis that growth of nodules larger than about 1 mm in diameter requires the ingrowth of arterial microvessels. The ingrowth of vessels may be initiated by hypoxia within small nodules as a result of restricted oxygen delivery due to tissue compression (Charts 3 and 4). Angiogenesis was recently shown to be triggered by hypoxia in macrophages (22). Thus, the ability to initiate arterial ingrowth may define a subpopulation of small nodules that can grow larger than 1 to 2 mm in diameter.

As nodules continued to grow from 2 to 10 mm in diameter, a progressive decrease in vascular fluid derived via the portal vein concomitant with an increase in fluid delivered via the artery was observed (Charts 3 and 4). Furthermore, the vascular fluid derived from the artery increased progressively from 40 to greater than 80% of the total as the nodules grew (Chart 5); however, the dramatic loss of fluid derived via the portal vein was only compensated for partially by the increase in fluid derived from arterial vessels, since the vascular space in nodules decreased from 70 to 40% of surrounding tissue as nodules grew from 2 to 10 mm in diameter (Chart 5). In vivo, small increases in arterial circulation may compensate for a loss in oxygen supply due to a relatively large decrease in portal circulation, since the concentration of oxygen in arterial blood is 2- to 3-fold higher than in portal blood (26).

These results and conclusions agree with the observation by Solt et al. (35) that large hyperplastic nodules have decreased blood flow via the portal vein in vivo and a report by Nilsson et al. (27) that ligation of the hepatic artery caused necrosis of 48 of 66 primary liver tumors in rats. Our conclusion that the progression from portal to predominantly arterial circulation is a function of nodule size is also consistent with the observation of Ackerman et al. (3) that delivery of radiolabeled albumin to small (about 0.16 g) metastatic Walker 256 carcinomas implanted on the surface of the liver via the portal vein or hepatic artery was similar to surrounding tissue; however, in large tumors (about 1.3 g), microsphere delivery via the hepatic artery increased 4-fold, whereas delivery via the portal vein decreased.

In several carcinogen treatment regimens, only a small fraction of foci become nodules (15, 29, 30, 33, 39); however, in the resistant hepatocyte model of Farber and coworkers (12, 36), all foci become nodules. The growth of foci to nodules may be a prerequisite for the development of some cancers, since carcinomas have been observed developing within hyperplastic nodules (13). In view of these data, Farber has suggested that progression from foci to nodules may be a multistep process, and that only a small subpopulation of foci has the ability to complete the necessary steps (12). Our data are consistent with the hypothesis that the progressive growth of small nodules requires the ingrowth of arterial microvessels. Nodules unable to arterialize may never grow larger than 1 mm in diameter, the upper limits of oxygen diffusion in liver. For example, tumor tissue transplanted in the rabbit cornea will not grow larger than 1 mm in diameter without arterial vascularization (13). Furthermore, drugs which inhibit angiogenesis inhibit the growth of metastases greater than 1 mm in diameter (38).

The shift from predominantly portal to arterial circulation may provide nodular tissue some autonomy from factors that normally regulate the replication of hepatocytes. For example, following arterIALIZATION, nodules will be exposed to lower concentrations of growth-inhibitory carcinogens (12) and endogenous compounds absorbed by the gastrointestinal tract and cleared largely in the first pass through the liver. It follows that arterIALIZATION of nodular tissue may be critical for the development of cancer.

REFERENCES

3. Ackerman, N. B., Lien, W. M., Kondi, E. S., and Silverman, N. A. The blood supply of experimental liver metastases. I. The distribution of hepatic artery
4. E. Farber, personal communication.


Microcirculation of Hepatic Nodules from Diethylnitrosamine-treated Rats

James G. Conway, James A. Popp and Ronald G. Thurman