ABSTRACT

Tumor cell proliferation is dependent upon concurrent growth of a supporting vasculature. This study aims to characterize the structural features of the microvasculature within a primary tumor model. There were 22 colon tumors induced in 16 rats by repeated administration of dimethylhydrazine. A cast of the microvessels was prepared by intraarterial administration of acrylic resin (Mercox). After corrosion of the tissue, the cast was examined by scanning electron microscopy.

Tumors 2.6 to 12.0 mm in diameter were examined. Within polypoid carcinomas up to 5.7 mm in diameter, there were two distinct vascular zones, a luminal vascular zone continuous with the vasculature of normal mucosa and a central zone continuous with the normal submucosa and muscularis propria vessels. Within both vascular zones, the organization of microvessels had the same general pattern as in normal mucosa. However, in tumors with diameters >5.7 mm, the vasculature was seen to be disorganized and of a greater density than normal.

In the smallest tumors, few morphological changes were seen in the individual microvessels when compared to normal. However, with tumor growth, there was elongation and increased diameters of the microvessels within the tumor. Microvessels within the luminal zone of the tumors which could definitely be traced to veins had diameters of 50 to 100 μm (compared to 12 to 30 μm for normal venules). Individual microvessels varied in diameter along their course forming saccular dilations in places. Networks of frequently Anastomosing microvessels were formed. Extravasation of resin occurred from some microvessels. Elongated vessels of uniform diameter which travel distances up to 2 mm without branching were seen and were probably arteries.

These appearances indicate that there are two distinct stages of development of the vasculature within primary tumors, an early phase where the tumor is supplied by the preexisting host microvessels, followed by a phase of proliferation of new vessels with abnormal morphological characteristics.

INTRODUCTION

Tumor cell proliferation is dependent upon concurrent growth of a supporting vasculature (1). In their studies of transplantable tumors, Gimbrone et al. (2) found that, after an initial prevascular phase during which tumor cells will proliferate, growth of an avascular tumor will stop when the tumor diameter reaches 1–2 mm. Further growth in the tumor cell population must be preceded by an increase in the microvessels that supply the tumor. This neovascularization has a marked effect on the rate of growth of the tumor. The development of the vasculature is also associated with the capacity of the tumor to metastasize (3). The stimulus for the development of the microvasculature appears to arise from the tumor itself (4) and a number of soluble factors, derived from both neoplastic and nonneoplastic tissues, are capable of inducing angiogenesis in experimental systems (5, 6).

Studies of the microvasculature within normal tissues have shown that the microcirculation has an important role in the specialized functions of that tissue and that the morphological features and spatial arrangement of the microvessels have adapted to facilitate those functions (7–9). Furthermore, the vessels are organized in a manner that results in efficient delivery of oxygen and nutrients to the tissue.

The morphology and spatial arrangement of the tumor vasculature have been studied almost exclusively in transplantable tumors. These studies describe a chaotic or disorderly array of capillaries within the substance of the tumor implant that is supplied by dilated arterioles and drained by large veins in the surrounding host tissues (10, 11). Capillaries within the tumor are tortuous and sinusoidal with diameters up to 200 μm. They form a dense vascular network which is profusely anastomotic (11). Thin and tapered capillaries were also noted at the edge of the necrotic center of the tumor (12). The sequential development of the microvasculature in tumor implant models has been described (11, 13) and indicates that while the tumor is nourished by the host vasculature initially, there is progressive dilation and tortuosity of the host venules with bulbous formations along their length. This is followed by sprouting of endothelial buds from these venules and from capillaries to eventually form a dense network of capillaries. With growth of the tumor implant, the relative vascular volume decreases and the center of the tumor becomes avascular and necrotic (10, 12).

Little is known of the characteristics of the individual microvessels and their spatial organization within primary in situ tumors. This has been noted by other authors (14). Furthermore, primary tumors arise within tissues which already have their own vasculature. Therefore, there are two possible sources of vascular supply within primary tumors. They may receive their vascular supply (a) by modification of the preexisting host vascular bed or (b) by the development of new vessels (angiogenesis). The relative role of each of these possible sources of vascular supply during tumor growth is not established. Therefore, the aims of this study are to determine the origin of the vascular supply within an experimental primary tumor and to characterize the spatial arrangement and morphological features of the microvessels within a primary tumor model.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (400–500 g) were housed in a temperature-controlled room with 12 h of light daily. They were fed Clark King GR2+ pellets and water ad libitum.

Tumor Induction. Colon tumors were induced by s.c. injections of dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO), at a dose of 20 mg/kg. Rats received weekly injections from the age of 4 weeks for a total of 24 weeks.

Tumor Detection. Following completion of dimethylhydrazine injections, colonoscopy was performed weekly to detect the presence of tumors. Under light ether anesthesia, the colon was examined using a flexible nasopharyngoscope (Fujinon NAP-F, Fuji Photo Optical Co., Ltd., Japan). The site, size, and number of colon tumors were recorded.

Vascular Casting. Vascular casts were prepared at least 24 h after colonoscopy. Each rat was anesthetized with methohexitone sodium (El Lilly & Co., New South Wales, Australia), 50 mg/kg i.p. The renal pedicles on both sides were then clamped through loin incisions. The thoracic aorta was exposed and a cannula (internal diameter, 1.57 mm) was inserted caudally and secured with ties. Blood was then washed out from the vascular system by infusing a filtered 0.154 M NaCl solution.
containing heparin, 10,000 units/liter (Weddel Pharmaceuticals, New South Wales, Australia), 1 μM papaverine (David Bull Laboratories, Victoria, Australia), and polyvinylpyrrolidone 40, 60 mg/liter (Sigma) at 37–40°C through the aortic cannula at a pressure of 150 ± 5 (SD) mm Hg. An incision of the right atrium allowed free efflux of the washout. When the effluent from the right atrium was clear (approximately 3–4 min after commencing the infusion) an acrylic resin solution, prepared by mixing 15 ml of Mercos CL-2B, 0.1 g of Catalyst-MA (Vilene Med. Co., Japan), and 5 ml of methylmethacrylate monomer (BDH Chemicals, Poole, United Kingdom) in a 20-ml syringe, was infused via the aortic cannula. The mean intraarterial pressure was measured in the first 5 rats via a cannula in the femoral artery. It was found that when a pressure of 200 mm Hg was applied to the casting medium in the syringe, this resulted in a mean arterial pressure in the rat of 110 ± 5 mm Hg. This reflects normal mean arterial pressure in the rat. When the resin was flowing from the right atrium, the aortic cannula and the inferior vena cava were clamped. The resin was allowed to fully polymerize at room temperature overnight. On the following day, the colon was excised and tumors were identified. Each tumor was then divided in half through its pedicle and in continuity with the surrounding colon.

One half of the tumor was fixed in 10% formalin overnight (12–18 h) for subsequent histological examination. The other half of the tumor was placed in a solution of 3.5 M KOH to digest the tissue away from the vascular cast. The resultant cast was dried at room temperature, mounted on an aluminum stub, gold coated, and examined in a scanning electron microscope (Cambridge; Stereoscan 250). The cast was photographed and examined using stereopairs. Measurements were made using the automatic micrometer scale at the bottom of the photographs.

Histology. The tumor half fixed in formalin was processed for histology by dehydrating in a graded series of ethanol solutions (50% absolute). It was then embedded in plastic (JB4 embedding medium) using a Polaron LM embedding kit (Polaron Equipment, Ltd., Watford, United Kingdom). Sections (5 μm) were cut and stained with hematoxylin and eosin.

Colon Tumors. Vascular casts of 22 tumors in 16 rats were prepared. There were 17 polypoid tumors the histologies of which revealed moderate or well-differentiated adenocarcinomas, 4 benign polyps, and one sessile adenocarcinoma.

RESULTS

The organization of the microvasculature within the normal rat colon has previously been characterized in detail (8). In this study, casts of the colon were prepared in 4 normal rats and our findings support those of Browning and Gannon (8). Briefly, it was found that in the mucosa of normal rat colon the spatial arrangement of the microvessels had a consistent pattern throughout the colon. Arterioles do not pass beyond the submucosa (Fig. 1). They branch at the border between mucosa and submucosa to supply the mucosal capillary network which drains into venules at the luminal surface only. These venules pass to submucosal veins without receiving further branches through the mucosa (Fig. 1). Capillaries within the mucosa are arranged in a regular honeycomb-like pattern around the mucosal glands (Fig. 2).

The polypoid adenocarcinomas ranged in diameter from 2.6 to 12.0 mm. The spatial organization of the microvessels in all of these tumors retained many features in common with the normal colon. In all tumors, two distinct vascular regions could be defined, but this feature was most obvious in the smaller tumors. There was a central region of larger vessels that were continuous with the vessels of the submucosa and muscularis propria layers of the adjacent normal colon. On the luminal aspect of this, there was an outer region of nutrient microvessels that were continuous with the mucosal layer of adjoining normal colon. This pattern is best seen in Figs. 3 and 5.

In the outer vascular zone of polypoid tumors up to 3.5 mm in diameter, the organization of microvessels had the same
general pattern as seen in normal rat colonic mucosa (Fig. 4). As in normal mucosa, capillaries in the outer zone of the tumors were supplied by arterioles that pass along the submucosa and divide into their branches at the border between the two vascular zones (Fig. 4). Capillaries then drain into venules mainly at the luminal surface. Within the central vascular zone of tumors up to 3.5 mm in diameter, tortuous, large diameter veins were the most obvious feature (Fig. 4). However, the spatial arrangement of the microvessels in this zone was also similar to that of normal colon.

Few changes in the morphological features of the individual microvessels were seen in tumors less than 3.5 mm in diameter. All microvessels were elongated but there was no apparent change in the diameter of arterioles which ranged from 15 to 25 μm. Capillaries had slightly greater diameters than normal (5-20 μm compared to normal, 5-8 μm), while venules were obviously of greater diameters than normal with a range of 15-70 μm (normal, 12-30 μm) (Fig. 4).

In the tumors >5.7 mm in diameter, the vasculature lacked features of the orderly structure seen in normal colon. This disorderly structure was characterized principally by variations in size of vessels, variations in direction, and increased numbers of interconnections without a perceptible pathway of flow (Fig. 8). The pattern was seen most prominently on the luminal surface and within the luminal zone of the tumor. Although quantitative studies have not been done, it gives the appearance of greater vascular density than the vasculature of normal colon and of smaller tumors. In the central vascular zone, the microvessels also showed a disorderly structure with marked variation in vessel diameters, irregularity in contour, and variations in direction.

In tumors >3.5 mm in diameter, a number of characteristic morphological features of the microvessels were seen. The diameters of the microvessels within the luminal vascular zone of the tumors varied over a wide range (5-100 μm) and in general were much greater than in the mucosa of normal colon (Fig. 5). The majority of microvessels ranged in diameter from 5 to 50 μm, while microvessels 50 to 100 μm in diameter were less frequent and often could be traced to veins. In all tumors that were approximately >3.5 mm in diameter, extravasation of the casting medium was seen to occur from the microvessels in the luminal zone. The greater proportion of extravasation occurred from microvessels near the surface of the tumor, so that resin was extruded into the lumen of the colon (Fig. 6). Smaller amounts of extravasation of resin could also be seen within the depth of the tumor. Many vessels were irregular along their length, varying frequently in diameter and giving rise to saccular dilations (Fig. 7). In places, microvessels anastomosed repeatedly with each other and formed a network of frequently anastomosing vessels (Fig. 9). Elongated microvessels of uniform diameters (40-50 μm) that travel distances up to 2 mm before giving any branches were seen and were probably arteriolar (Fig. 10).

Histopathological examination showed 17 of the polypoid tumors to be well or moderately well differentiated adenocarcinomas. There was no correlation between the degree of differentiation of the tumor and the nature of the vascular changes. Because of the presence of the casting resin within the lumen, the blood vessels were easy to identify in the histological sections. An abrupt transition between normal colon vasculature and abnormal tumor vasculature could be seen within the vascular casts of the larger tumors (Fig. 5). Examination of the histological sections of the corresponding half of the tumors revealed that the abrupt transition between the normal mucosal epithelial cells and the tumor cells correlated directly with the abrupt changes seen in the vasculature (Fig. 11). Within the
Fig. 5. Moderately differentiated adenocarcinoma of rat colon. Cross-section of polypoid tumor 5.7 mm in diameter. Bar, 1 mm. In this tumor there is obvious extravasation of resin from surface microvessels into the lumen of the colon (arrow). Two distinct vascular zones within the tumor, continuous with the vasculature of normal colon, can still be distinguished. In contrast to Fig. 3, all vessels within the outer zone of the tumor have greater diameters than normal. Within the central zone in this tumor, the microvessels are arranged in basket-like plexuses. Arrow, point of abrupt transition from normal colon vasculature to tumor vasculature at one edge of the tumor. This corresponds to the point of clear demarcation between tumor cells and normal colon mucosa seen on histological sections.

Fig. 6. Same tumor as Fig. 5. Bar, 50 μm. This view of the surface of the tumor shows that the regular honeycomb pattern seen on the surface of normal colonic mucosa (see Fig. 2) is no longer present. Instead vessels pass to the surface, loop over, and pass back into the tumor, after giving an irregular number of branches to adjacent microvessels. Extravasation of resin can be seen in the background (arrow).

Fig. 7. Same tumor as Fig. 5. Bar, 40 μm. Scanning electron microscopy of vascular cast demonstrating microvessels on the surface of the tumor. Vessel diameters range from 5 to 50 μm and thus are wider and more varied in diameter than normal colonic microvessels. Variations in calibre and frequent saccular dilations can be seen along their course.

Fig. 8. Well-differentiated adenocarcinoma of rat colon. Bar, 200 μm. This view of the vasculature on the surface of this 10-mm-diameter tumor demonstrates the disorderly structure of microvessels seen in these larger tumors. There is variation in size and direction of microvessels and the appearance of greater vascular density.

smaller tumors (up to 3.5 mm in diameter) there was obvious dilation of veins in the region of the submucosa invaded by tumor cells, but vascular changes within the tumor itself were not obvious on the histological sections, nor were there any vascular changes seen within the surrounding normal colon.

Four tumors were classified histologically as benign polyps.
Since there may have been malignant invasion in the half of the tumor that was corroded to examine the vascular cast, this classification is not conclusive. Nevertheless, there appeared to be no difference in the vascular structure of these tumors when compared to the carcinomas.

The one remaining tumor was a large well-differentiated adenocarcinoma 14 mm in diameter (Fig. 12). It differed morphologically from the other tumors in that the bulk of the tumor had invaded and expanded the muscularis propria. A central pedicle of large diameter vessels had not developed as in other tumors, and it was the only tumor in which an avascular area had occurred. On the luminal surface of the tumor there was a layer of microvessels continuous with the normal mucosal layer.
which had an organizational structure similar to that seen in normal mucosa.

DISCUSSION

The majority of the studies of the tumor microcirculation have been of transplantable tumors observed in transparent chambers in rodents (11, 13, 15–17). This technique allows direct visualization of the microcirculation in vivo over a period of time. However, the tumor can only grow in a lateral direction and is limited in size by being compressed by the chamber. Furthermore, the tumor is studied in an implantation site often remote from its tissue of origin and the tumor cell line has been passaged many times so that its morphological and proliferative characteristics may be quite different from those of the original tumor (18). In contrast, we have studied primary tumors that can be related to their tissue of origin.

The microvasculature of dimethylhydrazine-induced colon tumors in rats (19) and human colorectal tumors (20) have previously been studied using histological techniques to obtain morphometric data of the degree of vascularization within these tumors. These techniques, however, do not demonstrate the spatial organization of the vasculature within these tumors; neither do they reliably demonstrate all vessels within the tissue. Furthermore, it was not the aim of these studies to demonstrate the development of the vasculature within these tumors. In this study, microvascular casting was used since it will demonstrate the spatial organization and structural features of the microvessels within the tumor and will reliably cast the smallest microvessels due to the low viscosity of the casting resin.

In small tumors, the spatial arrangement of the microvessels had the same general pattern as the vasculature of normal colon and individual microvessels had similar morphological features but differed from normal colonic microvessels in being elongated and dilated. With tumors of increasing size, changes are seen in the spatial arrangement of the tumor vasculature and in the morphological features of the microvessels. Eventually, in the largest tumors the vasculature is completely disorderly and the individual microvessels are characterized by a number of abnormal morphological features.

These appearances indicate that there are two distinct stages of development of the vasculature within primary tumors. During the early phase of growth, the tumor is supplied by the preexisting host microvessels. These microvessels are then modified by the tumor but retain a similar organization to that in the normal host vascular bed. Then with further growth the tumor induces proliferation of new microvessels. These new vessels are clearly different in morphology and spatial arrangement from the host microvessels and would appear to be characteristic of tumor-induced microvessels. This finding in primary tumors correlates with the findings of many studies of tumor implants which have also shown that the tumor implant is supplied both by vessels recruited from the preexisting host vasculature and by new vessels resulting from tumor-induced angiogenesis (11, 13, 16, 21, 22).

Folkman et al. (1) were able to demonstrate that tumor cell proliferation is dependent on the concurrent growth of a supporting vasculature and that tumors induce angiogenesis from host vessels by release of soluble angiogenic factors. A number of angiogenic factors that are secreted by tumor cells, endothelial cells, and macrophages or that are stored in the interstitial matrix have recently been identified. The role of each individual factor and how they are interrelated is still to be determined (5). Some of these factors stimulate endothelial mitoty, others induce endothelial mitosis, and others cause both (23). Our results indicate that at least two types of angiogenic responses by the vasculature occur with the presence of tumor cells. In the early stages there is elongation and dilation of existing host microvessels, and at a later stage there is proliferation of new microvessels. It is possible that different angiogenic factors are acting separately in each of these two stages of development of the tumor vasculature.

Previous studies of the development of the vasculature of transplantable tumors showed that, after a phase of vascular proliferation and ingrowth of vessels into the expanding tumor mass, there is a further stage of blood vessel rarefaction and reduced vascular volume within the tumor (15, 24). Interestingly, in this study in only one tumor was an avascular zone seen.

Areas of hemorrhage within experimental tumors have been noted in several previous studies (10, 25), and carcinoma of the colon is well known to cause blood loss into the lumen of the colon (26). In this study, extravasation of the casting medium was seen to occur from microvessels on the luminal surface and within the depth of tumors >3.5 mm in diameter. This extravasation of resin may be the result of increased permeability of the tumor microvessels or may be due to hemorrhage. It is unlikely, however, that the resin is able to pass through endothelial cell pores or interendothelial cell junctions because of its physical properties. A large break in the integrity of the microvessel wall must precede resin extravasation. Therefore, this resin extravasation probably represents hemorrhage from the tumor microvessels.

The mechanisms responsible for hemorrhage in tumors are unknown although a number have been postulated. Endrich et al. (27) observed that hemorrhage occurred from enlarged veins and venules at the periphery of tumor implants. These vessels were thought to be mechanically fragile and damaged by the advancing tumor. Others have noted hemorrhage to occur near the necrotic center of tumor implants which is the furthest point from the arterial supply (10, 25). This may imply that necrotic tissue leads to damage to microvessels in this area in some way. In a further study, Endrich et al. (28) observed intermittent blood flow rates in different areas of tumor implants and noted that petechial hemorrhages developed in areas where flow was restored after slow or zero blood flow, thus implying hypoxic damage to microvessels. In this study there was no evidence of necrosis in any of the polypoid tumors >3.5 mm in diameter, despite the presence of resin extravasation. Van Den Breek et al. (29) found that extravasation of erythrocytes occurs through the tips of capillary sprouts which develop in the process of tumor-induced angiogenesis. Ausprunk and Folkman (13) further demonstrated that during migration of endothelial cells to form capillary sprouts, there was loosening of the cell junctions and dissolution of the basal lamina forming gaps in the blood vessel wall through which erythrocytes had extravasated. The association of hemorrhage with areas of vascular proliferation within tumors has also been noted by other workers (21) and is thought to be due to the presence of newly formed, immature blood vessels that are fragile and prone to rupture. Our results would support this, since resin extravasation was seen only from tumors in which there was clear evidence of the development of new blood vessels with abnormal morphological features.

Increasing tumor cell hypoxia and cell death with tumor growth has been a consistent finding in many studies (15, 28). Deficiencies of the tumor vasculature are believed to be responsible and a number of mechanisms have been postulated (30).
Although an avascular zone with corresponding tumor cell necrosis was present in only one tumor in this study, a number of possible inadequacies of the vasculature can be identified: (a) large diameter vessels are less efficient as exchange vessels because they have a lower surface area:volume ratio (31, 32); (b) although the outer vascular zone of the tumor is much thicker than normal mucosa, arterioles supplying this zone in the smallest tumors (<3.5 mm) appear not to increase their caliber in response to the presence of the tumor cell mass in the same way that capillaries and venules do. In larger tumors, elongated vessels which traverse relatively long distances before branching appear to be arteriolar and may be expected to have a greater resistance compared to normal arterioles; (c) increased vascular resistance within tumor microvessels may also result from the frequent irregularities seen along the course of the vessels and from their irregular branching pattern.

This study has confirmed that the developing vascular system within primary tumors has two components (22). This has implications in two main areas: (a) current models for examining the tumor microcirculation using tumor implants in the cornea or in transparent chambers will demonstrate the development of abnormal tumor-induced microvessels but do not demonstrate the early stage where the tumor is supplied principally by the preexisting host microvessels; (b) any study of a therapeutic approach which aims to control primary tumor growth through its direct action on the tumor microvessels must consider its effect on each of the two components of the vascular supply of the tumor.

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Microvascular Architecture of Experimental Colon Tumors in the Rat

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