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# The role of microvascular environment in the metastasizing ability of an experimental tumor

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**Summary.** A rapidly growing, locally very invasive and easily transplantable fibrosarcoma that was developed through chemical carcinogenesis in Balb/c mice in this laboratory several years ago did not metastasize into the viscera of its hosts when implanted into the subcutaneous connective tissue or skeletal muscle of syngeneic mice. When, however the same tumour was implanted into the liver or the kidneys of Balb/c mice it metastasized extensively into many different organs within 2 weeks of its transplantation. Evidence is presented that because of some unknown deficiency the cells of the fibrosarcoma under study are unable to penetrate through the endothelial wall into the lumen of the particular type of vessels which surround and vascularize the tumours in the subcutaneous connective tissue and muscle, and that, in contrast, they can easily cross into the lumen of the vessels that surround and vascularize them in the liver and kidney. Thus, this in vivo study indicates that the type of microvascular environment in which certain experimental tumours are transplanted can control their ability to accomplish vascular invasion, the first step of the metastatic process.

**Key words:** Neoplasia – Fibrosarcoma – Metastasis – Vascular invasion

## Introduction

Several years ago, a rapidly growing fibrosacoma was produced in this laboratory in the dorsum of Balb/c mice through the synergistic effect of two subcutaneously injected chemicals that are not carcinogens when given alone, i.e. tocopherol and soya oil (Constantinides and Harkey 1985). This fibrosarcoma proved readily transplantable every 3–4 weeks into the subcutaneous space of the dorsum of syngeneic recipients, and has now been maintained for just over 6 years (more than 80 transplantation cycles) since its creation. While originally rather differentiated (with spindle-shaped cells and some collagen production), the tumour became very anaplastic after the first few transplantations and is now very pleomorphic, with up to 18 mitoses per high power field and very little or no collagen production.

A puzzling feature of this tumour has been the fact that although it is highly aggressive locally, usually invading from its dorsal subcutaneous implantation site the entire length of the adjacent forelimbs down to the paws, it has never yet invaded any vessels or metastasized into the internal viscera of any of the more than 400 hosts that have been implanted with it during the 6 years of its existence.

One possible explanation for the lack of metastasizing ability was that for some reason the fibrosarcoma cells were unable to penetrate through the walls of the particular type of capillaries which they encountered in the subcutaneous environment and which are characterized by relatively thick, continuous (unperforated) endothelium. If this were the main barrier to metastasis, one might expect that (*a*) the implantation of this tumour into viscera with extremely thin-walled and perforated (sieve-like) capillaries such as the liver or the kidney would facilitate the entry of neoplastic cells into the blood stream and, therefore, metastatic dissemination, and (*b*) its implantation into another environment with relatively thick and continuous endothelial tubes such as skeletal muscle would, as in the case of subcutaneous connective tissue, lead to no vascular invasion and no metas-

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tases. The present study tested the above hypothesis.

## Materials and methods

*General plan of the study.* A tiny standard specimen of the fibrosarcoma produced and maintained in this laboratory was implanted into four different body sites of Balb/c mice, i.e. subcutaneous tissue, skeletal muscle, liver and kidney, and the vessel invading and metastasizing performance of the tumours that grew in these four different locations were compared with one another.

Vascular invasion was assessed by scanning histologically in step-serial sections all vessels within and around the primary tumour implants for the presence or absence of tumour cells within their lumen. In all animals that were killed 2 weeks, 3 weeks and 4 weeks after tumour implantation and were carrying implants with a diameter of about 5–8 mm, this scanning was carried out in groups of 4–6 sections (100 microns apart) obtained every 1 mm through every tumour implant and its surrounding host tissue. In the animals that were killed 7 h, 24 h, 3 days and 1 week after intrahepatic tumour implantation and were carrying implants with a diameter of about 1–3 mm, the scanning was done in sections obtained every 100 microns through every tumour implant and its surrounding tissue.

Metastasis was assessed by killing the tumour implant recipients at a given time interval after implantation, performing a detailed autopsy of every animal, and noting the presence or absence of grossly visible metastases in all organs. Metastases were easily identifiable macroscopically as ivory colored round or oval masses. All grossly visible metastases that occurred in the organs of 12 male mice that were killed 2 weeks after intrahepatic tumour implantation were examined histologically to confirm their neoplastic nature and to establish a correlation between their macroscopic appearance and their microscopic structure. In addition, since no grossly visible metastases materialized in the lungs of any of the mice bearing intra-hepatic or intra-renal tumour implants, several sections from both lungs of the first 6 animals killed 2 weeks after intra-hepatic implantation were examined histologically, in order to look for possible microscopic metastases in these organs.

The animals used in all experiments were adult male Balb/c mice, weighing 28–32 grams, except for two instances in which both male and female adult mice were used in order to see whether the sex of the hosts affects the vessel invading and metastatic performance of the tumour implants, namely a 4 week time interval after subcutaneous, and a 2 week time interval after intra-hepatic tumour implantation.

The donors of tumours for all experiments were adult male Balb/c mice that have been carrying the fibrosarcoma through serial subcutaneous transplantation since its creation in this laboratory.

Light microscopic studies of all primary tumour implants and of several of their metastases were performed on hematoxylin-eosine stained paraffin sections from tissues fixed in 10% buffered formaldehyde.

In addition, electron microscopic studies of microvessels within the primary tumour implant itself and in its surrounding host tissues were performed in 2 animals from each group, in order to find out whether the ultrastructure of the microvessels of the various host tissues changed or remained the same after invading (vascularizing) the fibrosarcoma implants. Tumour and host tissue specimens for these studies were pre-fixed in buffered formaldehyde, post-fixed in glutaraldehyde and osmium and embedded in epon; microvessels were first identified in 1 μ thick stained sections of the epon blocks with a light microscope, and ultra-thin sections were then cut from the microvessel-containing block areas for ultrastructural study.

*Subcutaneous tumour implantations.* Although no subcutaneous fibrosarcoma implants out of the more than 400 performed in 6 years ever metastasized, the tumour was implanted into the dorsal (interscapular) subcutaneous spcae of 40 mice which served as simultaneous controls to the 3 other types of implantation. These animals were killed 2 weeks, 3 weeks and 4 weeks after implantation in groups of 8, 8 and 24 mice per time interval, respectively. The subcutaneous implantation was as described previously (Constantinides and Harkey 1985) and can be summarised as follows: The tumour-bearing mouse was anesthetised with an intra-peritoneal injection of 0.05 ml of a 50 mg/ml nembutal solution; a tiny piece of viable tumour was then removed from it through incisional biopsy, quickly trimmed to a roughly 1 $mm^3$ cube-shaped specimen under cacodylate buffer in a petri dish and implanted subcutaneously through a small incision into the dorsum of a nembutal-anesthetised recipient mouse.

*Subcutaneous tumour implantations followed by transection of the tumour implants.* A group of 12 mice was set up in order to see whether cutting of vessels of established and vascularised subcutaneous tumour implants would enable these neoplasms to metastasize by giving tumour cells free access to the open ends of transected vessels. The animals received subcutaneous tumour implants as described in the preceding paragraph. However, 10 days after implantation, at a time when the tumours were fully vascularised (as shown by a previous pilot experiment on 6 animals), the mice were anesthetised again with nembutal, the skin over the tumours was incised and the tumours were transected through their center with a sharp razor blade; the two halves were then immediately re-apposed and sutured together again after slightly shifting their relative position to one another so as to ensure that the open ends of the transected vessels of each half did not meet with their counter parts in the other half, but faced solid tumour cell sheets; the skin incisions above the hemisected and sutured tumours were subsequently closed with interrupted sutures and the animals were killed and autopsied 3 weeks after the transection operation.

*Intramuscular tumour implantations.* A roughly 1 $mm^3$ cube-shaped fibrosarcoma specimen was implanted into the dorsal cervical muscle mass of 5 and into the extensor thigh musculature of 7 mice, and all 12 mice were killed and autopsied 3 weeks after implantation. The general procedure was the same as that described above for the subcutaneous implantation except that here the tumour specimen was deposited in the bottom of a longitudinal muscle incision about 60 seconds after the incision was made with a sharp razor blade, when the bleeding induced by the muscle incision stopped.

*Intrahepatic tumour implantations.* The tumour was implanted into the livers of 5 groups of mice that were killed and autopsied 7 h, 24 h, 3 days, 1 week and 2 weeks later, the number of animals per group being 6, 7, 6, 7, and 24, respectively. The implantation procedure was as follows: After nembutal anesthesia, the left (largest) liver lobe of the mice was pulled out of a roughly 1 cm long horizontal laparotomy incision; a 3 mm deep incision was then made into the center of the exposed liver lobe and, after waiting about 60 s for the bleeding to stop, a 1 $mm^3$ tumour sample excised from a subcutaneous fibrosarcoma carrier was inserted into the bottom of this liver incision; the left liver lobe was subsequently pushed back into the abdominal cavity and the laparotomy wound was sutured in two layers (muscle and skin). Special care was taken to avoid spilling

of tumour cells from the fibrosarcoma sample into the peritoneal cavity during the implantation procedure. The implant was cut from the most solid and cohesive (peripheral) part of the donor tumour, grasped with flat metallic forceps tips that completely covered it during transfer and deposited into the bottom of the liver incision – that was momentarily kept open with another forceps – without ever touching the liver surface or the incision lips; immediately afterwards the retracting forceps was withdrawn and the incision lips were allowed to close, burying the implant in the liver parenchyma.

*Intrarenal tumour implantations.* A 1 $mm^3$ fibrosarcoma specimen was implanted into the left kidneys of 12 mice that were killed and autopsied 2 weeks later. The procedure followed was as in the intrahepatic implantation, except that the tumour sample was deposited in the bottom of a 3 mm incision made from the lateral margin into the central parenchyma of the left kidney.

## Results

### *Subcutaneous tumour implantations*

No subcutaneously implanted tumour metastasized at 2 weeks, 3 weeks or 4 weeks after implantation, in agreement with the experience previously gained on more than 400 animals in 6 years in this laboratory; correspondingly, no tumour cells were ever found in the lumen of any vessel within or around the implanted tumour. These results were obtained in both male and female tumour hosts (see Table 1).

As with the intact subcutaneous implants, no metastases or vascular invasion whatsoever developed from the subcutaneous tumour implants that were transected 3 weeks before sacrifice in order to briefly expose neoplastic cells to open vessels (see Table 1). It should be noted that the transected subcutaneous neoplasms grew as well as their non-transected counterparts.

### *Intramuscular tumour implantations*

No intramuscularly implanted fibrosarcoma metastasized or penetrated into vessels 3 weeks after implantation (see Table 1). All of the intramuscular neoplasms took and they grew at about the same rate as the subcutaneous implants.

### *Intrahepatic tumour implantations*

Numerous grossly visible metastases in the form of ivory white spherical or ovoid nodules ranging in size from about $1 \times 1 \times 1$ to about $5 \times 5 \times 5$ mm, materialised in many organs and tissues of all the mice that received a tumour implantation in their left liver lobe and were killed 2 weeks later (see Table 1). These metastases developed in the pancreas, intestinal wall and mesentery, kidney, seminal vesicle in males, ovary in females, diaphragm, right liver lobe, mediastinal lymph nodes, anterior and posterior body wall and skin in the recipients of the intrahepatic tumour implants (see Fig. 1 for the microscopic appearance of typical metastases). No grossly visible pulmonary metastases materialised in any of the mice bearing intra-hepatic tumour implants and no microscopic metastases

**Table 1.**

| Type of implantation | Time post implantation | $n$[a] | Sex | Incidence (%) of vessel invasions | Incidence (%) of grossly visible metastases |
|---|---|---|---|---|---|
| Subcutaneous | 2 weeks | 8 | m | 0 | 0 |
| | 3 weeks | 8 | m | 0 | 0 |
| | 4 weeks | 12 | m | 0 | 0 |
| | 4 weeks | 12 | f | 0 | 0 |
| Transected 10 days after subcutaneous implantation | 4.5 weeks | 12 | m | 0 | 0 |
| Skeletal muscle | 3 weeks | 12 | m | 0 | 0 |
| Hepatic | 7 h | 6 | m | 0 | 0 |
| | 24 h | 7 | m | 0 | 0 |
| | 3 days | 6 | m | 0 | 0 |
| | 1 week | 7 | m | 29% | 0 |
| | 2 weeks | 12 | m | 100% | 100% |
| | 2 weeks | 12 | f | 100% | 100% |
| Renal | 2 weeks | 12 | m | 100% | 100% |

[a] Number of mice per group

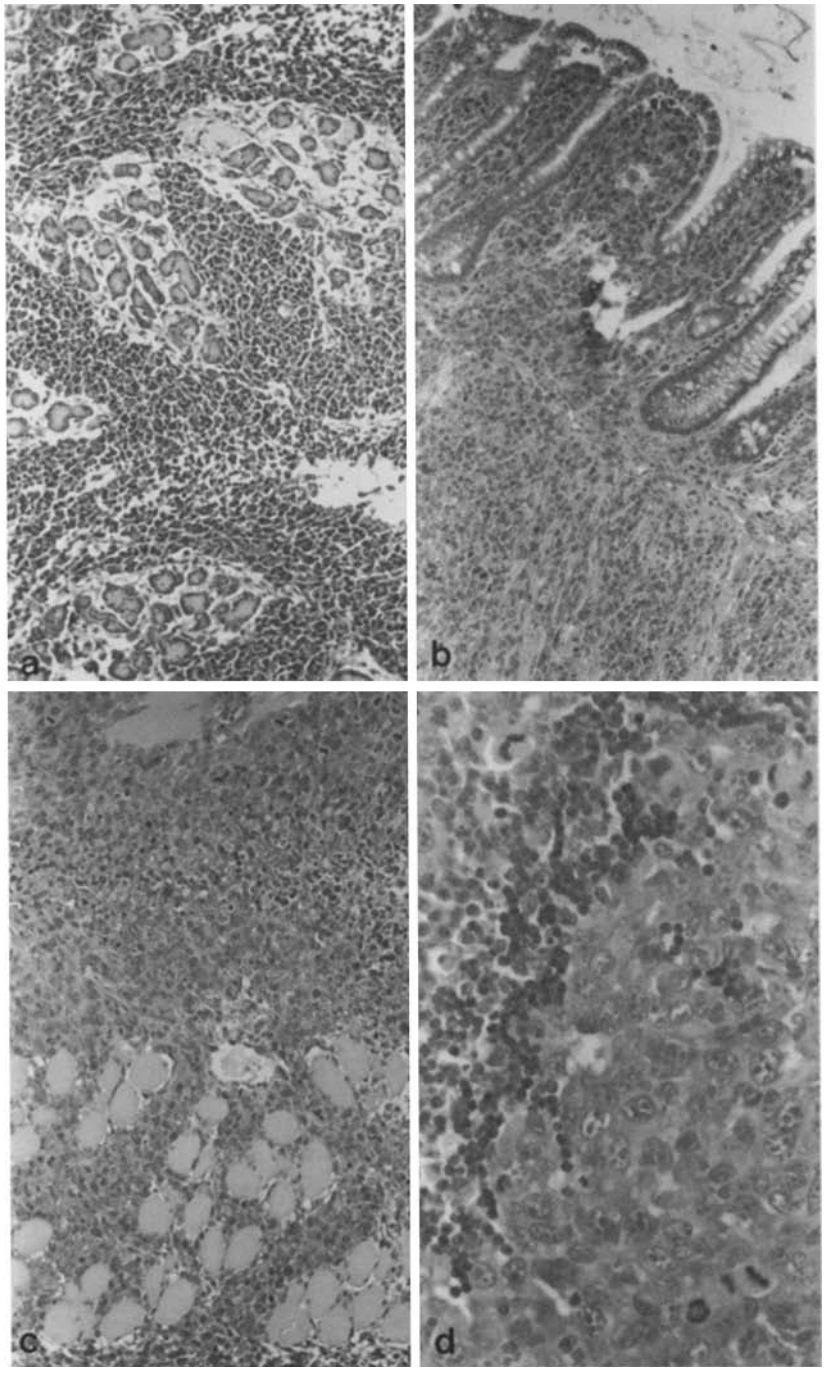


**Fig. 1. a** Metastasis of the fibrosarcoma into the pancreas. The tumour cell masses are pouring between and completely surround several groups of pancreatic acini. H & E, 90 × ; **b** Metastasis of the fibrosarcoma into the wall of the large intestine. The neoplastic cell mass, coming from the mesenteric side below, extends through the muscularis into the submucosa and lamina propria of the intestinal wall. H & E, 90 × ; **c** Metastastic fibrosarcoma in the muscles of the anterior abdominal wall. The tumour cell masses are pouring between the muscle bundles and pushing them apart. H & E, 90 × . **d** Mediastinal lymph node metastasis. Some lyphatic tissue is visible in the upper left. H & E, 360 ×

could be detected in any of the lung sections of the animals that were examined histologically. A minimum of 3 and maximum of 6 metastatic sites developed per mouse, and no significant difference could be seen in frequency, size or distribution pattern of metastases between male and female fibrosarcoma carriers.

Step-serial histological sections from each primary intrahepatic tumour implant and its surrounding liver parenchyma revealed unequivocal tumour cells, single or in clusters, within the lumen of both intra-tumour and peri-tumour microvessels in all mice killed 2 weeks and 29% of those sacrificed 1 week after implantation (see Figs. 2 and 3 for examples of vessel invasions by tumour). Since no vessel invasion by tumours was observed in any intrahepatic implant at 7 h, 24 h and 3 days after implantation, the first invasions appeared to

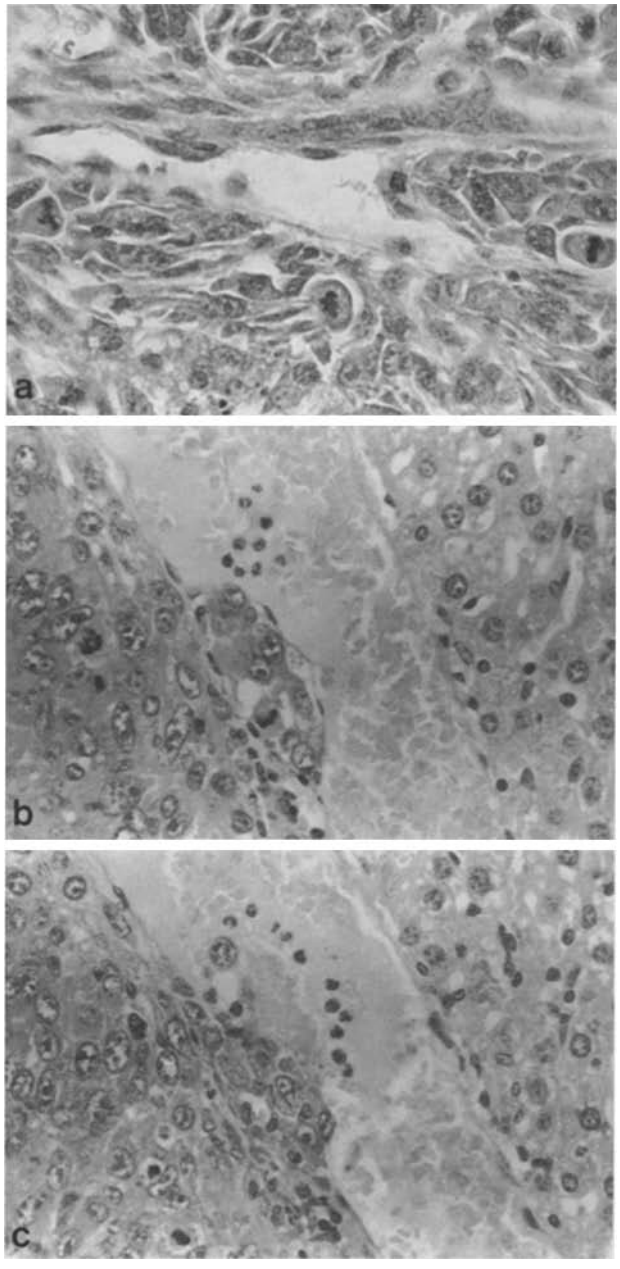


**Fig. 2. a** A cluster of tumour cells has entered and filled the right half of a thin-walled vessel within a primary tumour implant in the liver, 2 weeks after implantation. One of the invading tumour cells is in mitosis. H & E, 360 × ; **b** A thin-walled vein runs between the margin of a 2 week old primary hepatic tumour implant (on the left) and the liver parenchyma that surrounds it (on the right). A group of tumour cells (with one of them in mitosis) has penetrated into the subendothelial space of the wall of this vein from the left, lifting its endothelium and bulging into the lumen, while a group of 9 leucocytes has clustered in front of the imminent (or incipient) break-through point. H & E, 360 × ; **c** Same area as in 2B, just a step-serial section away. One tumour cell has clearly broken through the endothelium and is now floating in the lumen, a small thrombus appears to have developed at the point of tumour break-through and a string of leukocytes stays close to the thrombus and the invading tumour cell. H & E, 360 ×

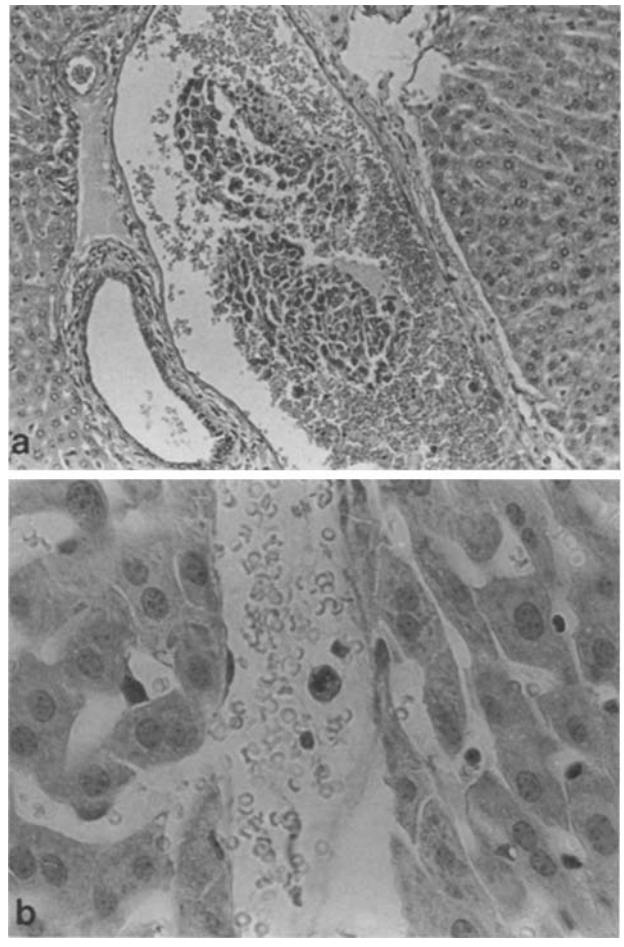


**Fig. 3. a** Two large clusters of dark fibrosarcoma cells lying among erythrocytes within the lumen of a thin-walled vein in the normal liver parenchyma close to a 2 week old intrahepatic tumour implant. H & E, 90 × ; **b** A single large round tumour cell with a bizarre mitosis is floating in the lumen of a thin-walled vein in the normal liver parenchyma close to a 2 week old intrahepatic tumour implant. Note that the invading tumour cell is several times larger than the normal blood cells that surround it. H & E, 360 ×

develop between the 4th and 7th post-implantation day. Although several of the vessel invasions were observed in the smallest visible microvessels (i.e. those with a diameter similar to that of normal sinusoids), most of them occured in venules larger than sinusoids with a diameter of approximately 50–150 μ.

In their earliest stages (7 h and 24 h post-implantation), the tiny intrahepatic tumour implants were surrounded by haemorrhages, fibrin strands, and some polymorphs, and as a result of the incision-induced interruptions and thromboses in the liver microvessels all around them, significant portions of the implants, as well as of the immediately adjacent hepatocytes, became necrotic. The haem-

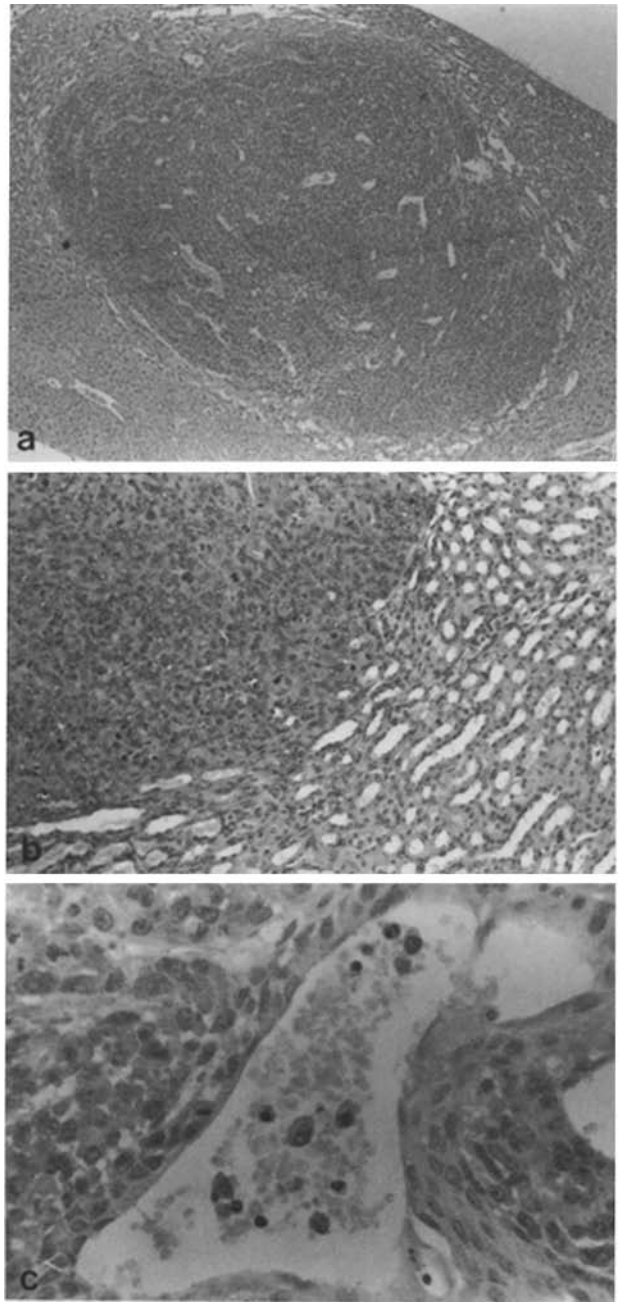


**Fig. 4. a** The whole intra-hepatic implant 2 weeks after implantation. The intrahepatic tumour implant is the vascularised dark ovoid mass buried within the paler parenchyma of the left liver lobe. H & E, 18,5 × ; **b** Boundary between intra-renal tumour implant and surrounding kidney parenchyma, 2 weeks after tumour implantation. H & E, 90 × ; **c** Several tumour cells are floating in the lumen of a thin-walled vessel within a primary intrarenal tumour implant, 2 weeks after tumour implantation. H & E, 360 ×

orrhages, fibrin, polymorphs and microvessel plugs had, however, largely disappeared by the third post-implantation day, and normal vascularised tumour and peri-tumour liver parenchyma were evident by the seventh day, when the first vascular invasions by the tumour were seen, and thereafter (see Fig. 4a).

All of the intrahepatic tumour implants took and grew. All of the implants at 1 week post-implantation, and all but 2 tumours at 2 weeks post-implantation remained surrounded by liver tissue and did not break through the mesothelial surface of the liver lobe in which they had been embedded; none spread into any neighboring structures by direct invasion during the observation periods of the present study.

*Intrarenal tumour implantations*

All intrarenally implanted tumours grew and had metastasized widely 2 weeks after implantation (see Table 1). The metastases were very similar in distribution pattern, color, shape, size and histological appearance to those of the intrahepatic implants. Microscopic study of sections through the primary intrarenal implants showed that tumour cells were visible within the lumen of intra-tumour and peri-tumour microvessels in all animals of this group (see Fig. 4b, 4c for a typical intrarenal tumour implant and vascular invasion). The intra-renal fibrosarcomata in 9 mice of the 12 mice that carried them remained embedded in the kidney, while those of the remaining 3 animals had broken through the surface of that organ at 2 weeks post-implantation.

*Electron microscopic observations on peri- and intra-tumour microvessels*

The ultrastructure of the liver sinusoids, the renal intertubular capillaries and the connective tissue and muscle capillaries that surrounded the tumour implants in their host tissues was as repeatedly described in most mammals (Constantinides 1974): The liver sinusoids had extremely thin, multiperforated (sieve-like) endothelial walls whose pores were all open, and they lacked a basement membrane while the intertubular renal capillaries likewise possessed extremely thin, multiperforated walls but some of their pores were sealed by ultrathin diaphragms and they were enveloped by a thin basement membrane; in contrast, the connective tissue and muscle capillaries had much thicker endothelial walls (about 6–16 times thicker) than those of the liver and renal capillaries, they were not perforated and they were both enveloped by a dense basement membrane.

Upon invasion (vascularisation) of the tumour implants, all of the above 4 types of microvessels

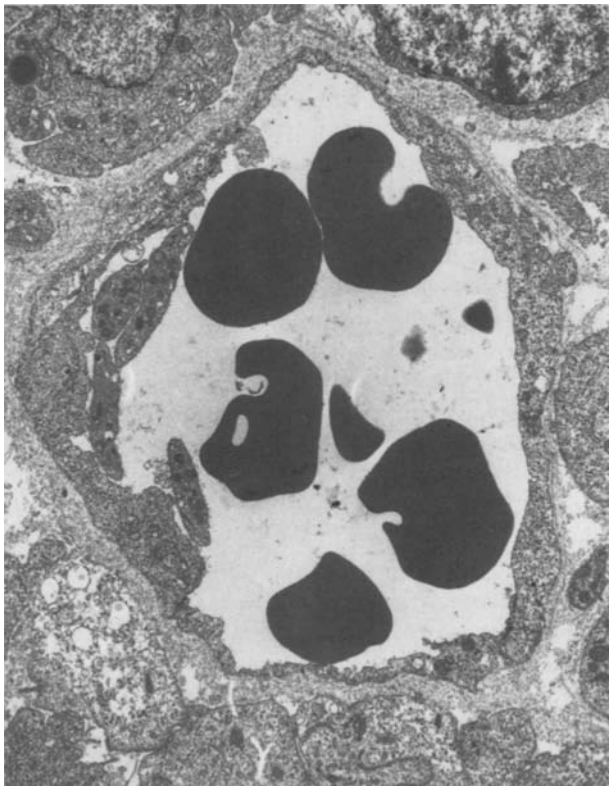

**Fig. 5.** Ultrastructural features of a typical microvessel deep within a primary hepatic tumour implant. Note the relatively thick and continuous (unperforated) endothelium that is rich in ribosomes and rough endoplasmic reticulum and is partly invested by a basement membrane (2700×). This appearance was characteristic of all microvessels that invaded the tumour implants, and in many cases the endothelium was even thicker, richer in ribosomes and ergastoplasm and completely invested by a basement membrane

underwent marked changes that were evidently the expression of the vigorous endothelial proliferation, intensive protein synthesis and dedifferentiation that materialises in all expanding capillary beds, whether this expansion occurs during embryogenesis, organisation of infarcts, organisation of thrombi or wound healing: Their endothelial cytoplasm became much thicker than it was before invasion of the tumour (reaching up to 10 or more times its pre-invasion thickness in the case of many microvessels), it became filled with polyribosomes and rough endoplasmic reticulum, and it lost its perforations – if it had any – before tumour invasion (see Fig. 5). As to the larger than capillary venules, they maintained within the tumours the continuous (unperforated), relatively thick (thicker than that of sinusoids) and basement membrane enveloped endothelium which they possessed outside the tumours.

## Discussion

This study has shown that the metastasizing ability of a chemically induced fibrosarcoma was controlled, among other things, by the type of host tissue or organ into which it was implanted. Thus, this neoplasm could not metastasize into any distant viscera when implanted into the subcutaneous connective tissue or skeletal muscle, but when implanted into the liver or kidney it metastasized into distant sites. The puzzling absence of any pulmonary metastases could be due to the well known "seed and soil" phenomenon, i.e. to metabolic peculiarities of the tumour itself that promote its successful metastasis into some organs and prevent it in others; thus, e.g. it has been found that certain platelet-aggregating tumour strains produced lung metastases while other strains that lacked this property produced only extrapulmonary metastases (Willmott et al. 1983).

While the rapidly growing literature on the mechanisms of tumour metastasis is controversial on several issues, there seems to be general agreement on the following 2 points: First, metastasis is a multi-step process involving the 5 stages of vascular invasion (penetration of tumour cells into the lumen of microvessels from outside), survival in transport across the bloodstream, arrest in certain distant vessels, extravasation (emigration from the microvessel lumen into the perivascular space) and successful post-extravasation proliferation. Second, the outcome at any of these stages depends on an interaction between factors that reside in the tumour itself and factors that reside in the host. Examples of tumour factors are collagenases that digest basement membranes, laminin that may facilitate attachment to host endothelium, platelet aggregators and procoagulants that can produce partial or complete thrombus sheaths around tumour emboli, enzymes that degrade fibronectin, proteoglycans and other components of host stroma, and supressors of certain host immune responses to tumours (see recent reviews by Liotta et al. 1980; Nicolson and Poste 1982; Nicolson and Poste 1983; Malinoff et al. 1984). Examples of host factors are tumouricidal or tumour-enhancing antibodies, tumouricidal macrophages and lymphocytes and their activators, and tumouricidal "natural killer" cells and their activators (see recent reviews by Fidler and Poste 1982; Nicolson and Poste 1983; Zoler 1983; Dennert 1985).

Our results show that the reason the fibrosarcoma cells could not metastasize from connective tissue and muscle was that they could not enter the lumen of the microvessels that surrounded and

vascularised them in these two environments, while the ease with which they metastasized out of the liver and the kidney was due to the fact that they could easily penetrate into the microvessels at these sites.

We do not know the reason for the dramatically different vascular invasion potential of the tumour in the two different environments which was documented in this study; we can only speculate about certain possible mechanisms that could produce such a difference, i.e. set up hypotheses to be tested by future studies.

One possible explanation for the different vascular invasion performance in the two different environments could be that the physical barrier to invasion was formidable in connective tissue and muscle – where the microvessel endothelium was relatively thick, continuous and enveloped in basement membrane – whereas it was enormously diminished or completely absent in liver and kidney – where the endothelium was extremely thin and multiperforated and its basement membrane was either completely lacking (as in the liver sinusoids) or very attenuated (as in the intertubular renal capillaries).

A closer scrutiny of our results, however, does not support such a simple, exclusively mechanistic hypothesis and suggests strongly that, while physical factors may have played some role in facilitating invasion of the peri-tumour capillaries in the liver and kidney, the factors that facilitated invasion of the intra-tumour capillaries and of both intra- and peri-tumour venules in the same organs were probably not physical. There are two reasons for this hypothesis: First, our electron microscopic findings showed that the microvessels which vigorously proliferated and invaded the tumours in liver and kidney had changed their original structure; within the tumours they were no longer thin and perforated but had developed a thick and continuous endothelium which generally represented just as great a physical barrier to invasion as that of the normal connective tissue and muscle microvessels. Second, the bulk of both intra- and peri-tumour vascular invasions in liver and kidney occurred into venules that were larger than sinusoids and intertubular capillaries (with a lumen diameter of about 50–150 μ) and whose endothelium was thicker than that of liver sinusoids and kidney capillaries, was unperforated and usually enveloped by a basement membrane.

It thus appears possible that the reason the fibrosarcoma cells could not cross into the lumen of the intra-tumour microvessels and the venules in connective tissue and muscle was that they lacked certain chemicals (glucoproteins, enzymes?) that are necessary for attachment to and penetration through the wall of these vessels, and conversely, that they could invade the intra-tumour microvessels and venules in liver and kidney because they happened to possess the chemical machinery required for penetration into these particular types of vessels. Such a hypothesis is in line with increasing evidence that, despite their morphological similarities, different segments of the mammalian endothelial tree exhibit different characteristics (permeability to macromolecules, toxicological vulnerability etc.) implying significant differences in the chemical make-up (surface glucoproteins, receptors, collagens etc.) of these various segments.

Another possibility is that our tumour was cytotoxic for liver and kidney but not for connective tissue and muscle endothelial cells, in line with Rössle's hypothesis of "individual host cell kill" as a mechanism for penetration of some very aggressive tumour cells through certain host cell epithelial or endothelial formations (Rössle 1949). This and all other possibilities will have to be explored by future studies in appropriate experimental settings and with appropriate techniques.

In essence then, our data indicate that the type of microvascular environment in which a tumour grows can be one of the important factors that control its vascular invasion and metastasis – at least for some experimental neoplasms, like that which was the object of this study.

One could argue that the metastasizing ability that materialised when the fibrosarcoma was implanted into the liver and kidney could very well be due to factors other than the physical or biochemical make-up of the microvessels in these organs, such as a higher temperature in these viscera than in the somatic periphery of the body, or the fact that when we made an incision into these organs in order to implant the tumour we unavoidably cut open many microvessels with the scalpel, and thus made it possible for the tumour cells to enter unimpeded into the open ends of the transected vessels and therefore into the blood stream.

We do not believe in the first possibility because repeated temperature measurements taken in the intraperitoneal cavity and dorsal subcutaneous space of several mice revealed a very small difference between the two environments in these furry, thermally well insulated animals: The average temperature in the subcutaneous space was 36.1° C and in the peritoneal cavity 36.3° C; the fact that the temperature in the abdominal cavity was very slightly (0.6%) warmer than in the subcutaneous space cannot explain the dramatic (all or nothing)

difference in metastatic potential between the tumours that were growing in these two different environments. Furthermore, hyperthermia of up to 42.5° C applied locally to tumours has been found not to increase their metastatic rate, or even to decrease it (Hahn et al. 1979; Hill and Denekamp 1982).

We do not believe in the second possibility for the following 4 reasons: First, our time studies on intrahepatic implants indicate that vascular invasion did not occur at the moment of implantation but started developing much later, sometime between the 4th and 7th post-implantation day, i.e. long after the microvessels cut during the implanting operation were sealed and the acute operative trauma was repaired. It is also important to note that the bleeding induced in the implantation bed by the liver incision stopped as a result of thrombosis in the severed vessels within about 60 s, and that the tumours were implanted only after haemostasis had occurred. Furthermore, the fact that about half the tumour cells and all adjacent hepatocytes became necrotic during the first 24 post-implantation hours is clear evidence of the haemostasis-induced cessation of circulation in the implantation area during that early period – a stasis that was evidently lifted only after the proliferation of new capillary networks re-established circulation around the implant and began promoting the ingrowth of intact capillary buds into the tumour itself at about the 3rd post-implantation day. Second, no vascular invasions or metastases whatsoever resulted trom the intramuscular implantations, in which numerous muscle microvessels were severed and led to a brief pre-implantation bleeding, just as in the case of the liver and kidney implants. Third, no vascular invasion or metastasis developed from any of the subcutaneous tumour implants that were transected when they were well vascularised in order to sever many of their microvessels and expose tumour cells to the open ends of these cut vessels. If a few tumour cells succeeded in entering the open ends of some transected vessels in the short interval before haemostasis sealed them, they represented a brief, episodic invasion that was evidently not sufficient for successful metastasis; such reasoning would be in line with recent quantitative findings that the overwhelming majority of tumour cells that enter the blood stream perish in transport (Nicolson and Poste 1982) – findings which imply that successful metastasis is more likely to result from a continuous, sustained shower of vessel entries than from a "one shot" invasion episode. Fourth, the documented abundant entry of tumour cells into the intra-tumour vessels of the hepatic and renal implants could not possibly be due to tumour cell entry into severed vessels, since the intra-tumour vessels were produced by the ingrowth of intact new capillary buds into the tumours after the trauma of implantation healed.

Finally, we can eliminate the possibility that a break of the primary tumours through the capsule of the liver and kidney into which they were implanted, leading to an "Abtropfung" and transperitoneal seeding of tumour cells, represented an important mechanism of metastasis of this neoplasm from its visceral implant sites. In the first place, such a breakthrough was documented in only a minority of cases and, furthermore, whenever it developed it occurred late (at the end of the second post-implantation week) i.e. when all primary tumour implants had already penetrated into intra- and peri-tumour vessels. Thus, while a terminal drop-off process could, in certain cases, have added some late and small metastases to those produced earlier by the main haematogenous spread mechanism, this did not affect the basic observation and conclusion of this study that our fibrosarcoma could invade the vessels of liver and kidney but not those of subcutaneous tissue or muscle.

What then are the specific defects of the fibrosarcoma cells under study that prevent their invasion of connective tissue and muscle capillaries and venules? Are the neoplastic cells unable to digest and cross the basement membrane of these vessels? Are they unable to attach to the outer surface of their endothelial tubes? Are they unable to open their interendothelial junctions from outside or to penetrate by destroying part of their endothelial cytoplasm? We shall attempt to answer these and other questions by experiments now in progress in which we are adding various chemical factors to subcutaneous fibrosarcoma implants hoping to help them overcome the barrier to their invasion of vessels. We also hope to get additional clues from a future electron microscopic exploration of the modes of penetration of our neoplastic cells through the walls of all the different types of peritumour and intra-tumour vessels which they encounter in liver and kidney. Such experiments are relevant not only to the fibrosarcoma of this study since it is well known that many other experimental tumours (chemically or virally induced) cannot metastasize from subcutaneous implantation sites.

The in vivo study of the factors that are needed by some experimental tumours in order to enable them to penetrate through the walls of somatic vessels may also help develop measures that prevent or delay the metastasis of some human sarco-

mata, most of which seem to have no problem invading connective tissue or muscle vessels. Such work seems particularly useful in view of the fact that, so far, the overwhelming majority of the experimental investigations in this area have been concerned with metastasis after intravenous injection of tumour cells, i.e. with the later, post-invasion stages rather than with the first step of the metastatic process.

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