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The Arrest of Circulating Tumor Cells in the Liver Microcirculation*

A Vital Fluorescence Microscopic, Electron Microscopic and Isotope Study in the Rat

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Summary. Previous studies have shown that thrombocytopenia reduces the lodgement of circulating tumor cells, thus indicating a role of platelets in tumor cell lodgement. In this study, a vital fluorescence microscopic technique, electron microscopy and isotope measurements were combined to analyse the very first phase (5 min) of tumor cell lodgement using a syngeneic, methylcholantrene induced fibrosarcoma. The mode of tumor cell arrest in the liver microvasculature of normal and thrombocytopenic rats was studied. In addition, tumor cell size and deformability were investigated in vitro. The in vitro experiments showed that the fibrosarcoma cells were very rigid. Both the vital microscopic and electron microscopic studies indicated that the tumor cells mainly became arrested by mechanical trapping in narrow liver sinusoids. Arrest of tumor cells by adherence to venular walls was a rare finding. Platelets did not seem to influence any of these initial phenomena of tumor cell lodgement. The isotope measurements, which showed no difference in the number of tumor cells lodged in the liver between normal and thrombocytopenic rats, further indicated that the initial arrest of the fibrosarcoma cells was not influenced by platelets.

Key words: Tumor cell trapping – Vital microscopy – Liver microcirculation

Introduction

Once a tumor cell has reached the blood circulation, the key event in metastasis formation is the arrest of the tumor cell (Hilgard 1978). This arrest occurs most frequently in the microvasculature (Zeidman 1961). The vital microscopic studies of tumor cell lodgement and metastasis formation in rabbit ear chambers by Wood and co-workers (Wood 1958; Wood et al. 1961) have profoundly influenced the concepts of secondary tumor spread. These investigators observed that microthrombi consisting of platelets, leucocytes and fibrin rapidly formed at the site of tumor cell arrest. Later studies, with other methods, have indicated that particularly the platelets play an important role in the lodgement of circulating tumor cells and metastasis formation (Jones et al. 1971; Warren and Vales 1972; Gasic et al. 1973; Ivarsson 1976).

In order to evaluate further the participation of platelets in the lodgement process we recently investigated the effect of thrombocytopenia on the lodgement of intravenously injected tumor cells in the rat lung (Skolnik et al. 1980b). The experiments showed that thrombocytopenia significantly reduces tumor cell lodgement, thus providing additional evidence in support of platelet involvement in this process. However, the mechnisms by which the platelets influence tumor cell lodgement still remain hypothetical. This is to a great extent due to the fact that the information available concerning the course of events in the lodgement process is mainly based either on in vivo observations in organs where metastases do not normally develop or on morphological studies.

In theory, tumor cell lodgement may be divided into two phases. The first phase should involve factors that cause the circulating tumor cells to *stop* at a certain location. The second phase involves the factors that make the arrested tumor cells *stay* and survive at these locations, making tissue invasion and metastasis formation possible.

In the present study, we have paid attention to the first phase of the lodgement process, especially to the mode of tumor cell arrest. The experiments were performed with a newly developed chamber technique which permitted vital microscopic observations of the

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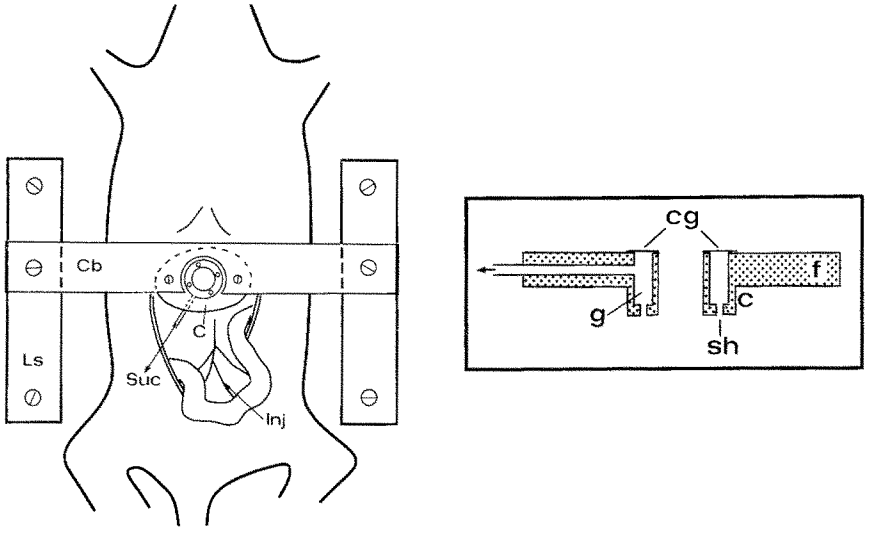

Fig. 1. Schematic drawing of the experimental set-up. A magnified cross-section of the suction chamber (*C*) is shown to the right. *Suc:* suction tube, *Inj:* needle for intraportal injection, *Cb:* crossbar connected to vertically adjustable supports (*Ls*), *cg:* cover glass sealing the top of the circular groove (*g*) in the collar (*c*) of the chamber, *f:* flange, *sh:* suction holes

behaviour of tumor cells in the rat liver microcirculation. The in vivo observations were combined with ultrastructural studies, quantitative isotope measurements and in vitro measurements of tumor cell deformability.

Materials and Methods

Hooded rats of the Lister strain, weighing about 200 g, were used. The rats were of the same age and sex in each experiment. Anaesthesia was induced with IM injection of penthobarbital (5 mg/100 g body wt.) and diazepam (0.4 mg/100 g body wt.). After induction of anaesthesia, the rats were tracheotomized, placed in a supine position and the abdomen was opened by a midline incision. A portion of mesentery was spread over a small rubber plate and a mesenteric vein cannulated near the bowel with a thin (∅ 0.3 mm) needle connected to a syringe by a piece of polyethylene tubing. The needle was fixed to the rubber plate by an artery clamp to keep it in position and prevent it from perforating the vessel wall.

The liver was exposed by gentle pressure on the thorax. A part of the left lobe was then immobilized by a plexiglass suction chamber (Fig. 1). This consists of an oval (max. ∅ 30 mm) flange, 2 mm in thickness, from the centre of which a circular collar (inner ∅ 6 mm, outer ∅ 10 mm) extends a further 2 mm. A cylindrical groove in the flange and collar connects four equally separated holes (∅ 0.5 mm) drilled through the bottom of the groove with a suction channel in the flange. The top of the groove is sealed with a cover glass glued to the upper surface of the flange. The cover glass is then cut at the inner margin of the collar so that the central aperture remains open for microscopic observations. The suction chamber is fixed with screws to a crossbar, attached to a vertically adjustable support on each side of the rat. During the experiments the liver and exposed intestines were kept moist by warm (37 °C) Tyrode solution.

Vital microscopic observations were performed with a Leitz vital microscope equipped with a low light level TV camera (Nightguard, Cotron Electronics Ltd, Coventry, England) and a video tape recorder. Incident light fluorescence microscopy was made using a Leitz Ploemopak illuminator, objektives ×6:0.18, ×11:0.25, ×23:0.45 and eye-pieces ×10 and ×25.

The tumor used was a syngeneic methylcholantrene induced fibrosarcoma (received from the Chester Beatty Research Institute, Sutton, Surrey, England). This tumor spontaneously metastasizes to lymph nodes and causes hepatic metastases when injected intraportally. Tumor cell suspensions were prepared as described by Ivarsson and Rudenstam (1975). The viability of the cell suspensions was about 90%. The tumor cells were labelled with ^{125}I-5-iodo-2-deoxyuridine and radioactivity measured according to Skolnik et al. (1980a). In addition to isotope labelling, the tumor cells were stained with a fluorochrome (acridine orange, 0.01%) so that they could be detected in the liver microcirculation by fluorescence microscopy. One milliliter 0.01% acridine orange was added to 10 ml tumor cell suspension which was immediately washed three times in Waymouth medium to remove excess dye.

In vivo Experiments

Twenty-two rats were randomly divided into two equal groups: Group 1, control animals; and group 2, animals injected IV with 0.5 ml antiplatelet serum 24 h before the tumor cell injection. This specific anti-rat-platelet serum, which was prepared in rabbits according to Ivarsson (1976), reduced the number of platelets to about 20% of the normal value.

After being placed on the microscope stage, each animal received 1 ml tumor cell suspension, containing 5×10^5 tumor cells, intraportally. During the infusion (30 s) the behaviour of the tumor cells in the liver microcirculation was observed in the microscope and recorded on video tape. Five minutes after completion of the tumor cell infusion, eight rats from each group were killed by exsanguination. The livers were removed, washed in tap water and stored in glass tubes for radioactivity measurements. The amount of isotope in the livers and in the injected amount of tumor cells was determined as counts per minute. The number of tumor cells in the livers could then be calculated.

The three animals remaining in each group were used for electron microscopic examination. These experiments were terminated – during continued light microscopic observations – by slow intraportal infusion of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 *M* sodium cacodylate, pH 7.2. After 5 min of fixation in situ an area (approximately 1–2 mm^2) of the liver, containing sinusoids with arrested tumor cells, was excised under a stereo microscope. The specimens were further immersed in the same fixative overnight and then postfixed in 1% osmium tetroxide for 1 h. The specimens were embedded in Epon. Sections were contrasted with uranylacetate and lead citrate.

In vitro Experiments

On the basis of observations made in pilot experiments in vivo, a deformability test of the tumor cells was included in the study. In this experiment, tumor cells were supended in rat plasma and sucked

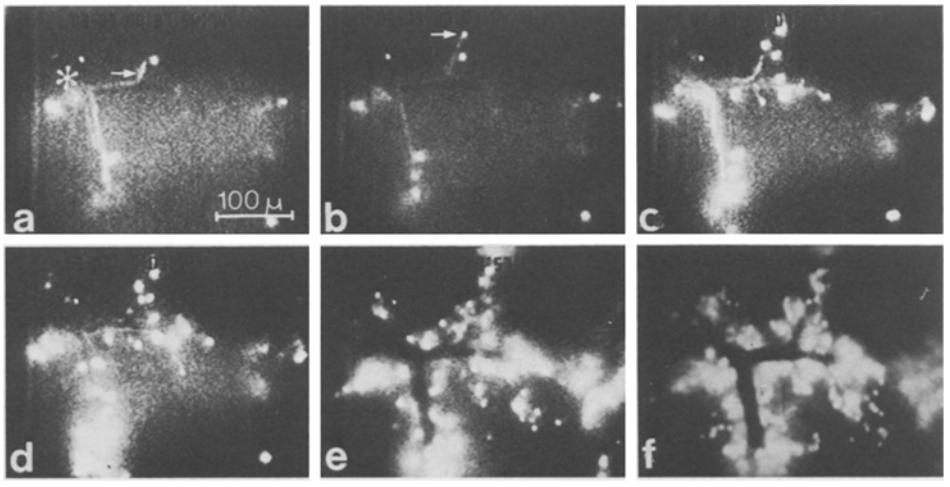

Fig. 2a–f. Sequence of photographs taken from a TV screen showing tumor cell (*white spots*) arrest in the liver sinusoids. A periportal vein emerges at the liver surface at the *asterisk* in **a** – the flow direction in the major branches of the periportal vein (best seen in **e** and **f**) is to the right and downwards. The abrupt arrest of tumor cells in the liver sinusoids is illustrated by the cell indicated by an arrow in **a** and **b**. In **a** the tumor cell moves so rapidly that it is shown only as a continuous trace due to the limited time resolution of the TV system – in **b**, 0.12 s later, the cell has been brought to a complete standstill

through a short glass capillary stenosis (min ∅ 8.0 µm) at a negative pressure of 100 mm H_2O. The time required for each of the 40 tumor cells to deform and pass through the stenosis was calculated from video tape recordings (for further details on the experimental procedure see Bagge et al. 1980).

Before the glass capillary experiments, the diameters of 87 freely suspended, i.e. spherical, tumor cells and 84 rat leucocytes were measured so that an appropriate diameter of the capillary stenosis could be produced. The capillary diameter to tumor cell diameter was chosen such that a comparison should be possible between the deformation data for the tumor cells and those previously obtained for normal leucocytes (Bagge et al. 1980). Leucocytes, which have well-defined rheological properties (Bagge 1975), are similar to freely suspended tumor cells both in respect to shape and general structure and therefore provide a suitable reference for studies on tumor cell rheology. The pressure level in the in vitro system was set at 100 mm H_2O to avoid permanent tumor cell plugging of the stenosis. The experiments were performed at room temperature (20–22 °C).

Statistical Methods

The statistical analyses were performed using the Student's *t* test and Wilcoxon's rank sum test.

Results

The suction chamber did not ensure full immobilization of the livers, but slight movements remained in synchrony with respiration. However, the intense fluorescence of the tumor cells (Fig. 2) still made it possible to study their flow behaviour in detail. It was also possible to estimate the quality of the blood flow (erythrocytes), whereas the technique did not allow identification of individual platelets.

Upon intraportal injection, the tumor cells first emerged in the periportal veins. Very few cells were observed to become adherent to the endothelium and to stay in the periportal veins or in the central veins. The rheological behaviour of the tumor cells was conspicuously different in the sinusoids where large numbers of tumor cells were arrested, either one by one or in rows of as many as five or six cells. The arrest of the tumor cells was very abrupt and usually occurred closer to the periportal veins than to the central veins (Fig. 2). With few exceptions, the tumor cells stayed at the initial site of arrest throughout the 5 min observation period. As a result of this trapping, the blood flow in the sinusoids was locally disturbed and redistributed. There were no phenomena suggesting formation of platelet aggregates in connection with the trapping of the tumor cells.

Although the majority of the sinusoids seemed to retain the tumor cells, some were wide enough to constitute pathways for rapid passage of the tumor cells into the central veins. Due to the high velocity of the tumor cells in these vessels it was not possible to calculate the number of cells passing unhindered through the liver microcirculation. The rheological phenomena described above were the same in both experimental groups.

The finding of tumor cell blockage of sinusoids in vivo was confirmed by electron microscopy. Thus, in both groups of animals the majority of the observed tumor cells (about 90%) was found to fill the sinusoidal lumina completely (Fig. 3a, b, c). Only about 10% of the tumor cells were seen to adhere to the endothelium in sinusoids with diameters larger than that of the tumor cells (Fig. 4a, b). Cells adhering to the endothelium in periportal or central veins were not observed. Although some of the tumor cell profiles (about 5%) were associated with aggregated platelets (only in normal rats; Fig. 4b) or leucocytes (Fig. 4c), most tu-

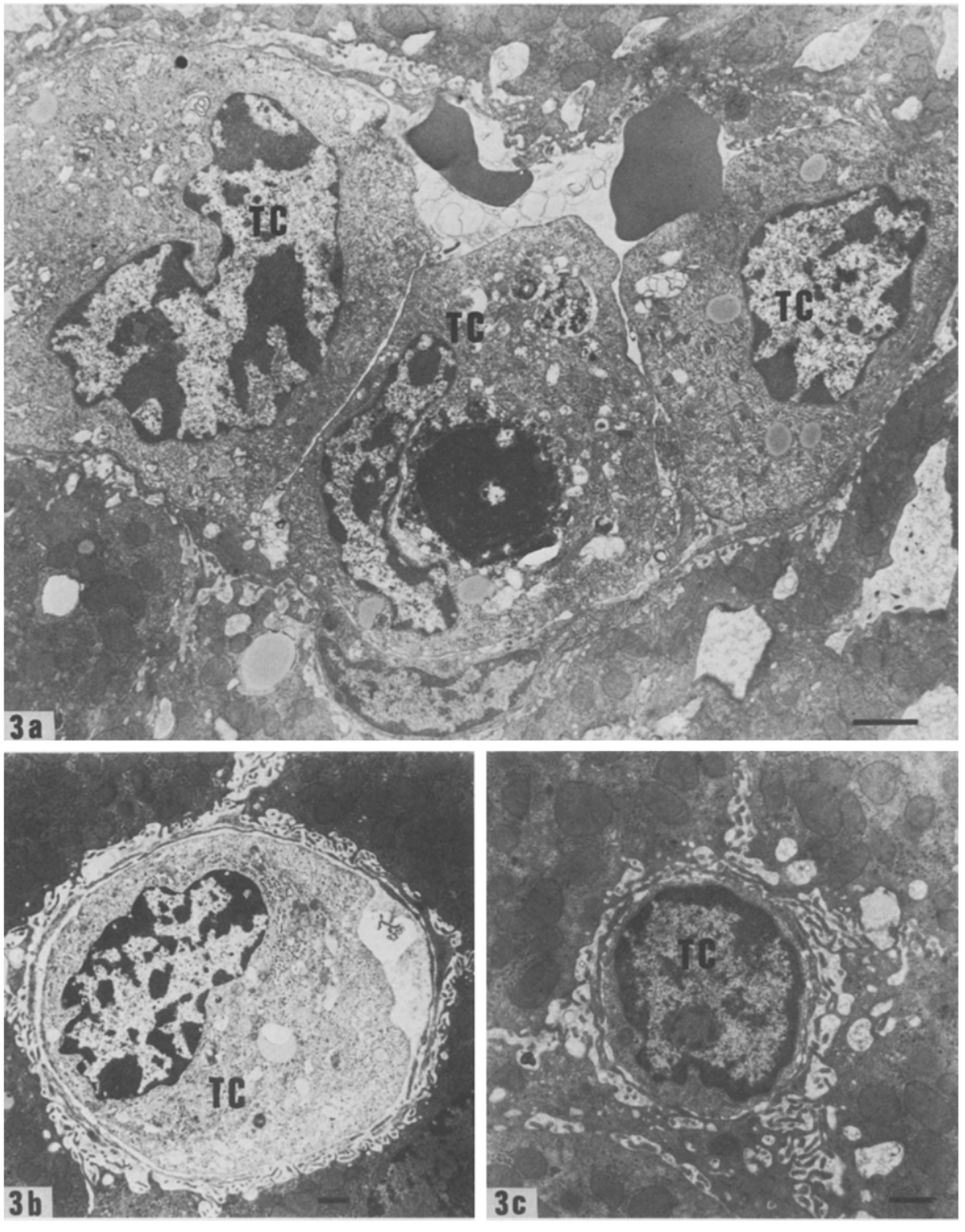

Fig. 3 a–c. Electron microscopy. Bar denotes 1 μm. **a** Three tumor cells (*TC*) are almost completely filling the lumen of this longitudinally sectioned sinusoid. **b** A tumor cell (*TC*) is completely filling the lumen of a transversely sectioned sinusoid. **c** A tumor cell (*TC*) jammed in a sinusoid, which has a smaller diameter than that in previous figure

mor cells did not display such an association. In some instances fibrin-like material was found in a sinusoidal lumen containing a tumor cell (Fig. 4d).

Pseudopods, originating from Kupffer cells and closely adhering to tumor cells, were observed in several instances (Fig. 4c, d), suggesting phagocytic activity. Disruption of tumor cells was not seen.

The in vitro studies showed that the tumor cells used in the in vivo experiments had an average diameter of 10.8 μm (range 7.5–15 μm). The average diameter of the leucocytes was 6.6 μm (range 6.0–8.8 μm). The mean deformation time of the tumor cells in the glass capillary stenosis was 9.8 s (range 0.7–78.9 s; S. D. 16.50 s); for a comparison with leucocytes, see Table 1.

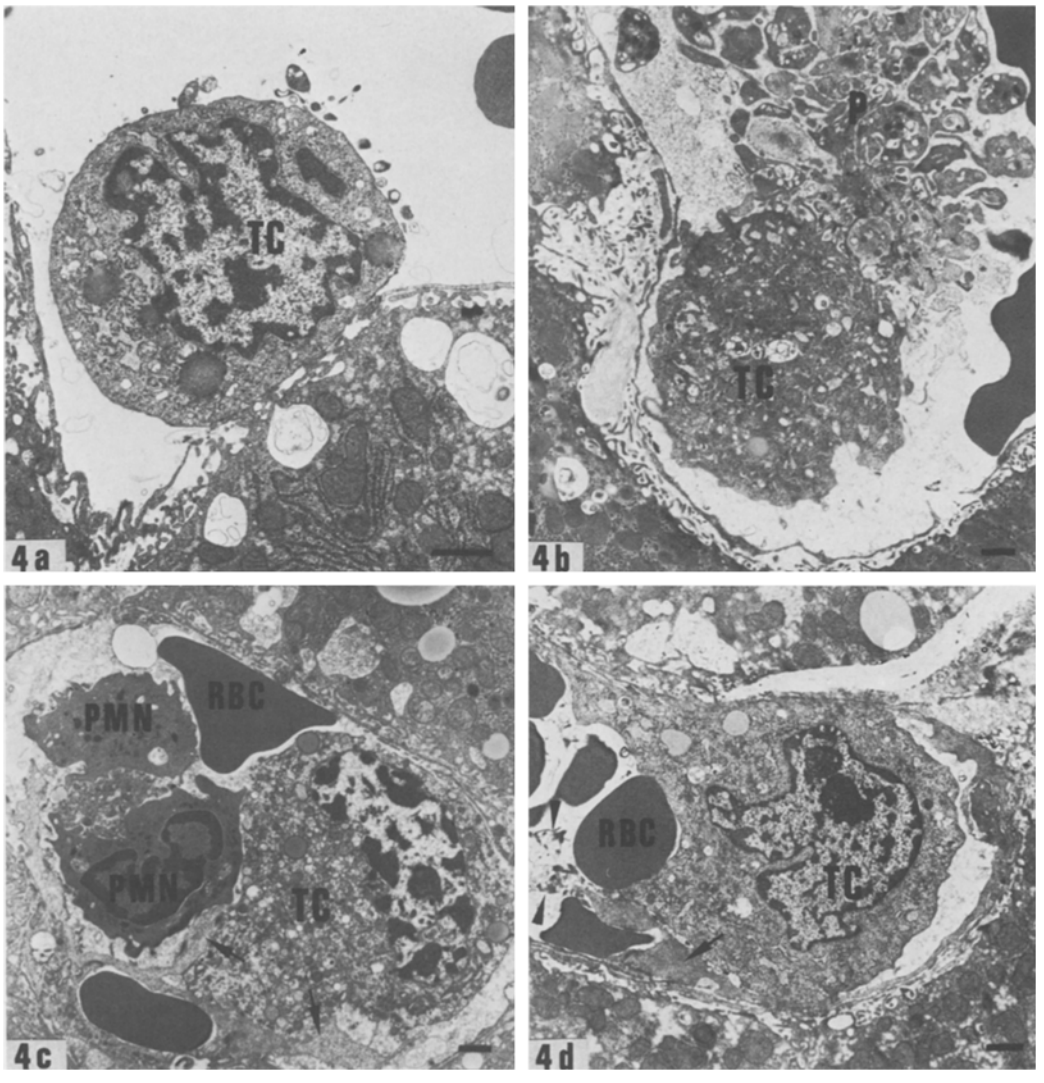

Fig. 4 a–d. Electron microscopy. Bar denotes 1 μm. **a** A tumor cell (*TC*) is adhering to the endothelial lining of a wide sinusoid (thrombocytopenic rat). **b** An aggregate of degranulated platelets (*P*) is in contact with a tumor cell (*TC*) (normal rat). **c** Polymorphonuclear leucocytes (*PMN*) and red blood cells (*RBC*) are present together with a tumor cell (*TC*) in a sinusoid. Cytoplasmic projections (*arrows*), in all probability pseudopods from a Kupffer cell, are closely applied to the tumor cell. **d** Fibrin-like material (*arrowhead*) is present in a lumen containing a tumor cell (*TC*) and red blood cells (*BRC*). A pseudopod (*arrow*) is in contact with the tumor cell

Table 1. Size and deformability of tumor cells and leucocytes

	Tumor cells	Leucocytes
Average diameter	10.8 μm (7.5–15)	6.6 μm (6.0–8.8)
Mean deformation time		
1. Pressure – 100 mm H_2O Minimal capillary diameter 8.0 μm	9.8 s (0.7–78.9) (SD: 16.50)	
2. Pressure – 30 mm H_2O Minimal capillary diameter 5.0 μm		0.59 ± 0.05 s [a]

[a] From Bagge et al. (1980)

The isotope measurements showed that only about 10% of the labelled tumor cells remained in the livers 5 min after intraportal injection. There was no statistically significant difference between the normal and thrombocytopenic animals (Table 2).

Discussion

Vital microscopy is the only experimental method that allows studies of the rheological behaviour of individual tumor cells in the microvascular bed. Earlier vital microscopic studies on tumor cell lodgement have been performed in low-differentiated tissues such as the me-

Table 2. Lodgement of ^{125}I-labelled tumor cells in the liver 5 min after intraportal injection of 5×10^5 cells

	Number of animals	Mean number of tumor cells in the liver
Control	8	52415 (SD 3299)
Thrombocytopenia	8	52186 (SD 4374)

sentery (Zeidman 1961; Gastpar 1977) or in the rabbit ear chamber (Wood 1958). In the present investigation we have extended the use of vital microscopy to observations of the mode of tumor cell arrest in the liver microcirculation. Of the organs known as common sites for metastases, the liver was chosen before the lung, which we have used in two previous isotope studies on the lodgement of circulating tumor cells (Skolnik et al. 1980 a, b), because exposure of the liver for vital microscopy is simpler and also permits survival of the animals. Although the method required staining of the tumor cells, this did not seem to affect the metastatic properties of the tumor cells. Thus, we found in pilot experiments that fibrosarcoma cells labelled with isotope (^{125}IUDR) and strained with acridine orange could still produce liver metastases after intraportal injection. A crucial point is to what extent other cells, primarily leucocytes, could have been stained when the tumor cell suspension was injected. However, in our experience acridine orange, when injected intravenously even at small concentrations, always instantly stains both leucocytes and endothelial cell nuclei. The fact that, in the present study, no fluorescent staining of the vessel walls was seen, indicates that the washing procedure effectively removed excess dye and excludes the possibility of leucocyte staining.

Both the vital microscopic and electron microscopic studies indicate that the initial phase of tumor cell lodgement in the liver microvasculature mainly consists of mechanical entrapment in the sinusoids due to pronounced cell rigidity. This rheological behaviour of the fibrosarcoma cells, which agrees with that observed also for other types of tumor cells (Zeidman 1961; Sato and Suzuki 1976), is strikingly similar to so-called leucocyte plugging (see Bagge and Brånemark 1977). Leucocyte plugging, which occurs in the normal microcirculation, is an important cause of intermittent flow in capillaries. The phenomenon is related to the driving pressure and dimensions of the microvasculature and the size, spherical shape and rigidity of the leucocytes. Thus, leucocyte plugging occurs whenever a leucocyte engages a microvessel which has a diameter smaller than the leucocyte. Due to the rigidity of the leucocytes their adaptation to the vessel lumen is slow (in comparison with that of erythrocytes) causing a temporary obstruction of the blood flow. In situations with low driving pressures the leucocytes may become permanently trapped in narrow capillaries (Bagge et al. 1980).

Freely suspended tumor cells are similar to leucocytes in shape (spherical) and general structure. The present study shows that the tumor cells have considerably larger diameters than leucocytes (as to leucocyte dimensions, see also Schmid-Schönbein et al. 1980). Further, the in vitro experiments show that the tumor cells are much more resistant to deformation than leucocytes. Since the average tumor cell diameter is approximatively twice that of the liver sinusoids ($5.5\ \mu m \pm 0.4$ SE; Reilly et al. 1981), large deformations are usually required before the tumor cells can pass through the sinusoids. Further, with increasing cell diameter the friction and adhesive forces increase because a greater surface area is in contact with the vascular endothelium. Considering these boundary conditions, mechanical entrapment of the tumor cells in the liver sinusoid should not be surprising. Still, the isotope measurements showed that the vast majority of the circulating tumor cells did not become arrested in the liver. In order to explain this discrepancy between theory and experimental results, one must assume that the tumor cells were shunted past the narrowest sinusoids in the liver. Rapid passage of tumor cells through wider sinusoids was in fact observed in vivo, although the number of passing tumor cells could not be quantitated.

Theoretically, mechanical entrapment of tumor cells should not require the presence of platelets. This notion is clearly supported by the present vital microscopic observations which showed no qualitative difference in the arrest of fibrosarcoma cells in the liver between normal and thrombocytopenic animals. Further, the electron microscopic studies only occasionally revealed the presence of platelets and fibrin in relation to tumor cells jammed in narrow sinusoids. The platelets did not seem to be responsible for tumor cell arrest through adhesion to the endothelium in larger sinusoids either. Adherent tumor cells, which were rather few, were found equally often in both normal and thrombocytopenic rats. In addition, the isotope measurements showed that the number of labelled tumor cells lodged in the livers at the end of the 5 min observation period was not affected by thrombocytopenia. Similar results have been obtained in studies on the initial phase of tumor cell lodgement also in the lungs, using B 16 melanoma cells (Gasic et al. 1973).

To summarize, the present results seem to indicate that the initial arrest in the liver of intraportally injected fibrosarcoma cells is not influenced by platelets. Since subsequent studies using the same tumor (Skolnik et al. 1983) have shown that thrombocytopenia reduces the hepatic lodgement 3 h after intraportal tumor cell injection, it seems that the previously reported

effect of thrombocytopenia on tumor cell lodgement (Gasic et al. 1973; Skolnik et al. 1980 b) must be exerted during the later phase of the process.

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