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Novel In Vitro Perfusion Model to Study the Interaction Between Coagulation and Blood-Borne Metastasis

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Abstract The association between cancer and hemostasis has long been studied in cell culture, animal models, and cancer patients developing thrombosis. The variety of biologic mechanisms involved in malignancy and metastasis makes the understanding of the relative importance of each mechanism difficult. We have developed a novel in vitro perfusion model that allows for the isolated study of the interactions between tumor cells and components of the hemostatic system under normal physiologic conditions. Segments of denuded umbilical cord or saphenous vein are cut longitudinally and mounted in a perfusion chamber under sterile conditions. Human breast cancer cells are perfused for 24 h under venous flow conditions with either whole blood (WB), platelet-rich plasma (PRP), platelet-poor plasma (PPP), or serum. Tissue samples are fixed and stained with hematoxylin and eosin as well as with pan-cytokeratin. Morphometric analysis is performed to quantify cancer cell adhesion. With PRP, this model maintains normal human physiologic conditions for the duration of the experiment. It differentiates between previously characterized high and low metastatic breast cancer cell lines. In addition, different vein tissue types do not alter tumor cell attachment. This model appears to be an accurate representation of the pathophysiology of in vivo metastasis. This model may serve as a useful bridge between cell culture studies and animal models. It may be a useful tool to elucidate the role of selected hemostatic systems in blood-borne metastasis and may potentially serve as a screening tool for the development of antimetastatic pharmaceutical agents. J. Cell. Biochem. 96: 700–708, 2005.

Key words: coagulation; metastasis; perfusion; vein segments

Since the initial observation by Trousseau in 1865, there has been an increasing body of clinical evidence establishing a link between cancer metastasis and a prothrombotic state, the latter frequently preceding the former. Hemostatic abnormalities are found in more than 90% of cancer patients and are clinically expressed in 11%–15% of these patients as venous thromboembolic episodes, postoperative venous thrombosis, or disseminated intravascular coagulation with hemorrhage and thrombosis [Bick, 1992; Rickles et al., 1992]. Up to 50% of patients with malignant cancer are found upon postmortem autopsy to harbor thrombosis. Thrombosis remains the second most common cause of death in hospitalized cancer patients [Ambrus et al., 1975a,b; Sack et al., 1977].

Studies have shown increases in fibrinogen/fibrin-related antigens and fibrinopeptide A in 60% of cancer patients, with levels increasing in conjunction with disease progression and poor prognosis [Yoda and Abe, 1981; Rickles et al., 1983]. Furthermore, there is an increase in thrombin generation as well as activated factor VIIa of the extrinsic coagulation pathway in patients with cancer [Constantini et al., 1998].

There also exists a large volume of in vitro and in vivo studies showing interactions between circulating tumor cells and various components of the hemostatic system. Upon intravasation from the primary site, tumor cells interact with platelets and other components of the

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hemostatic system [Mousa, 2002]. Platelets have been shown to be a requirement for the hematogenous dissemination of cancer [Gasic et al., 1973; Hilgard, 1973]. Platelets also "cloak" the tumor cells from the body's natural immune system, thus aiding in tumor cell survival [Trikha and Nakada, 2002].

Thrombin is another element from the coagulation cascade that plays a role in tumor metastasis. It is a potent platelet activator leading to exposure of the glycoprotein (GP) IIb/IIIa sites on platelets, which can also serve as connecting points between platelets and tumor cells [Felding-Habermann et al., 2001]. Activated platelets also release fibronectin and von Willebrand factor, resulting in increased adhesion. Certain cancers can induce thrombin generation and can be activated themselves by thrombin, thus resulting in a positive feedback mechanism [Nierodzik et al., 1991, 1992]. Tumor cells can activate thrombin generation via production of tissue factor (TF) by the cell itself or stimulating its production from host endothelial, monocyte, and macrophage cells [Lorenzet et al., 1983; Rambaldi et al., 1986]. It has been shown that the extracellular active TF/factor VIIa complex works synergistically with the TF cytoplasmic domain to enhance the efficiency of hematogenous tumor cell dissemination. Furthermore, TF can promote chemokine production from a variety of cell types, thereby promoting tumor cell infiltration [Mueller et al., 1992; Mueller and Ruf, 1998].

Both clinically and at the molecular level, there is strong evidence emphasizing an essential, although not fully understood, interaction between circulating tumor cells and the hemostatic system. However, it is still unclear which particular interaction or interactions play a major role in the metastatic process, and whether these interactions vary with respect to the cancer type.

To study the relationship between circulating tumor cells and the hemostatic system, we have developed a novel in vitro perfusion model that utilizes intact segments of human vein and cell cultured tumor cell lines. These studies, unlike any other in vitro models utilized to study metastasis, are performed under normal physiologic, sheer-stress flow conditions. The model permits the isolation of the individual pathways of interaction between tumor cells and the hemostatic system, allowing for the determination of each interaction's role in and relative importance to the spread of cancer.

METHODS

Perfusion Design

The custom-designed perfusion chamber, made of Plexiglas, is 12 cm long and 4.5 cm wide with a 1-cm-thick silastic base. The chamber has outlets on each end that can be connected via rubber tubing to a 250 ml flask containing the perfusate. The perfusion chamber is placed in a glove box to provide a sterile environment during perfusion (Fig. 1). The perfusion chamber, all surgical tools, glassware, and pump tubing (food-grade Norprene) are autoclaved prior to use.

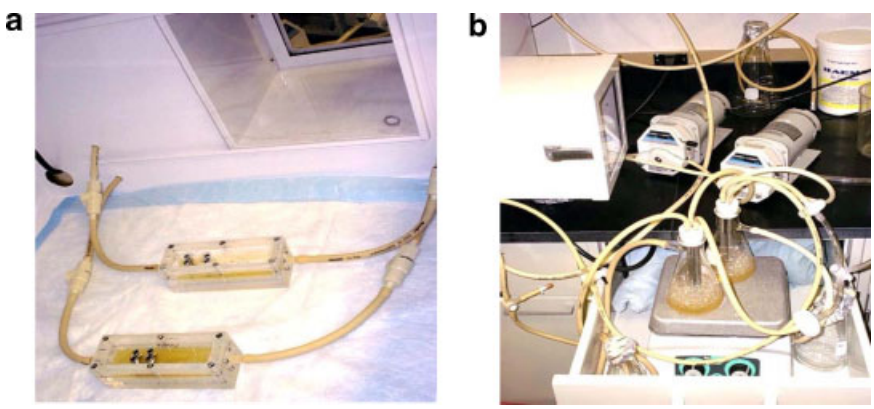

Fig. 1. Setup of the perfusion system. Matched parallel experiments can be run utilizing two chambers in which vein segments are from the same donor and platelet-rich plasma (PRP) perfusate is from a single donor. **a**: Two chambers, with umbilical cord vein segments pinned to the silastic base, are inside a sterile glove box. **b**: Perfusate is being pumped into the chambers from 2 individual flasks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tissue Harvesting

Following informed consent, fresh umbilical cord is obtained using sterile technique from the placenta following normal delivery via cesarean section from patients at Evanston Hospital. There are no criteria defined for participating patients except that they have a normal, low-risk, full-term, elective cesarean section delivery. Approximately 10 cm of the cord is excised. The cord is rinsed and wiped free of blood using cold 0.9% saline (Abbott Laboratories; Waukegan, Illinois) and sterile gauze. Then, using a syringe equipped with an 18 gauge Luer lock adapter, cold saline is used to flush the vein clean of blood. Two sections of umbilical cord (approximately 3 cm each) are cut longitudinally to expose the vein surface and are pinned to the silastic base of the chamber using reusable hypodermic needles (27 G $\times$ 1 inch). The vein surface is optionally denuded with 3–4 gentle strokes of a cotton swab depending on the different conditions to be tested. Any leftover vein is cut longitudinally and immediately fixed with neutral buffered formalin (NBF) as a control tissue.

Following informed consent, leftover saphenous vein is obtained at the end of a coronary artery bypass graft (CABG) procedure. There are no criteria defined for participating patients except that they have a CABG using a normal saphenous vein for the bypass. Using sterile technique, the extra vein from the operation is cut into two equal-length segments and pinned to the silastic base of the chamber at the four corners. There is no need to denude the vein segment due to the time lapse of several hours between vein harvesting and the end of the CABG operation, at which time the endothelial layer has already deteriorated. A small control section of vein is immediately fixed using NBF.

Cancer Cell Lines

Human breast cancer cell lines including MCF-7 (low metastatic propensity) and MDA-MB-231 (high metastatic propensity) were obtained from ATCC (Manassas, Virginia).

Perfusate Preparation

The perfusate is prepared with fresh whole blood (WB) collected in 3.2% citrate tubes, platelet-rich plasma (PRP), platelet-poor plasma (PPP), or serum, and it is supplemented with an antibiotic cocktail consisting of penicillin (50 U/ml), streptomycin (50 μg/ml), amphotericin B (1 μg/ml), and gentamicin (10 μg/ml). A 4 ml perfusate sample is collected and stored at -80°C for future analysis. Tissue culture cancer cells are added at a concentration of 0.2×10^6 cells/ml for perfusion.

Perfusion Procedure

The perfusate is continuously mixed and perfused at standard venous flow conditions (70 ml/min) for the 24 h duration of the experiment. It is bubbled gently with 5% CO_2/95% O_2. After 15 min and just prior to the termination of the perfusion period, a sample of the perfusate is collected to measure the pH, pCO_2, pO_2, glucose, and lactate. At the end of the perfusion, a 4 ml sample of the perfusate is collected and stored at -80°C for future analysis. The tissue segments are immediately fixed for 24 h and then sliced for processing and paraffin embedding. They are cut and stained with hematoxylin and eosin, as well as stained with pan-cytokeratin for morphometric analysis.

Morphometric Analysis

The initial attachment of a cancer cell in which a cell is bound to the vessel wall by a small amount of membrane and maintains its original shape is termed a contact cancer cell. The latter stage in which a cancer cell is more intimately bound to the subendothelium before penetrating is termed cell adhesion. The final stage in which a cancer cell has fully or partially infiltrated the subendothelial surface is termed cell penetration. Clusters of two or more cells are quantified as clusters and noted for the number of cells per cluster. By quantifying the percentages of cells in the contact, adhesion, and penetration stages, we can determine cancer cell attachment to the subendothelium, the first step in metastasis. In addition, the vein surface length is measured to calculate the number of cancer cells attached per unit length. Metastatic potential is characterized by calculating the number of cells attached per unit length and then normalizing for cancer cell concentration in the perfusate. Quantitative cell invasion and metastasis will be carried out in future studies.

Statistics

The cell attachment data from morphometric analysis are normalized to the exact cell concentration that was perfused over the tissue. A

two-tailed *t*-test is performed to determine significance of difference in cell attachment between groups.

RESULTS

Physiologic Measurements of the Experiments

The model has been able to maintain human physiologic pH, pCO_2, and pO_2 levels throughout the 24-h perfusion period (Table I). Over the course of the experiments ($n = 44$), pH levels rose, whereas pCO_2 and pO_2 levels both declined. However, all values at the beginning and end of the perfusion period are within normal physiologic range. Furthermore, data show a consumption of glucose and an increase in lactate throughout the experiment.

Microscopic Observations of the Experiments

For both human breast cancer cell lines (MCF-7 and MDA-MB-231) and in all perfusate types, adhesion of the cancer cells occurs only to the subendothelium and not to the endothelial surface (Fig. 2). In addition, different stages of interaction were observed, namely contact (Fig. 3), adhesion (Fig. 4), and penetration (Fig. 5). These varying stages of interaction can be seen with both individual and clustered cells. In WB or PRP, presence of microemboli—consisting of cancer cells, fibrin, and platelets—was further observed (Fig. 6). When serum or PPP is used as the perfusate, single cells are still able to interact with the subendothelium via pseudopod-like extensions leading to penetration of the subendothelial matrix (Fig. 7).

Comparison of Cell Attachment Between MCF-7 and MDA-MB-231

A comparison between the invasiveness of breast cancer cell lines MDA-MB-231 (high propensity to metastasize) and MCF-7 (low propensity to metastasize) revealed a higher attachment of MDA-MB-231 cells in PRP perfusate (Fig. 8). MDA-MB-231 cells attached at 66.80 ± 11.78 cells attached/millimeter/cell concentration (CA/mm/CC), while MCF-7 cells attached at 19.29 ± 3.22 CA/mm/CC. Statistics show a significantly greater attachment of MDA-MB-231 ($n = 14$) cells compared to MCF-7 ($n = 14$) cells ($P < 0.01$).

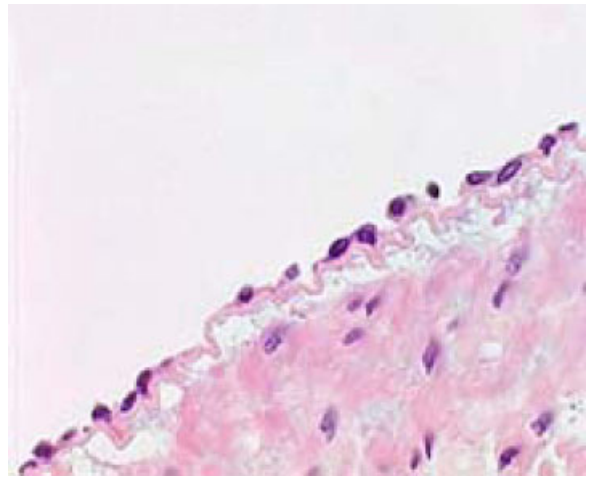

Fig. 2. A non-denuded umbilical cord vein perfused with platelet-poor plasma (PPP). No cancer cells are attached to the intact endothelium. Magnification 20×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Comparison of Cell Attachment Between Umbilical Cord and Saphenous Vein

A comparison between cell attachment data obtained from perfusions utilizing saphenous vein or umbilical cord segments for both MDA-MB-231 (Fig. 9) and MCF-7 (Fig. 10) cell lines was made using an unpaired *t*-test. The MDA-MB-231 cell line attached to umbilical cord ($n = 6$) at 57.55 ± 13.48 CA/mm/CC of cells and to saphenous vein ($n = 6$) at 51.12 ± 10.24 CA/mm/CC. MCF-7 cells attached to umbilical cord at 16.71 ± 4.55 CA/mm/CC and to saphenous vein at 17.55 ± 4.97 CA/mm/CC. Similar to

TABLE I. Physiologic Measurements Are Taken 15 min After the Commencement and at the End of the Perfusion Period

	Physiologic properties				
Time	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Glucose (mg/dl)	Lactate (meq/L)
15 min	7.351 ± 0.009	30.76 ± 0.859	209.1 ± 14.616	375.1 ± 8.323	2.22 ± 0.185
24 h	7.432 ± 0.030	22.97 ± 1.274	193.2 ± 9.387	359.8 ± 9.443	3.47 ± 0.192

All physiologic categories are within normal human physiologic ranges for both time points.
Each value is the mean ± the standard error for all values collected ($n = 44$).

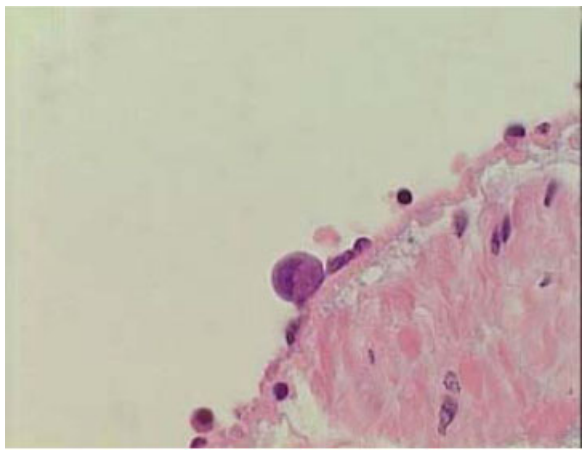

Fig. 3. A moderately denuded umbilical cord vein perfused with PPP. A large cancer cell is in direct contact with the subendothelium. The slight flattening of the cell at the sub-endothelial interface suggests the beginning of adhesion. Magnification 50×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MDA-MB-231, MCF-7 cells did not show significant difference in attachment between saphenous vein (n = 6) and umbilical cord (n = 6). Furthermore, both MDA-MB-231 and MCF-7 cell lines still maintained their invasiveness relative to each other. Based on image analysis and quantitative measurements of the adhesion levels, our initial data clearly showed that the greater the number of cells attached to sub-endothelium the greater the penetration and invasion.

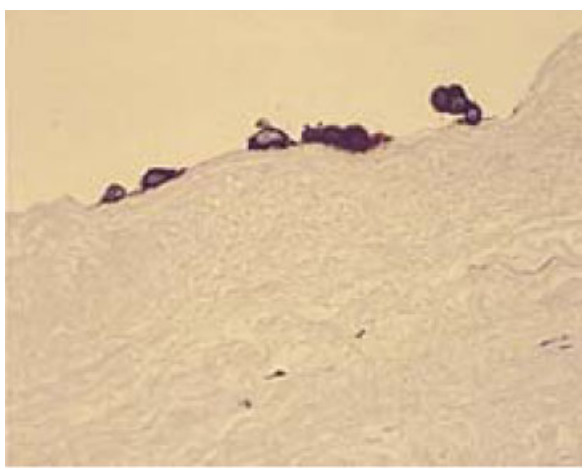

Fig. 4. A saphenous vein segment perfused with PPP. Flattened cancer cells attached to the subendothelium are in a state of adhesion. Magnification 50×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

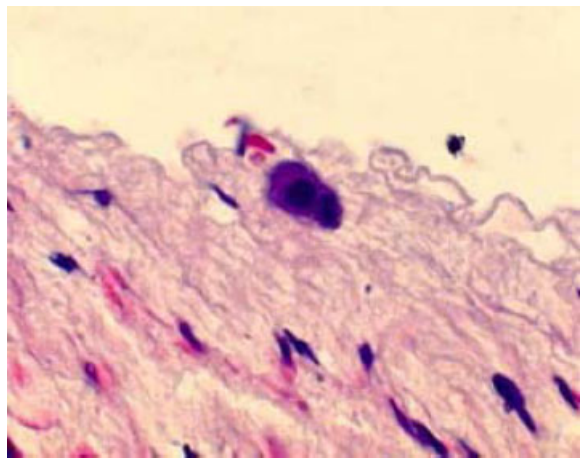

Fig. 5. A denuded umbilical cord vein perfused with serum. A cluster of two cancer cells have penetrated into and are embedded in the subendothelium. Magnification 100×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

There have been a variety of in vitro perfusion models and techniques used to study the hemostatic system and its interaction with human vessel walls. Baumgartner [1973] designed a perfusion model that allowed for the in vitro study of blood flow in relation to platelets, fibrin, and thrombi formation. The overall setup of our perfusion model mimics his design. Porter et al. [1996] pinned saphenous vein segments

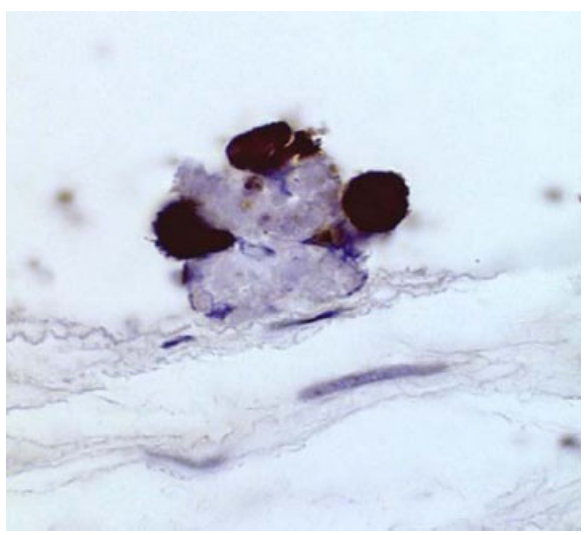

Fig. 6. A denuded umbilical cord vein perfused with whole blood (WB). A microembolus, composed of microfibrin, platelets, and three cancer cells, is anchored to the vein subendothelium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

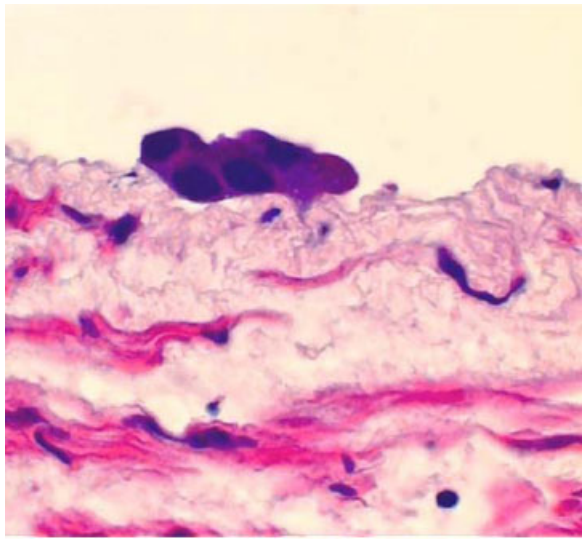

Fig. 7. A denuded umbilical cord vein perfused with human serum. A pseudopod-like extension is visible, indicating the beginnings of penetration. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

on silicone rubber tubing to study intimal hyperplasia. In our experiment, vein segments are pinned onto a silicone silastic base inside perfusion chambers. Labadie et al. [1996] described an antibiotic cocktail that combated infection while preserving proper saphenous vein vasomotor responses and endothelial cell morphology for perfusion periods up to 48 h. This antibiotic cocktail is included in our perfusate recipe. Turitto et al. [1983] devised a system to count platelet attachment to vessel walls. We have adapted this system for the identification and categorization of the various degrees of tumor cell attachment.

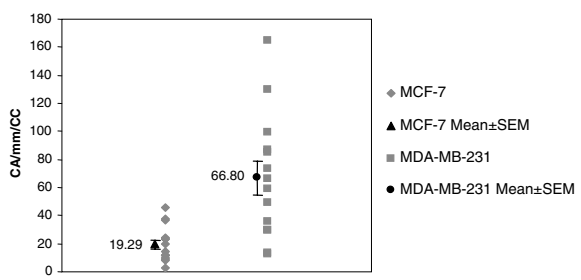

Fig. 8. Attachment of MCF-7 and MDA-MB-231 breast cancer cells to umbilical cord vein tissue replicates published data on the relative invasiveness of each breast tumor type. MCF-7: 19 ± 3.22 CA/mm/CC, n = 14. MDA-MB-231: 66.80 ± 11.78 CA/mm/CC, n = 14. A two-tailed *t*-test shows significantly higher attachment of MDA-MB-231 cells ($P < 0.005$). CA/mm/CC = cells attached/millimeter/cell concentration. Data represent mean ± SEM.

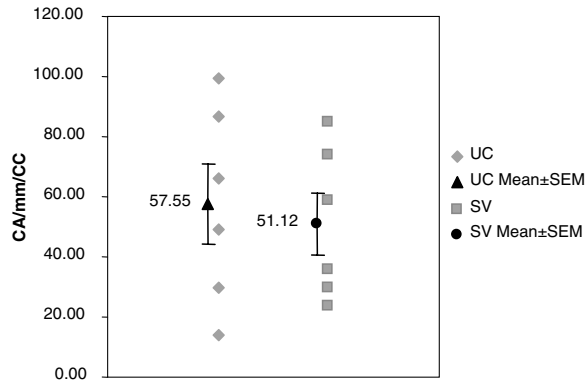

Fig. 9. MDA-MB-231 breast cancer cells attach similarly to both umbilical cord (UC) vein and saphenous vein (SV). UC: 57.55 ± 13.48 CA/mm/CC, n = 6. SV: 51.12 ± 10.24 CA/mm/CC, n = 6. A two-tailed *t*-test reveals no significant difference in attachment between the two groups ($P = 0.90$). CA/mm/CC = cells attached/millimeter/cell concentration. Data represent mean ± SEM.

As shown, we have brought together and adapted various aspects of previous designs to develop our perfusion model. Unlike other in vitro models utilized to study metastasis, this novel in vitro model allows for the study of the interactions between blood-borne metastasis, the hemostatic system, and the vein surface, while under normal physiologic, sheer-stress flow conditions. The physiological measurements and cell attachment results, obtained from experiments utilizing this perfusion model, are consistent and reproducible, and they appear to reflect human in vivo physiologic conditions.

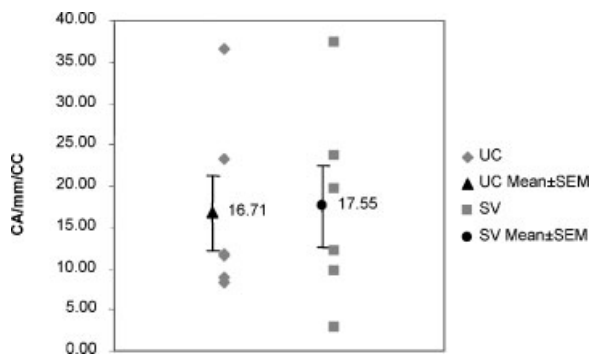

Fig. 10. MCF-7 breast cancer cells attach similarly to both umbilical cord (UC) vein and saphenous vein (SV). UC: 16.71 ± 4.55 CA/mm/CC, n = 6. SV: 17.55 ± 4.97 CA/mm/CC, n = 6. A two-tailed *t*-test reveals no significant difference of attachment between the two groups ($P = 0.99$). CA/mm/CC = cells attached/millimeter/cell concentration. Data represent mean ± SEM.

After initial testing using WB, PRP, PPP, and serum each as the perfusate medium, it was concluded that PRP should be used. This conclusion was derived from two key factors. First, platelets have previously been shown to be a requirement for metastasis and were therefore deemed necessary to the experimental design. Second, during perfusions using WB, maintenance of human physiologic conditions was especially difficult due to the presence of red blood cells. Therefore, PRP was deemed the more suitable perfusate medium, and all subsequent morphometric analysis data were obtained from experiments using PRP as the perfusate.

The physiologic markers that were measured all indicate that the perfusion system operates within standard human physiologic norms. The decrease in glucose and increase in lactate observed indicate occurrence of live cell respiration in the system. Staining with hematoxylin and eosin verifies the integrity of the tissue samples, allowing for rejection of data if necessary. Both perfusate physiologic measurements and tissue staining indicate tissue and circulating cell viability. Therefore, this perfusion model demonstrates an ability to maintain human physiologic conditions throughout the 24-h perfusion period.

The analysis of cell attachment for both MDA-MB-231 and MCF-7 breast cancer cell lines suggests replication of conditions for attachment of tumor cells to venous tissue. As reported, MDA-MB-231 cells attach with greater frequency than do MCF-7 cells. This result duplicates previously published in vitro and in vivo characterization of these two cell lines, showing MDA-MB-231 to be a more metastatic cell line than MCF-7 [Hijazi et al., 2000]. There is variation in the exact number of attached cells between trials; however, this can be attributed to differences of the content and interaction activity between tumor cells and the individual donor's blood.

Further confirmation of this model's ability to replicate in vivo results is revealed via microscopic observation. In vivo studies performed in the chick embryo have shown that tumor cells do not attach or weakly attach to endothelial cells [Mousa, 2000]. Rather, they attach via pseudopod-like extensions to the subendothelium at gaps in the endothelial layer. Consistent with the literature, this perfusion model demonstrates tumor cell attachment singly or in clusters to the subendothelium via pseudopod-like extensions.

Several studies have demonstrated that tumor cells form complexes with platelets and leukocytes in the vasculature, resulting in the creation of microemboli [Chambers et al., 1995; Chambers, 1999]. Microemboli allow tumor cells to arrest in the microcirculation for the subsequent attachment to and penetration of the subendothelium [Honn et al., 1992]. This perfusion model, when perfused with WB or PRP, shows microemboli formation consistent with previously published literature. This is noted using blood anticoagulated with sodium citrate, thereby suggesting the perfusion model is capable of procoagulant activity in the form of TF and/or thrombin.

Furthermore, when perfused with serum, this perfusion model still shows attachment of tumor cells to the vein surface. This suggests that tumor cells themselves express various linker proteins that allow the cell to attach to the subendothelial matrix in the absence of fibrin and platelets. Studies have shown that tumor cells express adhesion molecules that allow them to attach to subendothelial matrix components, such as laminin, collagen, and fibronectin [Ziober et al., 1996; Price et al., 1997]. Therefore, this perfusion model has again reproduced that which has been previously documented in published reports.

The comparison between saphenous vein and umbilical cord vein showed no difference in tumor cell attachment, thereby indicating that either vein type is acceptable for use in this model. The similarity in attachment is likely due to the fact that tumor cells attach to the subendothelium instead of the endothelial cells. Studies have shown that the extracellular matrix of all vascular walls is essentially composed of similar proteins, such as collagen, elastin, proteoglycans, and structural GPs [Jacob et al., 2001]. Furthermore, it has been determined that the endothelial cell layer is a relatively nonadhesive and nonthrombogenic entity for the cellular and macromolecular constituents of the blood [Gimbrone et al., 1997]. Therefore, essential attachment and penetration of a particular tumor type is likely equal among all vessels. It is rather chemokines and their relative expression that determine the preferential location of auxiliary tumor site growth, not the specific vessel type [Moore, 2001].

The similarity in attachment allows for the use of umbilical cord in our studies. Umbilical cord tissue provides greater flexibility in manipulating the conditions for experimentation. Full, partial, or no denudation of the endothelial cells is possible for umbilical cord tissue, whereas the saphenous vein, because it is obtained at the completion of surgery, has damaged or absent endothelium. Tumor cells can express growth factors and other signaling molecules that can activate endothelial cells to secrete factors that affect tumor cell metastasis and tumor cell interactions with the hemostatic system. Partial denudation can retain the interaction between tumor and endothelial cells while still providing abundant surface area for attachment.

As shown, this perfusion model can replicate in vivo physiology of circulating cancer cells. The model offers physiologically relevant data and more readily generates data than do in vivo methods. At the very least, a number of experimental variables could be eliminated through our model before animal experiments are performed, consequently allowing for more selective use of animals and thereby reducing the costs incurred with animal use. Furthermore, this model may avoid some of the limitations of animal models [Zucker, 1999]. Depending on the tumor phenotype, any one of the plethora of interactions between tumor cells and the hemostatic system may be more or less important. This point has been demonstrated by the perfusion model showing tumor cell attachment in the absence of fibrin and platelets, despite published reports stating that platelets are a requirement for the hematogenous dissemination of cancer. This shows metastasis to be controlled by a multi-faceted interaction among tumor cells, the hemostatic system, and the subendothelial matrix, all of which are yet to be characterized. With simple alterations of the experimental design (such as partial versus complete denudation of the endothelium; use of WB, PPP, PRP, or serum; or activation of cellular elements of the blood with either lipopolysaccharides or hemin), the isolation of different pathways of interactions between tumor cells and the hemostatic system can be studied. This approach is not always feasible in animal models.

The model also offers benefits of use compared to current in vitro techniques. Standard in vitro tools are static in design and serve more to study the factors that manipulate the tumor cell's microenvironment. Furthermore, there are many frequent temporary effects in small subpopulations that are responses to a given stimulus [Friedl, 1996]. In vitro models, with artificially processed extracellular matrix surfaces, inhibit or promote adhesion and migration in a uniform manner [Friedl et al., 1998]. Therefore, their ability to represent the physiologic and multidimensional nature of metastasis is limited. In some cases, such as the Matrigel outgrowth assay, this model may be more cost-effective than some in vitro models as well.

Potentially, this model may serve as a useful bridge between animal studies and cell culture. It can isolate for study several pathways of interaction among tumor cells, the hemostatic system, and the vessel walls, characterizing their importance in the process of extravasation, while producing physiologically relevant data. Ultimately, defining the relative importance of the multiple pathways of interaction between circulating tumor cells and the surrounding tissue may provide a better rationale for the development and screening of antimetastatic drugs. Future studies will be carried out to determine the correlation between cancer cell adhesion and invasion using improved software and imaging systems.

SPECULATIONS

This model appears to be an accurate representation of the pathophysiology of certain components of in vivo metastasis, and it may serve as a useful bridge between cell culture studies and animal models. It may be a useful tool to elucidate the role of selected hemostatic systems in blood-borne metastasis and may potentially serve as a screening tool for the development of antimetastatic pharmaceutical agents for use therapeutically or in chemoprevention.

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