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Tumor cell adhesion under hydrodynamic conditions of fluid flow

Review article

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Current evidence indicates that tumor cell adhesion to the microvasculature in host organs during formation of distant metastases is a complex process involving various types of cell adhesion molecules. Recent results have shown that stabilization of tumor cell adhesion to the microvascular vessel wall is a very important step for successful tumor cell migration and colonization of host organs. We are beginning to understand the influences of fluid flow and local shear forces on these adhesive interactions and cellular responses within the circulation. Mechanosensory molecules or molecular complexes can transform shear forces acting on circulating tumor cells into intracellular signals and modulate cell signaling pathways, gene expression and other cellular functions. Flowing tumor cells can interact with microvascular endothelial cells mediated mainly by selectins, but the strength of these bonds is relatively low and not sufficient for stable cell adhesions. Integrin-mediated tumor cell adhesion and changes in the binding affinity of these adhesion molecules appear to be required for stabilized tumor cell adhesion and subsequent cell migration into the host organ. Failure of the conformational affinity switch in integrins results in breaking of these bonds and recirculation or mechanical damage of the tumor cells. Various cell signaling molecules, such as focal adhesion kinase, pp60src or paxillin, and cytoskeletal components, such as actin or microtubules, appear to be required for tumor cell adhesion and its stabilization under hydrodynamic conditions of fluid flow.

Key words: Cell adhesion; fluid flow; integrins; selectins; shear forces.

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TUMOR CELL ADHESION DURING METASTASIS FORMATION

Once colorectal cancers have penetrated into the circulation or body cavities and carcinoma cells have been released from the primary tumor, they can be transported to near and distant organs where they must bind to host structures. The majority of circulating cancer cells delivered to various target organs

Abbreviations:
FAK – focal adhesion kinase; EC – endothelial cell; ECM – extracellular matrix; PDGF-R – platelet-derived growth factor receptor; MAPK – mitogen-activated kinase; PKC – protein kinase C; protein tyrosine phosphatase 1B – PTP1B; phosphatidyl-inositol-3-kinase – PI3-kinase; LN – laminin; VN – vitronectin; FN – fibronectin; ICAM-1- intercellular adhesion molecule-1; VCAM-1- vascular cell adhesion molecule-1; sLe^x – Sialyl-Lewis^x; HUVEC – human umbilical vein endothelial cells; Lu-ECAM-1- lung-derived endothelial cell adhesion molecule; mAb – monoclonal antibodies; C I – type I collagen; C IV – type IV collagen.

are trapped and rapidly and lethally damaged in the microvasculature, resulting in cell death and 'metastatic inefficiency' (1). To survive the circulating tumor cells must adhere to the vessel walls of distant host organs, and eventually penetrate the vascular wall to avoid blood shear forces and host defense mechanisms. The interactions between circulating tumor cells and host organs require specific cell surface structures, such as adhesion molecules. Successful cell arrest is dependent on the balance between adhesive and anti-adhesive forces, and the rate at which adhesive interactions are broken (2). The carcinoma cells must first establish adhesive interactions with the endothelium of the host organ. Endothelial cell (EC) adhesion molecules show organ-specific patterns of expression, and differences can be found for these molecules between microvascular EC from liver, lung and other organs, such as brain, spleen, or lymph nodes. Some of these adhesion molecules on the EC surfaces can act as receptors for circulating tumor cells, enabling these cells to establish stable bonds with the endothelium. However, once they attach to EC the tumor cells must induce morphological and functional changes in the EC in order to breach this structure. If they remain in the circulation, the environmental conditions in the circulation are toxic to tumor cells. Furthermore, the blood circulation creates shear forces on the adherent tumor cells, and these forces act against the newly formed adhesions and can break adhesive bonds. Therefore, tumor cells have to quickly stabilize adhesions to the vessel wall and extravasate. For the first task, the adherent tumor cells induce retraction of the EC allowing the tumor cells to interact with the underlying membrane matrix that serves as the backbone for the EC and a barrier between the circulation and the parenchyma. The interactions between tumor cells and the basement membrane are usually stronger than the tumor cell adhesions to EC. In addition, it has been found that shear forces caused by fluid flow in the circulation can induce cell signaling within the endothelium and the circulating tumor cells that can result in activation of various cell functions and modulation of metastatic properties.

MOLECULAR INTERACTIONS AND SIGNALING IN CELL ADHESION COMPLEXES

Functionally, cell adhesion to ECM or neighboring cells provides anchorage for the adhering cell in their environment. This mechanical linkage is a physiological requirement for tissue structure, cellular immunity and other functions involved in maintaining the integrity of the organism. However, anchorage from a biophysical context implies mechanical binding and its functional regulation, because all types of cell adhesions have a dynamic character and can and will be modified over time; however, large differences exist for the duration, speed and strength of these interactions.

Cell adhesions result in linkages to surrounding cells or the extracellular matrix (ECM), primarily basement membrane or interstitial matrix. Cell adhesion also establishes physical anchorage of cytoskeletal proteins to various types of supramolecular structures, such as focal adhesions, desmosomes, hemidesmosomes, or tight junctions that are associated with plasma membranes. These structures not only provide physical linkage, they also play a very important role in the regulation of various cellular functions, such as apoptosis, cell migration, growth regulation, cell differentiation, among others. The signaling pathways involved in these processes demonstrate an extraordinary array and complexity, including numerous protein-protein interactions, signaling pathways, and a number of different (secondary) cell adhesion receptors. Indeed many signals are interlinked with other cell surface receptors, such as growth factor receptors. For example, integrin-binding mediates activation and downstream signaling of the focal adhesion kinase (FAK), and this can be modulated by activation of platelet-derived growth factor receptor (PDGF-R) (3, 4).

Adhesion-mediated signaling can be generally considered in two opposite directions. Outside-in signaling can cause activation of intracellular functions through ligand-binding of adhesion molecules, such as modulation of protein tyrosine phosphorylation and activation of mitogen-activated kinase (MAPK), changes in the cytosolic pH, alterations of Ca^{++} influx, among others; whereas inside-out signal transduction

includes regulation of cell surface interactions, such as binding kinetics of cell adhesion molecules. Recent results provide increasing evidence of overlapping signaling events and feedback mechanisms within adhesion complexes. For example, in F9 teratocarcinoma cells Ser-phosphorylation of β_1-integrins appears to be required for the movement of this subunit to focal adhesion contacts (5). Focal adhesions, however, are an important factor in downstream signaling during cell adhesion. In addition, inhibitor-mediated blocking of Ser/Thr-phosphatases in human colon carcinoma cells was able to suppress their adhesion to ECM (6). Moreover, protein kinase C (PKC) associated with activated β_1-integrin subunits resulting in a modulation of the availability of these adhesion molecules at the cell surface in breast cancer cells which was related to the kinase activity (7). In a recent study, we have also found that inhibition of PKC resulted in a complete loss of adhesive interactions of colon carcinoma cells to ECM, whereas its stimulation enhanced adhesive properties of these cells under static and dynamic conditions (8).

Protein-protein interactions in adhesion complexes involve cell adhesion molecules, such as integrins, selectins or cadherins, cytoskeletal proteins, such as paxillin, α-actinin, vinculin, tensin, talin or actin, and signal transduction molecules, such as FAK, pp60src, pp130cas, crk, PI3-kinase, protein tyrosine phosphatase PTP1B, PTEN/MMAC1/TEP1, PKC, among others. The exact mechanisms of these complex interactions are only partially understood, and often results were obtained using in-vitro experiments where biophysical influences on cellular functions were lacking.

Controversial results have been reported on the involvement of various kinases or phosphatases in the regulation of cell adhesion processes (9–12). For example, cell adhesion of human fibroblasts to ECM components mediated by β_1- (collagen, laminin – LN) or β_3-integrins (vitronectin – VN, fibronectin – FN) demonstrated different sensitivity to inhibition of protein tyrosine phosphatases (9). The increased Tyr-phosphorylations of FAK, paxillin and Src-kinases that resulted from this inhibition were able to reduce β_1-mediated formation of new adhesive bonds and could reverse already existing cell binding, whereas β_3-integrin-mediated cell adhesion was less effected. The cellular machinery that regulates integrin avidity and binding kinetics, therefore, appears to be subunit-dependent. Although these pathways seem to have universal controlling mechanisms, differences between various β-subunits have been found that may be related to different signaling mechanisms for different integrin subunits (13). These differences lead to another problem that is related to in-vivo mechanisms. Since extracellular and basement membrane compositions are complex, including various matrix components, adhesive interactions in-vivo are likely to be polyligand rather than monoligand interactions.

The interactions of adhesion molecules with the cytoskeleton appear to be a very important regulator of the complex signaling events. For example, the integrity of the actin filaments and their ability to form stress fibers is required for the establishment of cell adhesions to ECM components. Disruption of actin filaments can completely abolish adhesive interactions, including initial cell adhesion, adhesion stabilization, cell spreading and migration in various cell systems (11, 14). This also results in loss of adhesion-mediated Tyr-phosphorylations of FAK and other focal adhesion proteins (15), that appear to be mediated by tyrosine phosphatases (11). In native integrins that are not occupied by their ligands the ability to interact with the cytoskeleton seems to be masked or suppressed; but after successful binding, Tyr-phosphorylation of various focal adhesion proteins, such as FAK, paxillin, Shc, Grb2, or pp60src occurs. These phosphorylation events are accompanied by a complex reorganization of cytoskeletal proteins and signaling and adapter molecules within focal adhesions. The cytoskeletal reorganization appears to occur as a hierarchy of accumulation of different cytoskeletal proteins to focal adhesion plaques (16–19). For example, simple monomeric ligation of integrins has minimal effects on focal adhesion organization and the phosphorylation status within these complexes. After clustering of β_1-integrins, along with FAK and tensin that can directly interact with these integrin subunits, the complexes accumulate at focal adhesions, resulting in phosphorylation of Src-kinases with subsequent downstream-signaling via the MAPK-pathway (13). In contrast, the binding of various other

cytoskeletal proteins, such as vinculin, talin or α-actinin requires integrin clustering and ligand occupancy. This may lead to cellular control of stable adhesion formation (18). The cytoskeletal reorganization only occurs if integrins are firmly bound to their ligands. In addition, the binding of actin and paxillin represents the next level of cellular organization requiring Tyr-phosphorylation (3, 17, 19). However, the activation of FAK can be dissociated from the assembly of focal adhesions in adherent cells and integrin activation following ligand occupation, as was shown for platelet adhesion to fibrinogen mediated by $\alpha_{IIb}\beta_3$-integrins (10).

The active role of cytoskeletal components in the regulation of cell adhesion is also supported by the fact that pharmacological modifications of cytoskeletal rearrangements, such as actin filaments or microtubules, can influence adhesive properties. For example, disruption of microtubules results in reduced adhesion of colon carcinoma cells to ECM components (15, 20). These effects are associated with modulation of cell signaling, such as Tyr-phosphorylation of the focal adhesion proteins FAK and paxillin (15). Actin filaments also appear to be involved in cell adhesion and its stabilization at various levels of cell-ECM interactions, such as receptor clustering, signal transduction and direct counteraction to shear forces. We found that when actin filaments were disturbed in colon carcinoma cells, there is a complete loss of specific adhesive interactions with ECM which occurred under both static and dynamic conditions. Initial cell binding, adhesion stabilization and further events, such as cell spreading, were completely abolished if actin filaments were disrupted. This has also been shown for other types of tumor cells, such as melanoma cells (21), and normal circulating cells, such as platelets (22).

The complex involvement of the cytoskeleton in adhesion stabilization, cell signaling and resistance to external forces is further complicated by the fact that morphological effects induced by shear stress appear to involve mechanisms that also depend on protein tyrosine kinase activity, intracellular calcium, and an intact microtubule network (23). These morphological alterations of circulating cells induced by shear forces are discussed in a separate section below. In conclusion, without secondary events involving the cytoskeleton the initial interactions between integrins and their ECM receptors appear to be unable to counteract even weak forces that act on the cells, for example, during washing in static adhesion assays or low flow rates in hydrodynamic experiments.

The adhesiveness of extracellular integrin domains is allosterically controlled by binding events in the cytoplasmic domains resulting in subsequent conformational alterations of these molecules across the cell membrane. For example, the structural basis of collagen recognition by $\alpha_2\beta_1$-integrins has been recently discussed (24). This integrin is expressed on various cell types, including platelets or tumor cells where it serves as a receptor for binding to collagen or LN. It has been demonstrated that $\alpha_2\beta_1$-integrins mediate cell adhesion under flow conditions similar to that found in the blood circulation to collagen-containing surfaces, such as found in subendothelial basement membranes exposed after endothelial injury (8, 25). Emsley et al. (24) compared the crystal structure of unbound and ligand-bound $\alpha_2\beta_1$-integrin and found extensive changes in metal coordination that were linked to major changes in the secondary and tertiary protein structure. These changes created a complementary surface for collagen binding. In-vitro studies under static conditions have shown that collagen binding of platelets can occur in a cation-independent fashion, whereas cations appear to be required under hydrodynamic conditions (26–28). These data support the hypothesis that a low-affinity and a high-affinity state of integrins exist. The low-affinity state is determined by a closed form of a scissors-like structure hiding the binding motifs for collagen. Comparable results with conformational changes in the tertiary structure have been found for the leukocyte $\alpha_M\beta_2$-integrin (29). Activation of the $\alpha_2\beta_1$-integrin may be accompanied by binding of regulatory proteins to the cytoplasmic domain of the integrin, resulting in a release of an α-domain and conformational alterations that enable high affinity collagen binding (24). These two activation states of integrins may correspond to the initial events of cell adhesion and subsequent adhesion stabilization. During cell adhesion to ECM components these two activation states can be differentiated under shear stress in a laminar flow chamber (8). In addition, using chimeric

$\alpha_{IIb}/\alpha_L\beta_{3/2}$-integrins it has been demonstrated that the regulation of cell adhesiveness is, at least for this receptor, not simply a switch between low and high affinity states of the integrin conformation but is further dependent on post-receptor events after ligand binding, including cytoskeleton and cytoplasmic signaling events (30).

EFFECTS OF FLUID FLOW ON CELLULAR FUNCTIONS

Fluid flow under physiological conditions of blood circulation can cause a variety of changes within the circulating cells and the endothelium. The most obvious influences are morphological alterations of cell shape and deformation with increased contact areas due to elongation of the attached cell (31). These responses to shear forces include functional alterations, such as activation of signaling cascades and induction of transcription or cell activation. On the other hand, shear forces can also be lethal to the circulating cells. It has been shown that shear forces in the physiological ranges can induce lethal damage in a high percentage of circulating tumor cells. For example, up to 70% of B16 melanoma cells were killed within one hour if these cells were exposed to shear stress (32). The sensitivity to lethal cell damage after adhesion to EC, however, appears to depend on the type of EC used in these studies. In addition, tumor cell adhesion to EC can induce degradation of EC with formation of gaps and exposure of subendothelial basement membrane or ECM. In-vivo the ability of tumor cells to survive the mechanical stress is a very important factor in determining the efficiency of formation of distant metastasis. The likely mechanism of loss of cell viability under flow appears to be the mechanical damage of the cell membrane by shear forces (33). The sensitivity of tumor cells to mechanical influences may also depend on cell cycle-related morphology (34).

In addition to the mechanical action of fluid flow, shear stress alone can induce functional changes in EC, such as release of activating cytokines, including interleukins IL-1 and IL-6, or vasoactive substances, such as nitric oxide (35, 36). Furthermore, expression of intracellular signaling molecules, such as c-fos or c-myc,

can be induced in EC by fluid shear stress (37). It has also been shown that different amounts of shear stress can induce modifications in the surface expression of adhesion molecules in EC. For example, using different shear stress conditions the expression of E-selectin was decreased following endothelial shear stress stimulation (38, 39); other studies, however, did not demonstrate such changes (40, 41). As a direct consequence of mechanical forces shear stress also upregulated the expression of the intercellular adhesion molecule-1 (ICAM-1) (39–42). However, contradictory results have been reported on the effect of shear stress on the expression of vascular cell adhesion molecule-1 (VCAM-1) in EC (39, 41–43). These differences may be caused by the different types of EC used, but also by differences in the magnitude and time of shear stress exposure. Changes in the expression of adhesion molecules on EC surfaces have been detected mostly after long-term exposure to a high shear force comparable to that found in the arterial system.

In addition to the modulation of cell adhesion molecules, mechanical forces can influence various other cellular functions. For example, circulating tumor cells can produce EC cell injury, resulting in exposure of the underlying basement membrane. Recent observations have shown that the production of reactive oxygen species by melanoma cells can cause morphological alterations in different types of EC, and radical scavengers were able to suppress these effects (44). Furthermore, shear stress caused by different flow rates within the blood circulation is able to modulate activation of the coagulation cascade and platelet aggregation. For example, the generation of plasminogen activator by tumor necrosis factor-activated EC was stimulated under high flow conditions, whereas low flow inhibited its formation (45). Additionally, flow rates in the physiological range can decrease consumption of nitric oxide by circulating erythrocytes (46). Moreover, fluid flow is able to induce or modify EC gene expression encoding various cellular proteins, such as Smad6, Smad7 or PDGF (47, 48). In leukemia cells mechanical agitation induced a transient increase in mRNA encoding various transcription factors. This induction was attenuated by serum or albumin which can protect against shear stresses in suspended cells. In these experiments even moderate mechanical

agitation was able to induce gene expression (49).

Cells from different tissues may detect fluid mechanical stress by similar mechanisms but respond differently (50). The diversity of responses could reflect the cell type and mechanical demands of the resident tissue, but cancer cells may adopt mechanisms that are not usually found in normal tissues. Changes in cancer cell cytoskeletal machinery have been shown to result in changes in protein tyrosine kinases that can initiate phosphorylation and modulation of signaling cascades (51). For example, it has been demonstrated that integrin-mediated traction forces can be selectively modulated by pp60src (52). In Src-deficient fibroblasts cell spreading on VN was inhibited, while the strengthening of linkages between integrin receptors and the force-generating cytoskeleton was significantly increased. In contrast, Src-deficiency did not affect FN-mediated activities (52). Liu et al. (53) demonstrated that exposure of fetal lung cells to mechanical forces resulted in increased pp60src activity, which was accompanied by increased Tyr-phosphorylation of 110–130 kDa protein(s). Thus pp60src appears to be part of a mechanosensory protein complex that may link integrins and the cytoskeleton. This linkage may also involve rapid pp60src phosphorylation in response to mechanical forces (50). In EC exposed to mechanical stretch Tyr-phosphorylation occurs with an increase in phosphorylation of FAK, pp130cas and paxillin. These phosphorylation steps appear to be necessary for morphological responses of the EC to mechanical forces (54).

BIOPHYSICAL FACTORS INFLUENCING ADHESIVE INTERACTIONS

In contrast to static cell adhesion, biophysical factors influence adhesion processes under flow conditions. Under dynamic conditions flow velocity determines available time for adhesion events and acts against adhesion once cell contact occurs. The forward-rate of receptor-ligand interactions during cell adhesion under the influence of shear forces appears to be more important than receptor affinity in the regulation of adhesive interactions (55). Two hydrodynamic parameters have been found to determine the efficiency of integrin-mediated cell adhesion: the level of shear stress and the intercellular contact duration (56, 57). For example, below a critical flow rate corresponding to the level of shear stress found in microcapillaries, contact duration predominantly limited adhesion efficiency of neutrophils. Above this critical shear stress (about 2–3 dynes/cm^2) the efficiency seems to be determined by contact duration and tensile forces (56). The initial areas of cell contacts to vessel walls are probably restricted because of the more or less spherical shape of flowing tumor cells, or initial cell contacts may occur via cell protrusions or filopodia. Therefore, possible alterations in cell shape and morphology of participating cells are important determinants of the total area of cell-cell or cell-surface contacts (2). However, circulating cells contain biochemically and biophysically active surfaces that tend to repel contacts, resulting in a higher separation distance to the vessel wall than calculations from fluid mechanics would expect (58). Additionally, receptor density on circulating cells determines the statistical probability of successful collisions resulting in adhesive interactions (59). Furthermore, cell flattening during adhesion enables adherent cells to avoid high shear forces within the center of the parabolic curve of fluid flow. These factors as well as the flow character (laminar under physiological conditions found in the microcirculation) are responsible for the strength of shear forces acting against adhesion (2).

Blood acts as a liquid, but it is a solution composed of non-liquid cellular and noncellular components. Its composition results in a viscosity that can vary depending on hematocrit, plasma protein composition, amounts of blood cells or their deformabilities (60). The viscosity determines shear forces acting on circulating or adherent cellular components. In addition, the shear stress depends on flow velocity, resulting in differences between the macro- and microcirculation (61). Once cells contact the vessel wall, changes can occur in the microviscosity along the vessel wall and ultimately in the vessel itself. For example, rolling of cells along the vessel wall can greatly increase the vascular resistance, modifying the blood flow and biophysical conditions that can affect adhesive interactions.

In patients with cancer the hemodynamics of blood can be altered by changes in blood viscosity and coagulation properties. For example,

blood viscosity and plasma protein concentrations were measured in 31 patients with a variety of visceral carcinomas. The mean whole blood viscosity was not elevated compared with normal controls because of a significantly lowered mean hematocrit; however, when hematocrit was eliminated as a variable, the mean whole blood viscosity was significantly elevated in the group with carcinomas. The most important factor determining the viscosity in these patients was the significantly elevated plasma viscosity that is mainly dependent on the plasma protein concentration. In patients with increased numbers of blood cells, such as in leukemia patients, the blood viscosity is also influenced by the numbers of leukocytes and their deformability (62). Since blood is a non-Newtonian fluid, and its viscosity increases markedly at low shear rates, these rheological abnormalities would be most important to cell-cell wall interactions at the low shear rates characteristic of microcapillary and venous circulation (63).

The resistance of adherent tumor cells to deformation by external forces or their rigidity is determined by intrinsic and extrinsic deformability factors. They include elasticity of adhesion receptors, viscoelasticity of the cell membrane and structural characteristics of the cellular components, such as cell volume/surface ratio, membrane shape and cytoskeletal arrangements (64). For example, Fritz et al. (65) recently demonstrated that the conformational structure of selectin-ligand interactions during leukocyte rolling on EC is affected by the presence of shear forces. Under static conditions the receptor complex is folded, resulting in a small distance between the cell membranes. In the presence of fluid flow, however, this complex becomes increasingly stretched depending on the amount of shear forces with a subsequent increase in the distance between the membranes. If shear forces exceed the strength of the cellular adhesive bonds, rupture of the selectin-ligand complex occurs, and both molecules return to their partially folded conformation (65). Rupture force and lifetime of the complexes are not constant, but directly related to the applied shear forces per time as a product of the intrinsic molecular elasticity and external shear stress (65, 66). The frequency of formation (on-rate) and dissociation (off-rate) of receptor-ligand binding determine the number of adhesion molecules that can contribute to the strength of adhesive bonds. Interestingly, selectins possess an about ten-fold higher off-rate compared to integrins, resulting in a lower resistance of selectins to external forces (67). The variability of cellular resistance to shear forces can result in loss of adhesive bonds or cell rupture leading to cell death. Thus the ability of adherent tumor cells to escape high shear forces or to modify their cellular deformability to resist high shear forces appear to be important determinants of tumor cell survival in the circulation and metastatic efficiency.

Cell-generated forces are important in many other cellular activities, such as cell adhesion, spreading and migration. For example, transformed human fibroblasts required less complex protein layers for successful cell spreading than their untransformed counterparts (68). In addition, earlier in the metastatic cascade tumor cells have to detach from the primary lesion, which requires loss of cell adhesions. It has been shown that increasing shear stress by enhancing fluid flow can result in increasing the rate of detachment of fibroblasts or various types of tumor cells from cell aggregates or monolayers (69). This ability to detach from a cell mass may be related to the metastatic potential of transformed cells (70).

The organization of actin enables cells to exert contractile forces against the ECM, allowing cell spreading and migration. These forces also generate stretching within the ECM and can result in its reorganization. For example, stretching of fibronectin exposes binding sites that can lead to increased self-assembly (71). Alternatively, cells can sense the restraining forces of the ECM structure at the attachment site. Such cells can then respond to increasing tension between ECM components and integrins by instituting a localized, proportional strengthening of the cytoskeletal linkages, allowing stronger forces to be exerted on the adhesive complex (72). Besides receptor occupancy, mechanical adhesion stabilization may be necessary for this ability to generate forces against the ECM. For example, it has been shown that vinculin promotes cell spreading by supporting the mechanical anchorage of integrins to the cytoskeleton independent from actin polymerization (73). In addition, microtu-

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bules also appear to be involved in the generation of contractile forces (15, 74).

Pharmacological disruption of microtubules can result in increased phosphorylation of focal adhesion proteins, such as FAK and paxillin (15, 75). Furthermore, disruption of microtubules can result in an increased amount of contraction of fibroblasts on collagen matrix due to phosphorylation of the regulatory domain of myosin light chains (76). The strength of adhesive bonds and resistance to external mechanical forces appear to be regulated by phosphorylation/dephosphorylation events. For example, the resistance-induced reinforcement of ECM-integrin bonds can be inhibited by the tyrosine phosphatase inhibitor phenylarsine oxide (72). This drug also reduces cell adhesion but not cell spreading of colon carcinoma cells to ECM components under the influence of shear forces (Haier, unpublished results). In contrast, modification of kinase activity, such as activation of PKC or Tyr-kinases, did not interfere with the ability of colon carcinoma cells to respond to restraining forces (72, 77).

MODELS FOR ANALYSIS OF TUMOR CELL ADHESION

Adhesive phenomena may influence many aspects of cellular behavior which can be investigated using a number of different experimental approaches (35, 78–80). Since cell adhesion under static or hydrodynamic conditions, cell spreading, migration and invasion are all related to dynamic formation of new adhesive bonds, their underlying processes are, at least in part, regulated by different cellular structures. For example, in cell spreading the ability of cells to resist slow deformation or to form shear-resistant adhesive bonds are independent properties characterized by different time scales (81). It is thought that although spontaneous cell agglutination results from weak adhesive interactions, cell spreading includes formation of strong adhesive bonds and cytoskeletal deformation. Cell migration also requires the additional establishment of tension within the cells for active movement. To discriminate these processes a number of models have been introduced that consider different steps of adhesive interac-

tions; however, there are certain limitations of experimental designs that must be considered.

In most conventional adhesion assays nonadherent cells are removed by aspiration after a period of static conditions (without shear forces) followed by repeated washing steps (application of shear forces). Thus these adhesion assays apply high shear forces only during the washing procedure, resulting in removal of cells that established only weak adhesive interactions with the underlying substrate. Various methods have been developed to avoid these shear forces for the measurement of unstabilized adhesions. For example, minimal shear forces were applied to adherent cells if the cells were removed by gentle shear forces in fluid environment. This can be accomplished by using gravity instead of washing to remove the loosely adherent cells. Using low shear forces resulted in a three-fold increase of lymphoma cell adhesion to ECM components compared to the conventional assays using aspiration and washing (82).

The most commonly used solution to mimic hydrodynamic conditions of blood flow in-vitro is the application of a parallel plate laminar flow chamber. The large width-to-height ratio of the chamber provides laminar fluid flow in a range comparable to physiological conditions within the circulation (35, 85, 103). For observation of the interactions between circulating cells and vascular surfaces interesting modifications of the laminar flow chamber have been developed. For example, using a special mirror system a side-view chamber allows direct measurement of the cell substrate length and cellular deformation under the influence of flow (31). Furthermore, artificial arterioles or venules have been introduced using small diameter glass capillaries (83). Although these structures are more rigid than the relevant physiological substrates, they more closely mimic the events in vivo.

Intravital microscopy or the in-vivo and real-time observation of circulating cells is of increasing importance for investigations on cellular interactions within the microcirculation. A common model is the dorsal skinfold window chamber in animals (84). This chamber is frequently used for observations of leukocytes or platelets within tumor microcirculation. Circulating metastatic tumor cells and their adhesive interactions with the vessel wall in distant host

organs can be investigated using intravital microscopy of commonly involved organs, such as liver or lung. Although circulating cells can only be observed at the surface of these organs, this model provides a useful tool for cell adhesion under physiological conditions of blood circulation. However, this model requires intravenous or intraarterial injections of relatively large amounts of cells to obtain observable events in the small microscopy window. A compromise is the application of isolated, perfused organs that allow more standardized procedures, but this procedure is limited in terms of its simulation of physiological conditions (85). For example, Al-Mehdi et al. (86) recently described a new model for intravital microscopy using observation of subpleural pulmonary microvasculature in-vivo or in isolated perfused organs which allows differentiation and exact localization of cellular adhesions to the host organ vessel wall.

Orthogonal polarizing spectral imaging is a new method for investigating events within the microcirculation. This technique is based on linear polarized light that becomes reflected by cells in the microvessels. This reflected light can be analyzed through a polarizer oriented orthogonal to the plane of the illuminated light where only depolarized photons scattered in the tissue contribute to the image. The optical response of these images is linear and allows quantitative spectrophotometry. This method also produces high-contrast images comparable to intravital fluorescence microscopy. A major advantage of this method is the small size of the probe that can be placed on the surface of solid organs under physiological conditions at otherwise inaccessible sites.

By combining computer simulation with actual adhesion experiments investigators can explore ligand interactions of a heterogeneous population of circulating cells under defined shear forces (87). In such studies microvilli-hard sphere models simulate the binding of single cells to their ligands. It has been demonstrated that these interactions occur as a forces balance between hydrodynamic, binding, and colloidal forces. Additionally, differences in binding characteristics in a circulating cell population have been found if the system was based on a homogenous distribution and identical number of cell surface receptors or if a Gaussian distribution of receptors was considered (87).

CELL ADHESION UNDER FLOW CONDITIONS

Homotypic cell adhesion of malignant cells occurs in the circulation, and this type of cell adhesion is likely to be a very important determinant of distant metastasis formation that is based on mechanical lodgment of tumor cell emboli in the microcirculation. But before tumor cells are released from primary locations there must be loss of cell-cell and cell-matrix adhesions. This loss of adhesive bonds can occur under low fluid flow conditions within veins and the lymphatic system, and it can also occur under high shear forces in the arterial vessels. Under these conditions, the strength of homotypic cell adhesion may be a significant determinant in the ability of a tumor cell or cell clumps to be released into the circulation (69). Our subsequent analysis of data on dynamic tumor cell adhesion, however, will focus on the formation of new adhesive interactions with the microvascular system in distant host organs.

In-vivo experiments in various animal models and investigating different host organs, such as liver or lung microvasculature, confirmed previous experimental results on specific interactions between circulating tumor cells and host organ vessel walls. In contrast to previous observations of entrapment of circulating tumor within small lumen microvessels because of size restrictions, intravital microscopy demonstrated that tumor cells mostly adhere within vessels of larger diameters, such as precapillary arterioles or postcapillary venules, leaving remaining lumen for continuos blood stream (86). These observations underline the importance of shear forces acting on tumor cells during their adhesive interactions within the host organ microvasculature. These differences between intravital observations and histopathological analyses may be explained by technical limitations of the later technique. Thin layer sections of tumor cells within host organ vessels do not represent whole cells and therefore can only partially show the relationship between tumor cells and hosting vessels resulting in an underestimation of vascular margins and real vessel lumen. Furthermore, histopathological investigation of tumors requires their removal from the blood circulation with loss of intravascular pressure that can cause collapse of small vessels and sub-

sequent artificial contact of tumor cells and vessel walls. In contrast, in-vivo models can analyze whole cells and blood vessels allowing three-dimensional pictures of adhesive interactions.

The role of cell surface molecules that mediate tumor cell adhesion under various conditions, including hydrodynamic conditions, is still in the process of being determined. An increasing number of reports have demonstrated the importance of shear forces in the regulation of these adhesive interactions (8, 32, 69, 88). For example, selectins are likely to play an important role in initial tumor cell adhesion to host organ endothelium, where they can mediate initial contacts and, at least in part, by cell rolling on EC cell surfaces. The kinetics of selectin-mediated interactions between circulating tumor cells or leukocytes and the vascular EC, however, suggest a lack of development of firm adhesions by these molecules (89).

Adhesion to ECM components of the basement membrane and adhesion stabilization appear to be mainly determined by integrin-mediated interactions. These results, however, can vary depending on the cell type, and other less well studied adhesion molecules are also likely to be important. In addition, several studies have revealed that the adhesion molecules involved in adhesive interactions between tumor cells and the vessel wall can vary if adhesion occurs under static or hydrodynamic conditions. For example, E-selectin has been shown to use different epitopes to bind to its target sialylated ligands, depending on the conditions of adhesion (90). HL60, Colo201 and CHO cell adhesion has been studied in detail and was found to be based on interactions of E-selectin with Sialyl-Lewis^x (sLe^x) under static or low shear stress conditions, whereas under high shear stress these interactions were mediated by sLe^a ligands. Using high fluid flow in a laminar flow chamber the capability of antibodies against sLe^x to block cell adhesion was reduced in these cells, and the susceptibility to sialidases was different compared to static conditions (90, 91). Similar results were obtained if mucin-like ligands were removed enzymatically from the cell surface (92). Using nonmetastatic and highly metastatic breast cancer cells it was demonstrated that an ICAM-1 mechanism may be involved in adhesion of poorly metastatic cells

to human umbilical vein EC (HUVEC), whereas adhesion of highly metastatic cells appeared to be mediated mainly by VCAM-1 and E-selectin (38). After a long-term exposure of HUVEC monolayers to arterial shear stress, the adhesive properties of both breast cancer cell lines were markedly changed compared to static conditions or after administration of short-term shear forces. In this case highly metastatic cells demonstrated decreased rates of adhesion, whereas nonmetastatic cells showed an increase in their rates of cell adhesion to HUVEC (38). A similar increase in tumor cell adhesion to HUVEC under flow conditions can be induced if the EC are activated by pretreatment with specific cytokines, such as IL-1β (91). In contrast, HUVEC inactivation by inhibition of prostaglandin I$_2$ reduced dynamic adhesion of colon carcinoma cells (91).

Direct attachment of tumor cells to uninterrupted endothelial cell monolayers mediated by non-integrin receptors has been observed, along with a direct role of integrin-mediated tumor cell adhesion to endothelial cells under flow conditions. In an example of nonintegrin adhesion receptors on colon carcinoma cells involved in endothelial cell adhesion under flow conditions Kitayama et al. (93, 94) reported controversial results with respect to the involved cell adhesion molecules. In these studies Colo201 completely arrested onto immobilized E-selectin in the presence of shear forces, and blocking of sLe^a or sLe^x decreased tethering of these human colon carcinoma cells to HUVEC under flow conditions. These results suggested that selectins alone may be sufficient to establish stabilized cells adhesions without the contribution of integrins (93). These results were obtained at very low flow rates under 1.5 dyn/cm^2, whereas at higher shear forces above 2 dyn/cm^2 only a few Colo201 cells were able to adhere to HUVEC monolayers. However, if HUVEC were grown to subconfluent monolayers on LN-coated surfaces, Colo201 cells exclusively adhered under flow conditions to the basement membrane that was exposed in the gaps between HUVEC. The binding of the Colo201 cells to the subendothelial basement membrane between HUVEC was mediated by β_1-integrins (94). The majority of these cells arrested to the exposed ECM components without rolling, results comparable to the data

above using HUVEC at low shear rates. In this study higher shear rates resulted in short term contacts of the Colo201 cells with the exposed basement membrane and subsequent detachment from the ECM components (94).

Some tumor cells do not appear to bind directly to endothelial cell surfaces but rather to underlying exposed ECM. This is supported by the observation that melanoma cells adhered to denuded arterioles, and this adhesion process was mainly mediated by FN-receptors on circulating malignant cells binding to subendothelial matrix FN. Intact arterioles did not support melanoma cell adhesion (95). MCF-7 breast cancer cells adhered more rapidly to ECM than to the apical surface of a confluent monolayer of EC (96). High adhesion rates of rat 13762NF mammary adenocarcinoma cells with different metastatic properties to target organ-derived subendothelial matrix were observed and correlated with their metastatic potential, whereas adhesion to the target microvessel EC occurred at lower rates and was independent from the metastatic behavior of the cells (97).

Two different patterns of tumor cell adhesion to the vessel wall under flow conditions have been described. In many carcinoma cell systems, such as breast cancer or colon carcinoma, cell rolling on EC surfaces has been found comparable with the behavior of leukocyte rolling on activated EC. In contrast, sarcoma and melanoma cells frequently adhered to EC without rolling. For example, B16-F10 human melanoma cells demonstrate cell arrest on EC monolayers without 'rolling'. However, in contrast to their permanent arrest on EC monolayers, many B16-F10 cells arrested only transiently on lung-derived endothelial cell adhesion molecule (Lu-ECAM-1)-coated surfaces, suggesting a lack in adhesion stabilization if these cells interacted only with this adhesion molecule (98). Various colon carcinoma cell lines, including HT-29 cells, demonstrated 'rolling' with short-term or transient stops on EC, and these interactions were inhibitible by monoclonal antibodies (mAb) against E-selectin (99).

We and others have found that specific and nonspecific tumor cell-ECM interactions may occur in the presence of flow comparable to the conditions found in the microcirculation. These interactions were distinctly different in cell lines with differing metastatic properties. Using human colon carcinoma cells we demonstrated higher rates of dynamic adhesion of poorly metastatic HT-29P cells to collagen compared to highly metastatic HT-29LMM cells. In contrast, we found high rates of static adhesion of HT-29LMM cells to FN, but poorly metastatic HT-29P cells did not show these properties. The advantage of HT-29P over HT-29LMM cells in collagen-mediated adhesion involved all phases of initial cell adhesion and adhesion stabilization, whereas the increased dynamic adhesion of HT-29P cells to FN was mainly limited to early events or initial adhesive contacts (14, 100, 101). Similar results were previously reported using other cell systems, such as melanoma and large cell lymphoma cells with different metastatic behaviors (102–104). In contrast, adhesion to the nonspecific substrate poly-L-lysine occurred in a completely different pattern.

Tözeren et al. (105) described initial adhesive interactions of HT-29 cells to ECM components under flow conditions, and that these cells finally adhered to immobilized LN, but not to collagen I (C I), collagen IV (C IV), or FN. These dynamic interactions with LN were mediated by the $\alpha_6\beta_4$ integrin and were inhibited by mAb against these integrin subunits. In contrast, we found cell rolling, sticking, stabilized adhesion and crawling of HT-29P cells to immobilized C I under flow conditions, and these specific interactions were inhibited by β_1-integrin mAb (8). Similarly, A549 lung carcinoma cells have been shown to adhere to FN in a shear-rate dependent manner mediated by integrins (88).

Although the reasons for different adhesive potentials of tumor cells with different metastatic behavior under shear forces remain unknown, numerous reports have described differences in the expression of cell surface receptors between cells of different metastatic potentials (97, 106–109). In addition to differences in adhesion, disparate effects on adhesion-mediated signaling events were also found (110).

Although the adhesive behavior of leukocytes or platelets is difficult to compare with tumor cell adhesion under flow conditions, interesting results on regulation and involved structures have been reported for adhesion of blood cells. Similar experiments using tumor cells have not yet been reported, but similarities may occur between the properties of tumor cells and blood

cells. For example, treatment of neutrophils with cytochalasins (to disrupt actin filaments) can convert their selectin-mediated rolling to stationary cell adhesion, and this was accompanied by an increase in cellular deformability (111). In circulating leukocytes inhibition of shear stress-sensitive K^+-channels and reduction of extracellular Ca^{++}-levels induced retraction of pseudopodia of adhering cells resulting in increased rolling on EC and detachment from the endothelium (112).

Under flow conditions adhesive interactions have to counteract wall shear stress or WSS. The cytoskeleton appears to be involved in this process in two ways. First, the cytoskeleton is the most important cellular structure that can distribute mechanical forces throughout the cell for maintenance of cellular structure and integrity (22). Second, cytoskeletal integrity and adhesion stabilization appear to be related to signal transduction within the cell that leads to structural changes, such as formation of focal adhesions and increased binding affinity of integrins to ECM components.

Most of the data on the importance of cytoskeletal components for maintenance of cell structure and morphology under flow conditions were obtained using EC; however, it is likely that the cytoskeleton is also involved in cell structure and morphology of circulating cells as well. For example, it was shown that disruption of different cytoskeletal components affects the ability of circulating cells to maintain cell shape, structure and deformability, probably through changes in cytoskeletal stiffness and intracellular viscosity (113). Intracellular traction forces appear to become focused in the nuclear regions of the cell, suggesting the involvement of various cytoskeletal components in the mediation of these internal cellular mechanical stresses (114). Disruption of actin filaments, microtubules or intermediate filaments resulted in changes of all these parameters, and combinations of drugs that disrupt actin, microtubules and intermediate filaments had synergistic effects on cytoskeletal stiffness and viscosity but did not affect permanent cell deformation (113). Also, in attached cells, such as fibroblasts the internally generated forces against ECM appear to depend on the stiffness of the matrix components. For example, it has been shown that fibroblasts grown on stabilized collagen gels generate traction forces, whereas cells attached to floating gels did not show changes in the formation of stress fibers (115). This variable regulation of adhesive interactions in relationship to the rigidity of adhesion substrates can also be regulated by signal transduction events, such as protein Tyr-phosphorylation and formation of focal adhesions (116) that will be discussed below.

Using a parallel plate laminar flow chamber we compared the effects of cytoskeletal disruption on dynamic cell adhesion of human HT-29 colon carcinoma cells to collagen under static conditions (15). Disruption of actin filaments completely inhibited all types of adhesive interactions. In contrast, impairment of tubulin polymerization or disruption of intermediate filaments resulted in different effects on static and dynamic cell adhesion. The latter treatment did not interfere with dynamic cell adhesion, but under static conditions it partially reduced adhesion rates. In contrast, under dynamic adhesion conditions increased initial adhesive interactions between HT-29 cells and collagen were found after disruption of microtubules, and the adherent cells demonstrated extensive crawling on collagen surfaces. Under static adhesion conditions, however, disrupting microtubules did not affect cell adhesion rates (15).

As described above, hydrodynamic shear forces can modify intracellular signaling, such as Tyr-phosphorylation of focal adhesion proteins. Therefore, it is reasonable to assume that kinases and/or phosphatases are involved in the regulation of integrin binding in the presence of wall shear stress. For example, involvement of kinases has been suggested for $\alpha_2\beta_1$-integrin-mediated platelet adhesion to collagens under flow conditions (117). We could also demonstrate that pp60src is likely to participate in the early events of adhesion stabilization. Transfection of HT-29 cells with pp60src antisense oligonucleotides did not modify adhesive properties to collagen I or IV under static adhesion conditions; whereas using hydrodynamic conditions in a laminar flow chamber we found a slight reduction in early adhesion events and an even greater difference in adhesion stabilization rate. The transfected cells showed a significant reduction in their ability to withstand shear forces and stabilize adhesive bounds. These changes correlated with the cellular levels of pp60src

(Haier, unpublished results). As possible mechanisms of pp60src involvement, the adhesion-mediated Tyr-phosphorylation of other focal adhesion proteins FAK and paxillin were investigated. HT-29 cells were exposed to fluid flow, resulting in an increase in Tyr-phosphorylation of of FAK and paxillin. The hyperphosphorylation of paxillin was accompanied by a shift in its electrophoretic mobility in transfected but not in parental cells. Increased phosphorylation of FAK and paxillin were induced by static adhesion, however, this increase was similar in untreated cells and cells with reduced pp60src expression (unpublished results). Upon activation pp60src can be localized on actin-filaments present in focal adhesion plaques, at adherens junctions between cells and also in microfilament bundles (118, 119). Activation of pp60src can result in conformational alterations of the cytoskeleton and induce its association with cytoskeletal proteins (120). The interactions of pp60src with cytoskeletal proteins also correlated with integrin-mediated platelet aggregation (121). Thus it has been suggested that translocation of pp60src to the cytoskeleton and its association with the cytoskeleton may regulate tyrosine phosphorylation of cytoskeletal proteins (122, 123), which can then modulate cytoskeletal architecture during cell-ECM interactions (121, 124). Furthermore, pp60src appears to be involved in mechanotransduction of hydrodynamic shear forces to the cytoskeleton during cell adhesion to ECM components. In addition, it is likely that other kinases and possibly phosphatases also participate in the detection of and cellular responses to mechanical forces.

Mechanical shear forces are not limited to fluid flow in the blood or lymphatic circulation. They can also occur if metastasizing tumor cells attach to and invade serous epithelial layers, such as the peritoneum. For example, using agitation to stimulate peritoneal fluid dynamics and shear forces in vivo, the attachment of human ovarian tumor cells was found to occur to the ECM but not to epithelial cells. Although adhesion also occurred without shear forces, it did so at a lower rate than it did with agitation. The cells exhibited a more rapid and firmer attachment to ECM than to the mesothelial cells or to plastic. As assessed by phase-contrast microscopy tumor cell attachment was re-stricted to areas of exposed ECM in wounded mesothelium. Morphologic alterations of the mesothelium induced by tumor cells were observed with the use of scanning electron microscopy and immunohistochemical staining. The observations documented disruption of intercellular junctions leading to retraction of mesothelial cells, exposure of underlying ECM, and subsequent attachment and proliferation of tumor cells on ECM (80). A similar sequence of events was seen previously with tumor attachment to and penetration of endothelial cell monolayers (125).

Tumor cell adhesion to distant organs occurs in the presence of various circulating cells and soluble plasma proteins. It is known that these environmental factors can influence cellular behavior, including adhesive interactions. For example, platelets have been found to interact with tumor cells, EC and ECM components (126–128). Recalling the influence of shear forces on platelet function, platelet effects on tumor cell adhesion should be considered under more physiological conditions of fluid flow. For example, when melanoma cells resuspended in human blood were perfused over thrombogenic surfaces, such as collagen, in a parallel plate flow chamber, cell adhesion was modified by the presence of platelets. Comparable to other cell systems melanoma cells demonstrated differences between static and dynamic cell adhesion to the ECM. Moreover, melanoma cells were found to associate with thrombi resulting in stabilized tumor cell arrest (128). Inhibition of platelet activation decreased melanoma cell adhesion (129). Furthermore, it was found that the interactions between platelets and melanoma cells were active events specifically mediated by $\alpha_v\beta_3$- or $\alpha_{IIb}\beta_3$-integrins, and these interactions did not occur as a result of passive and nonspecific melanoma cell entrapment by preexisting thrombi (129–131). This model demonstrated that direct adhesive interactions between circulating malignant cells and ECM or EC are important factors in the establishment of stable tumor cell adhesions and the resistance to shear forces under flow conditions (130, 132). Although tumor cell adhesion can occur under static conditions without binding of platelets, under the influence of fluid flow these adhesive bonds were only established if platelets interacted with the melanoma cells (132). This is

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supported by in-vivo results indicating that a lack of platelets or their impaired function can reduce metastasis formation in a variety of cell systems (133). Under static conditions, the presence of platelets did not affect the adhesive properties of A549 lung carcinoma cells to EC or ECM components. Flow conditions, however, were able to enhance adhesive interactions of these cells resulting in higher rates of adhesion to ECM. However, this was dependent on the intensity of shear stress applied (134).

CONCLUSIONS

Tumor cell adhesion during formation of distant metastasis is a complex process that is influenced by a variety of factors, including the unique properties of the tumor cells themselves, the characteristics of the host organ vascular system, the presence of other circulating cells, such as lymphocytes and platelets, and biophysical factors. As described above, most of the research on metastatic tumor cell adhesion has

been done without consideration of these latter factors, especially biophysical factors. In living organisms, however, biophysical factors, including external forces of any kind, pressures within tissues or the blood circulation or thermal factors can modify biological processes and cellular responses. Therefore, our understanding of basic biochemical and biophysical processes during tumor cell adhesion has to be translated into systems that reflect in-vivo conditions more accurately. They must certainly consider the influences of biophysical factors during these processes.

The localization and extravasation of circulating tumor cells at distant host organ sites is a complex process that involves several types of adhesion molecules with apparently distinct functions. It is a dynamic and highly regulated biophysical process that includes interactions between tumor cells, microvessels and circulating host cells (135). Reviewing the current data on tumor cell adhesion there seems to be no doubt that different types of adhesion molecules are involved in the formation of adhesive
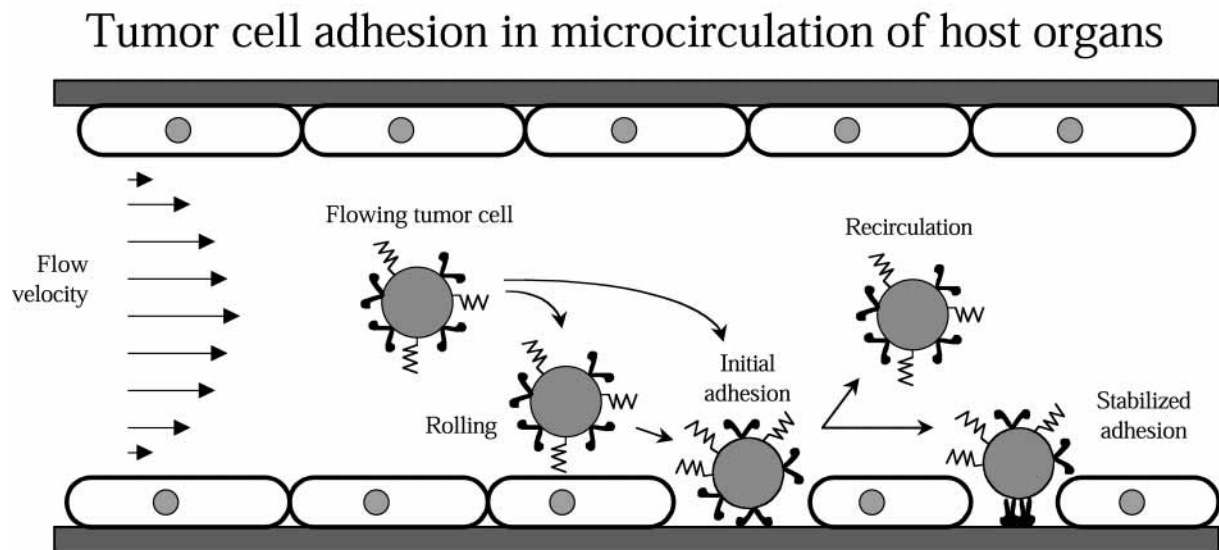

Fig. 1. Model for tumor cell adhesion to microvascular vessel walls within the circulation. Laminar fluid flow causes shear forces that act against adhesive bindings. Flowing tumor cells can interact with microvascular endothelial cells mediated mainly by selectins that have a high elasticity, but the strength of these bonds is relatively low and not sufficient for stable cell adhesions resulting in rolling of the tumor cells with a reduced speed along the endothelium. This results in enhanced probability for integrin-mediated adhesion. However flowing tumor cells can also achieve direct integrin-binding. Initial integrin-mediated adhesion is characterized by a low affinity state. If high affinity of integrin-binding can be established rapidly stable tumor cell adhesion occurs with subsequent signaling for cell spreading, migration and extravasation. However, failure of the conformational affinity switch in integrins results in breaking of these bonds and recirculation of tumor cells or cell damage by shear forces.

bonds between circulating tumor cells and the microvascular vessel wall in host organs. Although similarities can be found between the processes whereby normal blood cells (leukocytes, platelets) interact with vessel walls and extravasate, differences do appear to exist in the types of adhesion molecules involved and in cell signaling pathways regulating the affinity, avidity and interactions of these molecules in tumor cells.

Although the amount of data are limited and the experimental designs differ considerably, the evidence discussed above may be summarized in a hypothetical model of tumor cell adhesion in the presence of shear forces caused by fluid flow that corresponds to in-vivo conditions during the formation of metastases (Fig. 1). Similar to the adhesion of leukocytes to microvascular walls (136) tumor cell adhesion can be divided into a number of steps that are required and are influenced by fluid shear forces: (a) initial tumor cell contact with the vessel wall; (b) initiation of adhesive bonds; (c) adhesion stabilization; and (d) cell flattening and spreading. These steps occur sequentially, and clear distinctions between the different processes, involved molecules and cellular functions cannot always be made. Subsequently, tumor cells must bind to subendothelial ECM and penetrate this structure, and these events appear to involve different sets of molecules than those used for initial cell adhesion and stabilization to the endothelium.

To our knowledge cell margination, as it has been described for leukocytes, has not been observed with tumor cells. One reason could be the larger diameter of tumor cells that provides relatively close contact with the microvascular EC. Most tumor cells possess larger diameters than the average microvessel diameter; thus tumor cells must be deformed even more than lymphocytes during the process of capillary passage. This may provide tumor cells with ample contact with the endothelium to establish adhesive bonds. Since tumor cells can recirculate to sites other than the first capillary encountered, shear forces in the microcirculation must be enough to cause detachment and recirculation of many if not most tumor cells.

Biophysical as well as biochemical events in the microcirculation are important in tumor cell arrest, adhesion and adhesion stabilization. Although tumor cell adhesion to EC might be en-hanced by cytokine-mediated activation of the EC, considerable adhesive interactions can also occur to nonactivated or 'resting' EC. Changes in electrical surface charges and/or surface glycosylation of tumor cells and EC may, however, modify the molecular distance between circulating cell and vessel wall molecules, resulting in statistically different probabilities of successful interactions between surface molecules. The distance between adhesion molecules on the circulating tumor cells and their ligands on the vessel wall may influence the next step during the establishment of stable cell adhesions. If the distance is large, only adhesion molecules with relatively long extracellular domains can reach their ligands and bind successfully. This may be important for selectins, but not for integrins. Differing results have been found for the requirement of selectin binding to EC during tumor cell adhesion within the microvasculature. The aforementioned problem may provide an explanation for this phenomenon. The role of selectins could be twofold. Binding to these adhesion molecules can reduce the distance between adjacent cell surfaces allowing contact of different adhesion molecules with shorter extracellular domains, such as integrins, to interact with their ligands. Since selectins have extracellular domains that have intramolecular elasticity, these molecules can be stretched. Although the strengths of selectin bonds are much less than those involving integrin-ligand interactions, selectin-mediated binding allows some resistance to shear forces. This can result in a reduction in the speed of cell movement and increased time available for interactions between other adhesion molecules and their ligands, especially those required for further steps of adhesion. This phenomenon has been observed in leukocytes where it is known as 'cell rolling' and seems to be required for stable cell adhesion.

As discussed above, in tumor cells surface structures electrical charges and cell size differ from leukocytes. This may allow these cells to come into closer contact with surface structures of microvessel walls, and it could enable cell adhesion without its mediation via selectins. The small number of studies published on this topic suggests that, in general, some types of carcinoma cells may depend on selectin-binding to EC. However, under certain circumstances integrin-mediated adhesive interactions can occur

without selectin-binding, comparable to the situation observed in malignant cells from ectodermal or mesodermal origins. Independent of the involvement of selectins in initial cell contacts, integrin-mediated cell adhesion must occur to enable stable cell adhesion that can resist shear forces. These integrin-mediated interactions occur in two distinct steps. First, integrins bind to their ligands at low affinity. This low affinity state seems to be the primary condition of unbound integrins, and this low affinity binding has to switch to a high affinity state in order to stabilize cell adhesion. The most important integrin family that mediates these events appears to be the β_1-group, although other integrins, such as β_3-, β_4-, β_5- or β_6-integrins may also contribute to these interactions. The switch from low affinity to high affinity seems to involve active cellular responses and signal transduction. Although the detailed mechanisms are not known yet, Tyr-phosphorylation of focal adhesion proteins, such as FAK, paxillin or Src, seems to be involved in this regulation. Furthermore, transglutaminase-catalyzed cross-linking between integrins and ECM components between different ECM ligands may be required for formation of stable tumor cell adhesions (95, 104). Certainly an important step in the process of stabilizing adhesion is the recruitment and concentration of adhesion components or clustering of receptors and their ligands into dense adhesion structures. This step may set in motion the attachment of cytoskeletal components and other molecular structures that help to stabilize the adhesion complex or focal adhesion plaque.

It will be challenging to identify tension-responsive molecules/mechanisms and signaling pathways that mediate the affinity switch of receptors and the formation of adhesion complexes. If the switch from low to high integrin binding affinity does not occur, adhesion stabilization may not be established, and the tumor cell eventually detaches and recirculates. Currently, it remains hypothetical whether other adhesion molecules, such as VCAM, CD44 or ELAM-1, are also required for adhesion stabilization or subsequent events, such as cell spreading and flattening. At a minimum, these molecules can provide additional stability to the cell adhesion complex, resulting in an increase of resistance to shear forces. From in-vitro re-

sults, the time required for adhesion stabilization is in the range of a few seconds. During this time cytoskeletal reorganization takes place including the formation of focal adhesion plaques. However, it is still unknown whether these focal adhesion plaques are required for adhesion stabilization or vice versa, but some evidence supports the first hypothesis. Downregulation of the expression or functional inhibition of key focal adhesion proteins, such as FAK or Src, can significantly reduce adhesion stabilization. In addition, close contacts between tumor cell surfaces and ECM components can enable binding of other cell surface receptors, such as growth factor receptors, to their ligands bound to the ECM, resulting in further modification of downstream signaling and cellular behavior. Furthermore, formation of focal adhesion plaques appears to occur as a stepwise recruitment of signal transduction and cytoskeletal proteins, suggesting the involvement of early acquired molecules in the regulatory tasks of adhesion. Molecules recruited later appear to be involved in adhesion-mediated downstream signaling and subsequent cellular responses (3, 13). Once adhesion stabilization occurs, tumor cells undergo remarkable changes in cytoskeletal organization resulting in cell flattening, spreading and increased cell surface that is in contact with the vessel wall.

The model discussed above is based on a review of current results on tumor cell adhesion under the influence of shear forces mediated by fluid flow. However, since many of the details have not yet been investigated under hydrodynamic conditions or were obtained using non-malignant cells, there are some limitations of this model. Additional studies are necessary to elucidate the role of biophysical factors in the adhesion of circulating tumor cells and to identify tension-sensitive molecules/mechanisms and signaling pathways involved in the regulation of adhesion affinity and its stabilization.

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