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MORPHOLOGICAL STUDY OF THE INTERACTION BETWEEN M21 MELANOMA AND LYMPHATIC ENDOTHELIUM

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ABSTRACT

In order to study the interaction between melanoma and lymphatic endothelial cells (LECs) and to investigate the mechanism of lymphatic metastasis, M21 melanoma cells were seeded on the confluent LECs monolayer and the alterations of both cells were observed. The results showed that tumor cells could both adhere by pseudopodia to LECs at the site near the intercellular junction and on the apical surface. The adhesion of the melanoma cells induced the endothelial junction dissolution and endothelial retraction, which allowed the passage of the tumor cells through the opened gap and attached to the subendothelial matrix, then the tumor cells invaded and migrated under the LECs monolayer. These findings suggest that tumor cells could metastasize through the lymphatic vessel by destroying intercellular junctions or the LECs directly.

The metastasis of neoplastic cells at sites distant from or nearby their primary tumor is responsible for the majority of deaths caused by cancer. The lymphatic system is the major pathway for cancer cell dissemination and the involvement of lymph nodes is the most important indicator for the poor prognosis. Recently, greater emphasis has been focused on the relationship between lymphangiogenesis and lymphogenous metastasis (1-4). However, information about the interaction between the tumor cells and LECs is scarce, and the sequence and mechanism of the event remain obscure. Using an in vitro model, we have for the first time observed the adhesion, invasion and migration of M21 melanoma cells during interactions with the lymphatic endothelial monolayer.

MATERIALS AND METHODS

Isolation and Culture of the LECs

A bovine (newborn) thoracic duct was obtained immediately after slaughter and kept in Hanks (4°C). LECs were isolated using the method described by Johnston and Walker (5) with modification: both ends of the thoracic duct were cannulated with catheters. The vessel was flushed with PBS to clear the lumen, and then filled with a solution of collagenase (0.02% in PBS) and incubated for 15 minutes (37°C). The digested LECs were flushed with M199 medium into a 15ml centrifuge tube and centrifuged at 200g for 10 minutes. After removing the supernatant, the faint cell pellet was suspended in M199 medium with 15% fetal bovine serum, 50ng/ml bFGF, 10ng/ml heparin, 100μg/ml penicillin, 100μg/ml streptomycin, and seeded in a 1% gelatin coated multi-well plate. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2.

LECs were subcultured at confluence after treatment with 0.25% trypsin-0.02% EDTA (1:1) and maintained in the medium

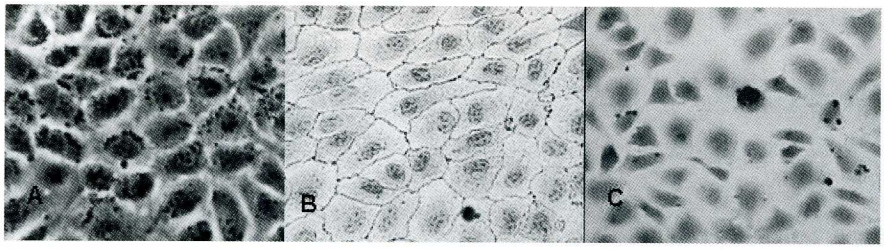

Fig. 1. Light micrographs of LECs. A: phase contrast light micrograph. B: $AgNO_3$ staining. C: staining for factor VIII-related antigen. x200.

above. Cells used for the adhesion study were passage 3 to 5 and grown on gelatin coated glass coverslips (placed in the wells).

Characterization of the LECs

The LECs were stained with silver nitrate using the method of Poole (6) with modification: Confluent LECs grown on the cover slip were treated with 5% glucose for 1 to 2 minutes, 0.4% $AgNO_3$ for 1 minute, rinsed with 5% glucose for 1 minute, then treated with 1% NH_4Br and cobalt bromide for 3 minutes and rinsed with 5% glucose, fixed in 4% formalin for 20 minute, rinsed in PBS and stained in Diff-Quik. The slips were rinsed, dehydrated in alcohol and infiltrated with xylene before mounting with gum.

To detect Factor VIII-related antigen, an immunohistochemical method was used: The LECs grown on the cover slips were washed in PBS and fixed in 95% alcohol for 20 minutes, treated with 3% H_2O_2 for 30 minutes and normal goat serum for 30 minutes. Then they were incubated with rabbit antiserum to human Factor VIII-related antigen (first antibody) over night (4°C) and a goat anti- rabbit IgG (second antibody) conjugated with biotin for 1 hour at room temperature followed by SABC for 30 minutes. Finally, the cells were colored with DAB and re-stained with hematoxylin.

Adhesion and Scanning Electron Microscopy

0.5ml M21 melanoma cells (about 1-2 x 10^4) were seeded on the confluent LECs monolayer grown on the cover slips and incubated for different intervals. The slips were rinsed in PBS, fixed in 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide for 1 hour and dehydrated in an alcohol series to 100%, then infiltrated with anyl acetate and dried at critical point using CO_2. The cells were coated with Gold-Palladium and examined by HITACH S-570 scanning electron microscopy.

RESULTS

Morphology of the LECs

Under the phase contrast microscope, the LECs were characterized by a cobblestone appearance (*Fig. 1A*). The reactions of silver nitrate were deposited at the intercellular space of adjacent cells and outlined the intercellular borders (*Fig. 1B*). Almost all of the LECs exhibited an intense staining reaction to Factor VIII-related antigen in the perinuclear region (*Fig. 1C*).

Adhesion of Tumor Cells

The adhesion or attachment of M21 to LECs occurred in a few minutes. About 40% of the melanoma cells adhered to or transmigrated through the LECs monolayer in the first hour. Under the SEM, we counted at random 100 melanoma cells on one slip and found that most (93 cells) of the seeded tumor cells attached to the LECs monolayer

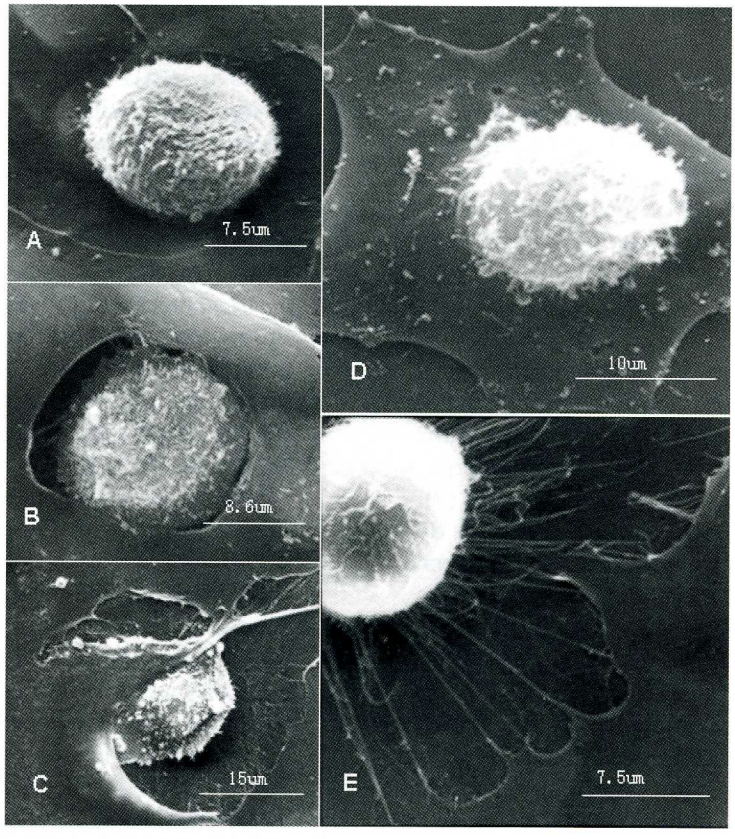

Fig. 2. Scanning electron microscopy (SEM) views illustrating the attachment, invasion and migration of M21 melanoma cells after seeding on the LEC monolayer. A: A melanoma cell attaches at the endothelial junction xH4000. B: A melanoma cell penetrates the LEC monolayer through the destroyed rupture x3500. C: A melanoma cell migrates under the LEC x2000. D: A melanoma cell attaches onto the apical surface of LECs, many membrane bulbs protruding from the basolateral surface of the melanoma can be seen x3000. E: A melanoma cell attaches to the adjacent LECs by numerous long and fine microvilli x4000. (A,D,E 15 minutes; B 30 minutes, and C 1 hour after tumor cell seeding).

at or near the junction region between the adjacent endothelial cells (*Fig. 2A*). Only a few of them (7 cells) adhered directly onto the dorsal surface of LECs (*Fig. 2D*).

Tumor cells just attached to LECs remained spherical in shape (*Fig. 2A*). Many microvilli could be found on the surface especially at the contacted area; some of them were very long and fine (*Fig. 2E*). These microvilli adhered to the endothelial surface or to the substrate exposed between the adjacent LECs. At this initial stage, the LECs still retained their intact features; the intercellular junctions were not destroyed.

Retraction and Disruption of LECs

The attachment of tumor cells to LECs induced endothelial intercellular junction dissolution and endothelial retraction at the site of adhesion, consequently enlarging the intercellular gap and exposing the underlying extracellular matrix (substrate). At the site that tumor cells adhered to the dorsal surface of LEC, the endothelial cell membrane was dissolved with a hole-like capture, which allowed the tumor cells to come into complete contact with the substrate (*Fig. 2B*).

Invasion and Migration of Tumor Cells

After endothelial retraction or hole-like disruption, the tumor cells penetrated through the monolayer to the underlying substrate and spread on it. They extended plate-like cellular process or pseudopodia into and migrated between the endothelial cells and substrate (*Fig. 2C*).

DISCUSSION

To disseminate and successfully establish a secondary tumor at a distant site from the primary one, the tumor cells must undergo a complex sequence of interrelated events (7). The basic steps necessary for tumor cells to extravasate or intravasate include attachment or adhesion to the endothelium and disruption or penetration through or between endothelial cells. Although much is known about this process in hematogenous metastasis, little is known about the steps in lymphogenous metastasis. The prevailing view has been that tumor cells passively enter lymphatics between intercellular junctions owing to the specificity in their structure. But in our results, the melanoma cells can adhere and cause direct damage to the LECs (cytoplasmic membrane and cellular junction dissolution and retraction which enable the larger melanoma cell to penetrate through the vessel) (8). The findings demonstrate the active mechanism by which tumor cell entry involves adhesive interactions with LECs.

In the present study, the initial attachment of tumor cells involves tumor cell membrane-LECs membrane interactions evidenced by their specific membrane structures. In this stage, the tumor cells emit characteristic extensions or process especially at the contacted sites. But only when the attachment is stable do the long and fine microvilli disappear. The microvilli may take part in both fixation and migration of tumor cells on the LEC surface. The LECs may play an important active role in the adhesion, for example, soluble factors constitutively expressed by LECs may facilitate tumor cell invasion of lymphatic vessels. Activation of LECs by VEGF-C or other factors produced by the tumor could promote the release of chemokines. Research has demonstrated the critical role of the chemokine/receptor system (such as CCRs/ CXCL12, CCL19 or CCL21) in mediating tumor cell homing in lymphogenous metastasis (9).

The mechanisms whereby the majority of the seeded cells adhere at or near intercellular junction region are not clear. This may be a mechanical process due to the gravity of tumor cells and the irregular surface of the LEC monolayer. Before or during initial adhesion, the seeded tumor cells can roll on the LEC surface to a lower site just located between the adjacent endothelial cells (7). On the other hand, the cytokines involved in this stage may be rich in the peripheral membrane of the flattened endothelium, or the melanoma cells may prefer to adhere to the exposed subendothelial matrix (substrate) (2,10).

In contrast to many blood-borne metastasis studies, we find some tumor cells adhere directly to the dorsal surface of LECs, dissolve the cytomembrane to form a hole capture and then penetrate through it. This indicates that the bridge between both types of cells by surface components (adhesive molecules) is strong enough to transport the tumor cells. Whether this phenomenon can occur in vivo where the tumor cells are exposed to shear stress needs further study.

After initial adhesion, the deformation of tumor cells and the retraction of LECs at the contacted sites suggest that both types of cells play an important and active role in this step. The integrity of the LECs endothelium, which may resemble the endothelium of the blood vessel, is mainly dependent on the organization of intercellular adherent junctions formed by homotypic interaction of a transmembrane protein, such as Desmoplakin, VE-cadherin, PECAM-1, which forms a complex to an intracellular protein network (actin cytoskeleton) (11-12). The initial attachment can cause these

molecules to redistribute or disappear from the attachment site, which leads to local dissolution of the interendothelial junction and reorganization of the actin skeleton and microfilaments and, consequently, retraction of the LEC (8). Cancer cells derived bioactive lipids and reactive oxygen intermediates (hydrogen peroxide, hydroxyl radical) and enzymes (plasminogen activation, matrix metalloproteinases) may also participate in destroying the intercellular junction (7).

Unlike the common finding in blood-borne metastasis studies (8,14), we never observed the endothelial monolayer to reform and eventually reestablish extensive intercellular junctions after the tumor cells invaded, spread and migrated under the LECs. We think that the disruption, at least at the cytomembrane (hole capture), may be persistent as we found no evidence to support that a destroyed LEC is capable of self-repairing.

Following the further development of techniques for the isolation of LECs and the identification of their molecular properties, better understanding of the lymphatic endothelial properties and how they may be altered in cancer should open a new door to therapeutic interventions.

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