

MICROPATTERNED HYALURONAN SURFACES PROMOTE LYMPHATIC ENDOTHELIAL CELL ALIGNMENT AND ORIENT THEIR GROWTH

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ABSTRACT

The implant of a biocompatible device capable of guiding lymphatic vessel regeneration in patients who underwent removal of lymph nodes might contribute to restoring an efficient lymphatic drainage and help to prevent the occurrence of lymphedema. The aim of this study was to evaluate whether a microstructured surface could provide a guidance for the growth of cultured lymphatic endothelial cells. The presence of microstructures on a surface permits the control of cell adhesion, migration, proliferation, and differentiation. We report here that lymphatic endothelial cells align on microstructures of alternating hyaluronan and aminosylanized glass stripes obtained by photoimmobilization. Cells consistently spread and proliferate only on aminosylanized glass. They orient parallel to the longitudinal axis of the stripe. A pattern of alternating stripes of aminosylanized glass uniformly covered by elongated cells and of hyaluronan devoid of cells eventually forms. The presence of α_v -integrins along cell borders of cells in search of contact with each other and at the leading edge of migrating cells, sites where new focal adhesions are presumably formed, indicates that integrin-mediated adhesion to the substrate guides cell migration along the microstructure. Micropatterned surfaces of hyaluronan thus proved to adequately orient the growth of cells allowing

the regeneration of lymphatic endothelium in the desired direction.

Removal of lymph nodes in cancer therapy, particularly for breast cancer, often followed by radiotherapy, may lead, after a variable period of time, to the occurrence of lymphedema. The reconstruction of interrupted lymphatic vessels is a great challenge to prevent this disabling condition. The aim of this *in vitro* study was to evaluate whether lymphatic endothelial cells can be induced to grow in a desired direction as a model for lymphatic vessel regeneration.

The extracellular matrix *in vivo* does not provide a smooth surface. Actually, most of the extracellular matrix proteins are irregularly arranged. These irregularities probably play a pivotal role in guiding cell orientation and growth. Also *in vitro*, the topographic clues of the substrate greatly influence cell behavior. It has been shown that different types of cells, e.g., macrophages or fibroblasts, orient along fibronectin strands and grow faster under these conditions than on a uniform layer of the same molecule (1). Based on techniques developed in the field of electronics, it is now possible to guide cell growth by the use of artificial devices with a microstructured surface. The presence of microstructures on a surface permits the control of cellular behavior in terms of adhesion, migration, proliferation, and differentiation

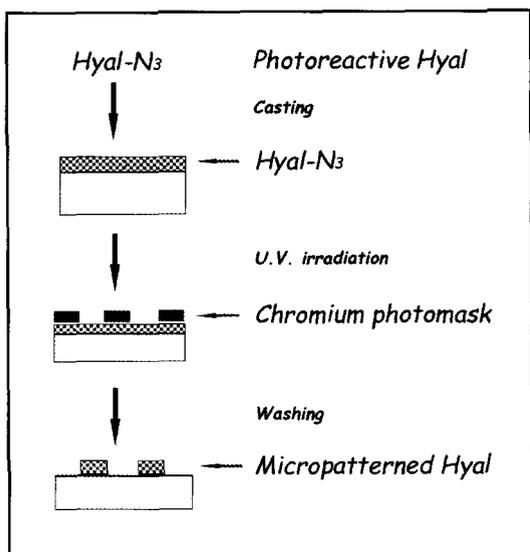


Fig. 1: Scheme of the photoimmobilization process. Hyal = hyaluronan, Hyal-N₃ = hyaluronan conjugated with azidoaniline.

and the manipulation of two fundamental external signals: cell-substrate and cell-cell interactions in order to attain a pattern of highly oriented and differentiated cells.

Microstructures can be produced with different patterns. We theorized that a stripe pattern might be ideal for our purpose. Microstructures with this pattern have in fact already proved capable of promoting cell alignment and orientation (2).

Microstructured surfaces with a stripe pattern were produced by photoimmobilizing hyaluronan onto aminosylated glass. Hyaluronan was chosen because it is one of the main components of the normal extracellular matrix where it is present in soluble form. From the interstitium, hyaluronan is transported to the lymph nodes via lymphatic vessels (3). Blockage of regional draining lymphatics impairs the degradation of hyaluronan, which then stagnates in skin (4). Lymphatic endothelium possesses a receptor for hyaluronan, LYVE-1, a homologue of the CD44 receptor on blood endothelium (5) and antibodies to LYVE-1 are commonly used as lymphatic markers (6,7).

Cell-substrate interaction is mediated by integrins, heterodimeric transmembrane glycoproteins that link the cells with the extracellular matrix (8,9). We previously reported that lymphatic endothelium expresses the α_v -integrin subunit (10), defining receptors for vitronectin and other RGD containing proteins, including fibronectin, which is upregulated in angiogenesis (11,12) and that lymphatic endothelial cells in culture possess the molecular cascade that is responsible for signal transduction and cell adaptation to extracellular matrix solicitations (13). We report here that lymphatic endothelial cells grown on microstructured surfaces also express α_v integrins at presumed sites of newly formed contacts with the substrate.

MATERIALS AND METHODS

Lymphatic endothelial cells were obtained from bovine thoracic duct by collagenase treatment (0.05% collagenase, Worthington, type II, in medium for 15 min. at 37°C) as previously described (14) and cultured on uncoated plastic 24 well multiwells (Falcon) until confluent. Culture medium was Dulbecco's Modification of Eagle's MEM containing 20% FBS, 3% ECGS and 50 μ g/ml gentamycin. At confluence, cells were trypsinized and resuspended in the same medium with 10% FBS and no ECGS. A 200 μ l drop of cell suspension containing 7×10^3 cells was gently pipetted onto the hyaluronan-coated microstructures.

The microstructures were obtained by photoimmobilization (Fig. 1) as previously reported (2). Briefly, hyaluronan was rendered photoreactive by conjugation with 4-azidoaniline. A drop of this photoreactive solution was deposited onto an aminosylated glass coverslip and allowed to dry in the dark. The coverslip was then irradiated for 1 min. with UV light through a photomask with slits of 50 μ m. The covered polymer was not photoimmobilized by the UV light, and it

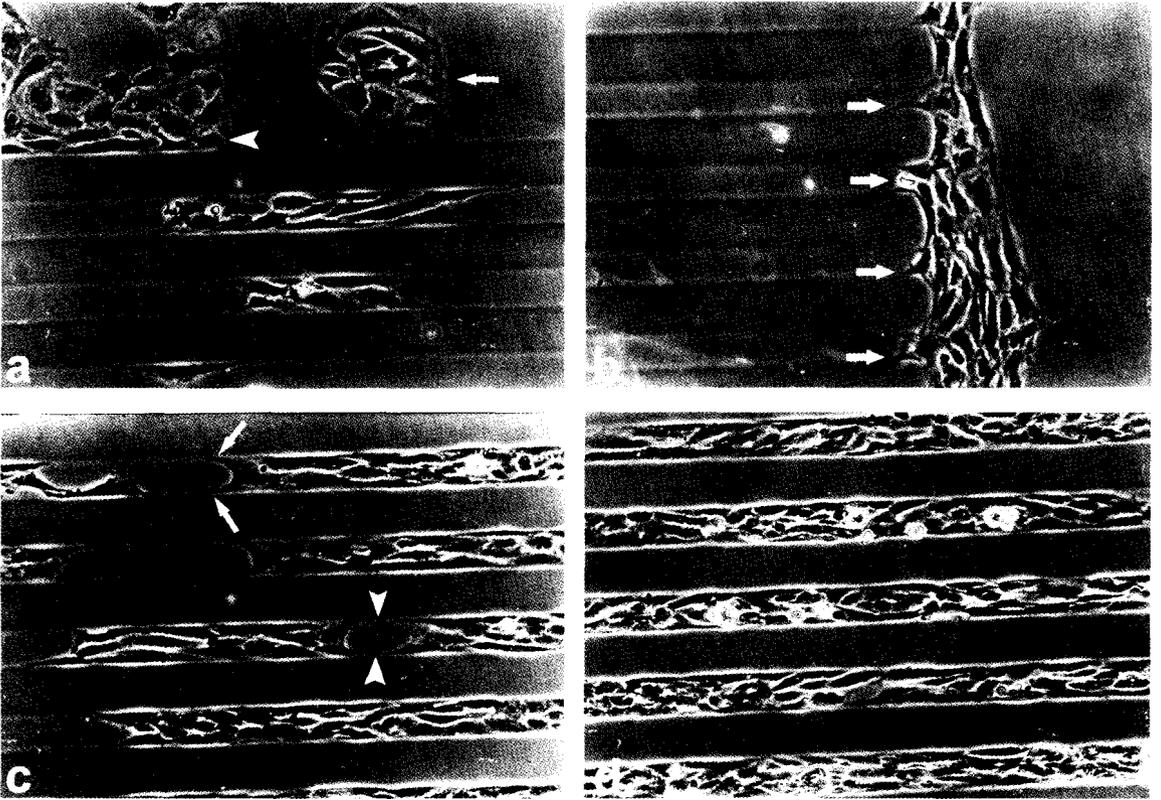


Fig. 2: a) Border between the microstructured area and the uniform layer of aminosylated glass. Irregular colonies of polygonal cells (arrow) on the uniform layer of aminosylated glass. The cells in the row in contact with the microstructured area, align parallel to the stripe (arrowhead); b) LEC entering the microstructured surface (arrows) from the surrounding aminosylated glass; c) LEC growing on the aminosylated glass between hyaluronan stripes send processes to make contact with the stripes (arrows) and with other cells (arrowheads); d) an alternating pattern of hyaluronan and confluent but still elongated LEC eventually forms. (orig. mag. x16)

was easily removed by washing with distilled water. As a result, a pattern of alternating stripes of aminosylated glass and photo-immobilized hyaluronan was obtained. The microstructures were sterilized with ethanol prior to use. Cell growth and orientation were evaluated by phase contrast microscopy.

Integrin expression was evaluated by the use of a polyclonal antibody to the α_v -subunit of integrins (Chemicon, diluted 1:20 in phosphate buffered saline containing 0.01% Triton to permeabilize the cells). The reaction was detected using a FITC-conjugated secondary antibody.

RESULTS

Phase Contrast

LEC usually adhered and spread onto microstructured surfaces within 24 hours after seeding. The coverslip contained a micropatterned area surrounded by a uniform layer of aminosylated glass (Fig. 2a). This allowed comparison of the behavior of cells which had adhered onto the aminosylated stripes versus those that had adhered on the uniformly aminosylated layer.

Cells that had plated on aminosylated

glass surrounding the microstructures formed irregular colonies of polygonal cells. When one of these colonies came into contact with the microstructure, the row of cells growing along the border of the microstructure aligned parallel to the hyaluronan stripes. A cobblestone monolayer of polygonal cells eventually colonized the whole area surrounding the microstructure. At this time some "pioneer" cells started entering the microstructured surface (*Fig. 2b*). Upon entering the microstructure they behaved like the cells that had plated from the beginning onto the microstructure.

The cells that had plated onto the microstructure consistently adhered and grew only in the aminosylated glass between the hyaluronan stripes where only a very thin layer of polysaccharide was present (*Fig. 2a-d*). No cells were ever found on the hyaluronan stripes. As soon as the cells spread, they sent processes to contact each other and to establish contact with hyaluronan stripes (*Fig. 2c*). All cells in the microstructured area, isolated or in groups, oriented along the longitudinal axis of the stripes. Cells maintained an elongated morphology and avoided the hyaluronan stripes up to and even at post-confluence. A pattern of alternating stripes of glass covered with a uniform layer of cells and hyaluronan devoid of cells was eventually observed (*Fig. 2d*).

Immunohistochemistry

Integrin clusters were observed along cell borders of isolated cells (*Fig. 3a*), at the leading edge of migrating cells (*Fig. 3b*) and also at regions of cell contact (*Fig. 3c*). They appeared as fluorescent dashes distributed at regular intervals.

DISCUSSION

When LEC were grown on microstructured surfaces, the cells that had plated onto the microstructured area consistently aligned with their major axis parallel to the stripes.

The cells that had plated outside the microstructured area, upon contact with the stripes, also aligned. Whether this is due to mere topographical reasons (hyaluronan stripes are approximately 250 nm high), or if electric or chemical forces also play a role, is yet to be determined. A higher "step" of 800 nm did not prevent bovine aortic endothelial cells from preferentially growing on top of sulphated hyaluronan stripes obtained with a different technique: laser ablation (15). In the same study, we also tested the behavior of bovine aortic endothelial cells on alternating stripes of hyaluronan (not sulphated, as in the present report) and aminosylated glass. The cells spread only on the glass substrate, as in the present study, but they did not align, rather assuming a polygonal shape. The present microstructures obtained by photoimmobilization are therefore preferable to guide lymphatic proliferation.

The reason why lymphatic endothelial cell do not grow on hyaluronan stripes is unknown. Nevertheless, it has been demonstrated that immobilized hyaluronan is not a good substrate also for other cell types, including fibroblasts, chondrocytes and melanocytes (16) which hardly adhere and grow on the polysaccharide. Hyaluronan, besides being a normal component of the extracellular matrix, is successfully used in clinical practice as a film to reconstruct the dermis after severe burns. The photoimmobilization process does not alter the chemical structure of hyaluronan but probably renders it less apt to interact with some types of cells. In particular, the absence of cells on hyaluronan stripes may be due to the particular conformation assumed by the immobilized polysaccharide, which does not expose the minimal hexasaccharide and decasaccharide sequences necessary to bind the CD44 (17) and presumably the LYVE-1 receptor.

What renders aminosylated glass so apt to promote LEC adhesion and proliferation is more easily explained by the fact that the process of aminosylation renders the glass very hydrophilic and thus favors

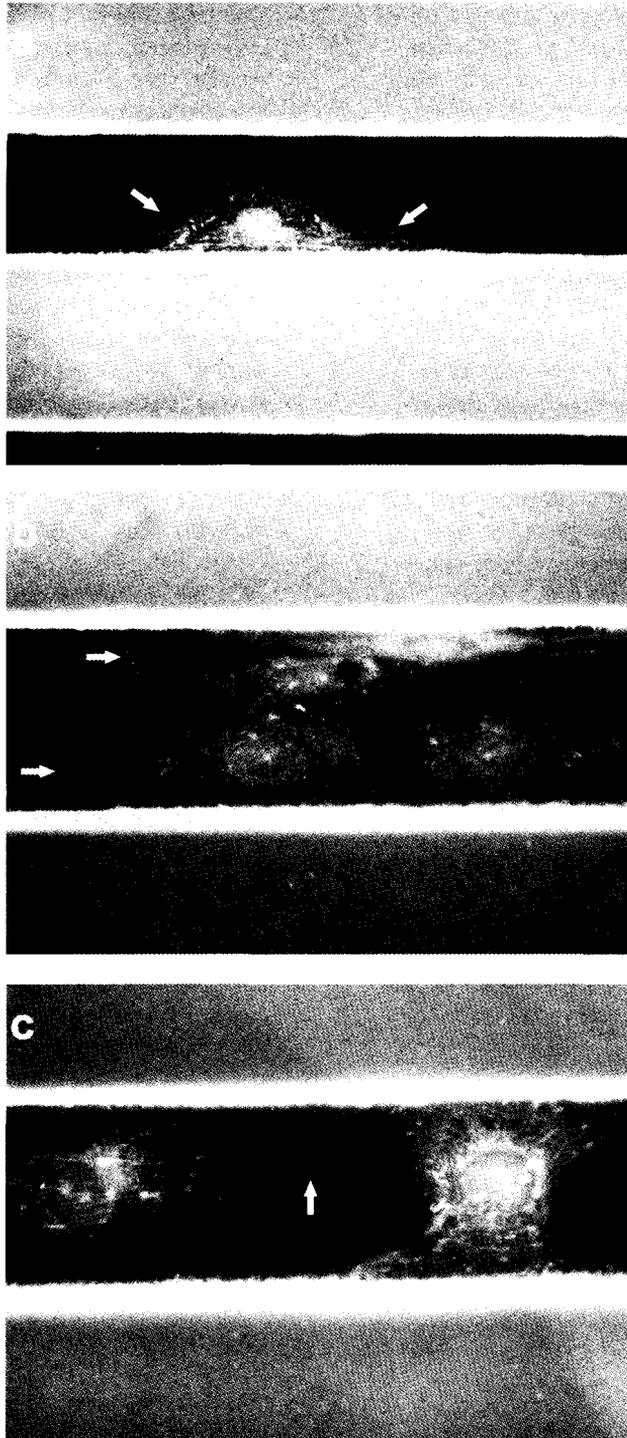


Fig. 3: Alpha-v integrin expression in LEC cultured on microstructures. Integrins are recognizable as short fluorescent dashes (arrows) regularly distributed: a) along cell borders (orig. mag. x40); b) at the leading edge of migrating cells (orig. mag. x60); and c) where cells contact each other (orig. mag. x60).

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adsorption of serum proteins (18). Among the proteins that are known to promote cellular attachment, fibronectin and vitronectin are normally present in serum. They are adhesive glycoproteins containing an RGD sequence which can be recognized by integrins. We here report that LEC grown on microstructures are immunoreactive for α_v -integrins. The presence of integrin clusters along cell borders of LEC in search of contact with each other and at the leading edge of migrating cells, i.e., sites where new focal adhesions are presumably formed, indicates that integrin-mediated adhesion to the substrate guides LEC orientation and migration along the stripes. This raises the possibility that fibronectin and/or vitronectin adsorbed to aminosylated glass may be responsible for integrin-mediated adhesion of LEC. It has, however, been recently reported that while both fibronectin and vitronectin are able to adsorb to aminosylated surfaces from pure solution, in the face of competition from other serum components, fibronectin is unable to adsorb (19). Under our experimental conditions, in which cells are grown in the presence of 10% serum, vitronectin is therefore a more likely candidate in promoting LEC adhesion to aminosylated glass. This, of course, does not exclude the participation of other molecules and does not imply a preferential role of vitronectin in mediating the interaction of lymphatic endothelium with the extracellular matrix *in vivo*.

Our model does not aim at reproducing the *in vivo* situation but rather at providing a device capable of guiding regenerating lymphatic endothelium. Hyaluronan coated microstructures proved effective in this respect by orienting LEC growth in the desired direction. Much work is needed before a bio-compatible microstructured device can be realized and tested in experimental animal models to verify whether functional lymphatic vessel regeneration may be induced in the attempt to reconstruct interrupted lymphatic routes.

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