Chapter 35

Examination of the Role of Galectins and Galectin Inhibitors in Endothelial Cell Biology

Kitty C. M. Castricum and Victor L. J. L. Thijssen

Abstract

The growth of new blood vessels is a key event in many (patho) physiological processes, including embryogenesis, wound healing, inflammatory diseases, and cancer. Neovascularization requires different, well-coordinated actions of endothelial cells, i.e., the cells lining the luminal side of all blood vessels. Galectins are involved in several of these activities. In this chapter, we describe methods to study galectins in three key functions of endothelial cells during angiogenesis, i.e., endothelial cell migration, endothelial cell sprouting, and endothelial cell network formation.

Key words Angiogenesis, Endothelial cell, Migration, Network formation, Sprouting, Galectin

1 Introduction

Blood vessels are part of the infrastructure of an organism, as they facilitate the transport of molecules and cells throughout the body. The vascular infrastructure is formed during embryogenesis by two main processes, vasculogenesis and angiogenesis. Vasculogenesis refers to the early de novo formation of a primitive vasculature by angioblasts. During further development, new vessels are continuously sprouting from the existing capillaries in this vasculature by a process called angiogenesis [1]. Angiogenesis is a complex process that involves different well-coordinated and stepwise activities of endothelial cells (EC), i.e., the cells that cover the luminal side of blood vessels. The angiogenesis cascade is initiated by the activation of the EC by pro-angiogenic growth factors like vascular endothelial growth factor (VEGF). Once activated, supportive cells (pericytes) detach from the capillary and supportive structures (basal membrane and the underlying extracellular matrix) are degraded by proteases. Next, the endothelial cells start to proliferate and migrate in the direction of the proangiogenic stimulus. The growing EC sprouts form primitive tubular structures that are eventually

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stabilized by the deposition of a new basal membrane and the recruitment of pericytes. This allows the EC in these newly formed capillaries to return to a quiescent state [1, 2].

As evident from their pleiotropic functions in cell biology, several galectins are involved in different steps of the angiogenesis cascade. This is not restricted to physiological angiogenesis during, for example, wound healing and embryogenesis, but extends to different pathologies that are characterized by dysregulated angiogenesis, including cancer [3–5]. Galectins have been shown to activate EC and to promote angiogenic signaling in EC. In addition, galectins have been associated with EC adhesion, migration, and proliferation during angiogenesis [6–9]. Thus, galectins are now recognized as regulators of angiogenesis and even as targets for angiostatic therapy [3]. In this chapter, we provide methods to assess the effects of galectins on endothelial cell migration, network formation and sprouting in vitro. Apart from studying the direct effects of galectins, these methods can also be used to determine the effects of galectin-blocking compounds.

2 Materials

2.1 Migration

1. Endothelial cells (see Note 1).
2. Flat bottom 96-well plates (Costar) (see Note 2).
3. Pipetman with 1.0–10 mL sterile pipettes.
4. 10–1000 μL pipettes with sterile tips.
5. Phosphate-buffered saline (PBS).
6. 0.2% gelatin (Merck) in PBS.
7. Recombinant galectins and/or galectin inhibitors.
8. Humidified 5% CO_2 incubator at 37°C/99.5 °F.
9. A 96-well pin tool scratcher (see Note 3).
10. UniversalGrab 6.2 software (DCIlabs) for image acquirement.
11. Image analysis software, e.g., ScratchAssay 6.2 (DCIlabs), ImageJ or Photoshop.

2.2 Network Formation

1. Flat bottom 96-well plates (Costar).
2. BD Matrigel basement membrane matrix (seeNote 4).
3. Endothelial cells (see Note 1).
4. Recombinant galectins and/or galectin inhibitors.
5. Humidified 5% CO_2 incubator at 37°C/99.5°F.
6. UniversalGrab 6.2 software (DCIlabs) for image acquirement.

2.3 Sprouting

1. Endothelial cells (seeNote 1).
2. μ-Slide eight-well Ibidi plate.
3. Nonadhesive square petri dishes (10 × 10 cm).
4. Methocel medium: RPMI with 20% methocel (Sigma-Aldrich) and 10% heat-inactivated human serum (in-house produced).
5. Sprouting medium: 10% fetal bovine serum (FBS), 0.1% Heparin (Leo Pharma), 14.8% Methocel, 62.3% PureColl (Sigma-Aldrich), 8.3% 10 × 199 complete (Gibco/ThermoFisher), 4.5% NaOH.
6. Humidified 5% CO_2 incubator at 37°C/99.5 °F.
7. UniversalGrab 6.2 software (DCIlabs) for image acquirement.
8. Image analysis software, ImageJ.

2.4 Special Equipment

1. Inverted microscope equipped with a camera. We use a Leica DMI3000B microscope equipped with an automated xyz-stage and a Hitachi 1.4 Mb GiGE color camera.

3 Methods

3.1 Migration

1. Coat a flat bottom 96-well plate with 50–80 μL 0.2% gelatin/PBS for at least 30 min at 37°C/99.5°F or 2 h at room temperature. Aspirate gelatin before seeding the cells. Alternatively, the wells can be coated with the recombinant galectin of interest in PBS (optimal concentration should be determined empirically).
2. Seed cells and grow to confluence in 2–3 days (seeNote 5).
3. Scratch the confluent monolayer with a 96-well pin tool (see Note 3).
4. Aspirate/drain the culture medium.
5. Carefully wash cells one time with 100 μL PBS.
6. Apply the appropriate medium containing recombinant galectin or galectin inhibitors (seeNotes 6 and 7).
7. Take images of the scratch at t = 0, t = 2, t = 4, t = 6, and t = 8 h (seeNote 8 and Fig. 1a).
8. Measure wound width or scratch area with automated Scratch-Analysis software. Alternatively use ImageJ (use straight line selection tool to measure wound width or free hand selection tool for measuring scratch area) or Photoshop (use ruler for wound width measurement or wand tool for scratch area). Calculate the percentage of wound closure or remaining wound width/area (% of t = 0).

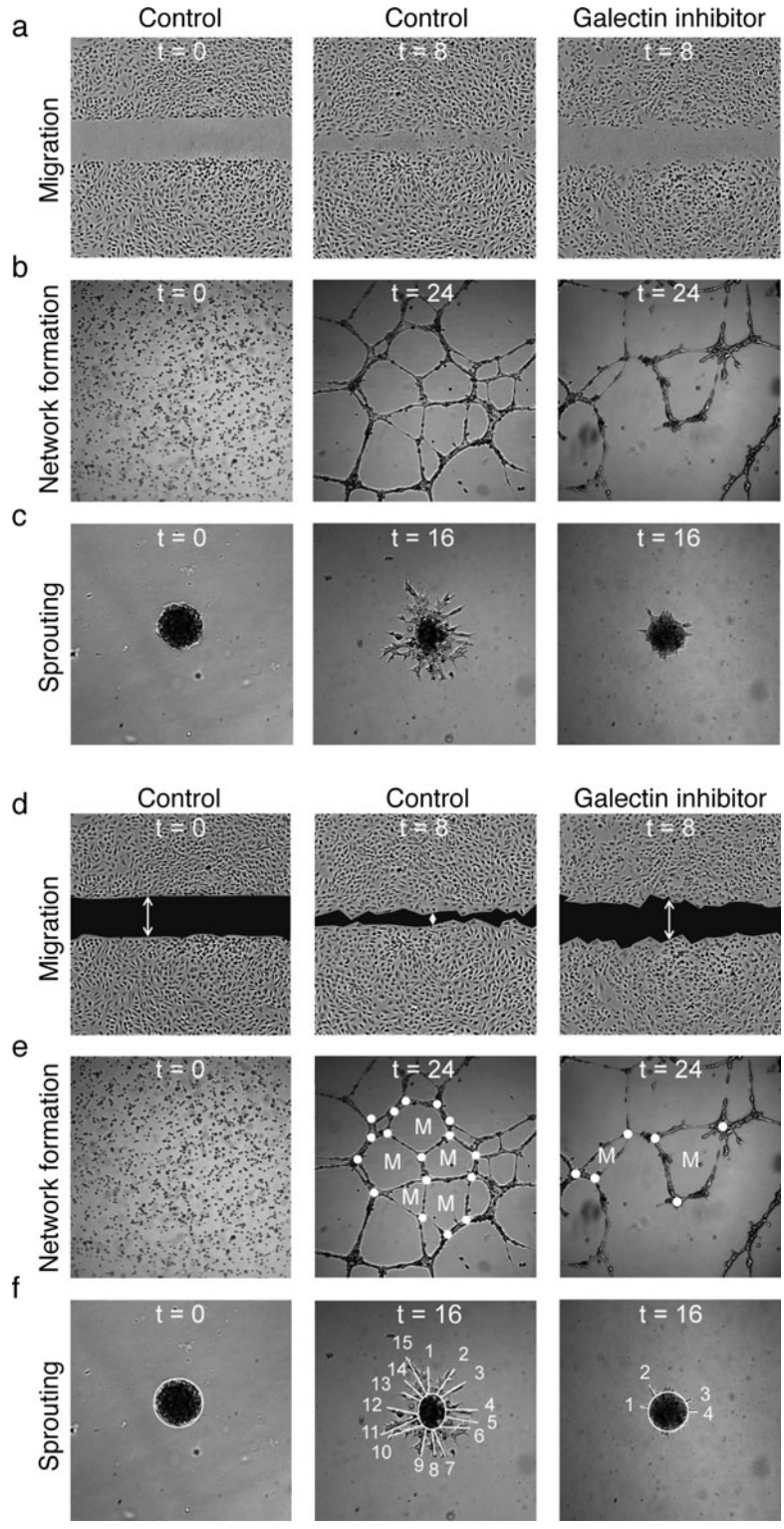

Fig. 1 In vitro angiogenesis assays. Representative pictures of migration (a+d), network formation (b+e), and sprouting (c+f) of nontreated (control) and galectin inhibitor-treated (e.g., anginex 5 mg/mL) HUVEC. The first panels represent the

3.2 Network Formation

1. Coat each well of a flat bottom 96-well plate with 40 µL Matrigel (see Note 4) and incubate at 37°C/99.5°F for 30 min.
2. Apply the required number of cells in 50 µL medium per well (see Note 9). Add recombinant galectin of interest or galectin inhibitors (e.g., lactose or anginex) to the medium.
3. Incubate overnight in a humidified incubator at 37°C/99.5°F, 5% CO_2.
4. Acquire images using a microscope with a camera system.
5. Endothelial cell network formation can be quantified by counting the number of branch points, number of meshes (see Fig. 1b).

3.3 Sprouting

1. Harvest cells and resuspend to a final concentration of 40,000 cells/mL in methocel medium (see Note 10).
2. Distribute 25 µL drops with a multichannel pipet on the lid of a nonadhesive 12 × 12 cm square petri dish (see Note 11).
3. Add 10 mL PBS to the bottom of the petri dish to prevent evaporation of the drops.
4. Turn the lid with drops carefully upside down and place on the bottom dish containing PBS.
5. Incubate overnight in a humidified incubator at 37°C/99.5°F, 5% CO_2.
6. Add 100 µL sprouting medium per well to a µ-Slide eight-well Ibidi plate (see Notes 12 and 13).
7. Incubate the plate for 30 min at 37°C/99.5°F until the sprouting medium is solidified.
8. Harvest the spheroids using a 1-mL pipet and centrifuge for 1 min at 1200 rpm.
9. Remove supernatant and resuspend the spheroids carefully in sprouting medium at approximately 30 spheroids/100 µL and transfer 100 µL per well (see Notes 12 and 13).

Fig. 1(continued) starting conditions at t = 0. The second and third panels indicate typical results at the end point of the experiment of nontreated and galectin inhibitor-treated cells, respectively. The panels e-fillustrate different ways of analyzing the respective results: (d) For analysis of migration, wound width (white arrow), or wound area (in black) can be measured at different time points and compared with t = 0. (e) Network formation can be quantified by counting the number of meshes (M) and branch points (white dots). For clarity only part of the image is scored. (f) Sprouting is analyzed by measuring the sprout length (white lines) and counting the number of sprouts

10. Incubate the plate for 30 min at 37°C/99.5°F until the sprouting medium is solidified.
11. Apply 100 μL medium, containing recombinant galectin proteins and/or galectin inhibitors on top of the sprouting medium (see Notes 13 and 14).
12. Incubate in a humidified incubator at 37°C/99.5°F, 5% CO_2 for 16–24 h and take pictures of the spheroids (see Note 15 and Fig. 1c).
13. Analyze sprout length and sprout number per spheroid using, e.g., ImageJ or Adobe Photoshop (see Note 16).

4 Notes

1. Different sources of endothelial cells are available [10] Human umbilical vein endothelial cells (HUVEC) are frequently used, and these cells can be isolated in house or purchased from a commercial source. Apart from HUVEC, we also use the EC lines EC-RF24 (HUVEC origin) and HMEC (human microvascular endothelial cells). For network formation and sprouting assays, we generally use HUVEC while migration assays can be performed with any source of EC. If required, cells can be transfected with galectin-targeting siRNA or galectin expression constructs according to standard protocols. All EC are cultured in RPMI1640 (Lonza) supplemented with 10% fetal bovine serum (FBS), 10% human serum, 1% L-glutamine, and 1% penicillin/streptomycin. At confluency, passage the cells 1:3.
2. We prefer the use of Costar flat bottom plates since plates from other suppliers sometimes contain markings on the back of the plates due to the production process. These markings can interfere with the scratch analysis.
3. A pin tool scratcher is preferred since this gives low variability and high reproducibility in wound width. We use the Peira HTSScratcher. However, it is also possible to apply a scratch manually using a small pipetting tip, e.g., 10 μL tips. Take a fresh tip for each well to avoid increasing scratch width due to cells that stick to the tip.
4. Matrigel is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains several extracellular matrix proteins that facilitate endothelial cell adhesion and migration. However, standard Matrigel also contains several growth factors that stimulate the angiogenic activity of endothelial cells. Therefore, depending on the research question, e.g., test effect ofinhibitors on galectin-induced network formation or sprouting, it is better to use growth factor reduced Matrigel. Note

that Matrigel stock solutions should be kept at temperatures below 4 °C/39.2°F to prevent solidification. Thawing should therefore be performed on ice in a fridge (this takes approximately 1 h).

5. It is important to start with a confluent monolayer of cells in order to induce unidirectional migration. For RF24 cells, seed 15,000 cells/well, and for HUVEC/HMEC, use 5000 cells/well.
6. Evaluation ofinhibitory activity can be performed in normal EC culture medium. To evaluate stimulatory activity, low serum (0.5% FBS and/or 0.5% human serum) can be used. Furthermore, we have observed a biphasic activity of galectin-1 depending on the concentration [8, 11]. Therefore, we suggest using a broad concentration range to optimize your assay.
7. Always include proper controls, e.g., Sutent (Sunitinib malate) (Sigma-Aldrich) for inhibition or Fibroblast Growth Factor-Basic (bFGF) (Sigma-Aldrich) for stimulation of migration. Use as directed by the manufacturer.
8. For analysis, it is important that the images of the scratch within the time series are taken at the exact same position. If no appropriate software and automated xyz table are available to automate this, make sure to mark the culture plate in such way that the area in the well where the images are taken can be easily found. For example, prior to plating the cells, use a scalpel to make a scratch on the backside of each well in the plate. Make sure that the scratch is perpendicular to the direction of the "wound." The perpendicular scratch can then be used to identify the region where the wound width is measured.
9. The optimal number of cells used in this assay should be individually determined for each cell line. Too high cell numbers will result in robust branches or confluent areas at the center of the well. Too few cells will yield incomplete network structures. We generally use ±20,000 HUVEC per 96-well.
10. Dilute to 40,000 cells/mL to obtain 1000 cells/25 µL drop. Prepare 60 drops (60,000 cells in 1500 µL methocel medium) per test condition since not all drops will successfully form a spheroid.
11. Use reverse pipetting technique to avoid air bubbles disturbing the spheroid formation.
12. When transferring the spheroid suspension, make sure the whole well is covered by pipetting the suspension "swirl-wise," starting in the middle of the well. Avoid air bubbles.
13. The experiment can also be performed in a 24-well culture plate. In that case, the volumes in steps 6, 9, and 11 should

be adjusted to 200 µL, 200 µL, and 500 µL, respectively. The concentration of galectin depends on the activity. For example, for galectin-9, we used 500 nM [11].

14. As a positive control for the induction of sprouting, add 100 nM of the γ-secretase inhibitor dibenzazepine (DBZ) to the medium. As a positive control for inhibition of sprouting, 10 µM Sutent can be added to the medium.
15. For reliable analysis of sprouting, at least 10 spheroids per treatment condition should be photographed and analyzed.
16. Sprout number and/or length can be assessed manually in Adobe Photoshop by using the "count tool" or "ruler tool" in Image analysis. ImageJ also provides similar tools or even plugins to analyze sprouting.

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