Evaluating VEGF-induced vascular leakage using the Miles assay

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Abstract

Before the endothelial mitogenic activity of the Vascular Endothelial Growth Factor A (VEGF) was described, VEGF had already been identified for its ability to induce vascular leakage. VEGF-induced vascular leakage has been most frequently studied *in vivo* using the Miles assay, a simple yet invaluable technique that has allowed researchers to unravel the molecular mechanisms underpinning vascular leakage both for VEGF and other permeability inducing agents. In this protocol, a mouse is intravenously injected with Evans Blue dye before VEGF is administered locally via intradermal injection. VEGF promotes vascular leak of serum proteins in the dermis, enabling Evans Blue-labelled albumin extravasation from the circulation and subsequent accumulation in the skin. As the volume of dye extravasation is proportional to the degree of vascular leak, it can be quantified as a proxy measurement of VEGF-induced vascular leakage.

Key words

VEGF, Miles assay, vascular permeability, vascular leakage, Evans blue, oedema

Running title: VEGF-induced *in vivo* vascular leakage

1 Introduction

All blood vessels are lined with an endothelial monolayer that regulates fluid and solute exchange between the circulation and vascularised tissues. Several proteins, such as histamine or the vascular endothelial growth factor A (VEGF), disrupt the endothelial barrier, thereby increasing vascular leakage and causing tissue oedema [1]. First applied in the 1950s to guinea pigs [2], the Miles assay has emerged as the principal technique for studying vascular leakage *in vivo* [3]. Now invariably performed on mice, especially to exploit their potential for genetic manipulation, the Miles assay quantifies vascular leakage through the assessment of Evans Blue-labelled albumin extravasation from the dermal vasculature. In the initial steps of the procedure, Evans Blue dye is introduced into the mouse circulation by intravenous injection. Subsequently, VEGF or another permeability inducing agent is administered to the skin of the mouse via intradermal injection, thereby promoting endothelial barrier disruption and leakage of Evans Blue dye into the skin. In principle, the quantity of Evans Blue dye that accumulates in the skin is proportional to the degree of vascular leakage. Thus, after the mouse has been culled, Evans Blue dye can be extracted from the skin and its abundance measured. Comparing the abundance of Evans Blue dye extracted from VEGF-injected skin versus vehicle-injected skin in the same animal provides a normalised readout of VEGF-induced vascular leakage, which can then be compared between experimental groups to determine how a variable influences vascular leakage.

As the Miles assay requires relatively few reagents and consists of only a small number of steps, it is often favoured for its simplicity compared to other techniques that measure vascular leakage. Moreover, the Miles assay can be used to compare vascular leakage between different strains of genetically modified mice or coupled with

pharmacological treatments to identify novel inhibitors or molecular mediators of vascular leakage [4-6].

2 Materials

All solutions should be prepared in a laminar flow cabinet and sterilised by passing through a 22 µm filter syringe.

2.1 Mouse preparation

Electric trimmer.

Isoflurane.

Weighing scale for rodents.

2.2 Injections

Pyrilamine maleate: 4 µg/µl in 0.9% saline

Evans Blue dye: 1 % Weight/Volume Percentage Concentration (w/v) in 0.9 % saline

VEGF solution: 2.5 ng/µl recombinant mouse VEGF-164 in phosphate buffered saline (PBS)

1 ml and 350 µl syringes

30 G needles

Mouse restrainer

2.3 Dye extraction/ quantification

Deionized formamide

Cork/polystyrene board

Scalpels

Spectrophotometer

3 Methods

3.1 Mouse preparation

1 Mice must be shaved 24 – 48 hours before step 3.2. Anaesthetise the first mouse using isoflurane (3% isoflurane for anaesthetic induction, 1.5-2 % isoflurane for anaesthetic maintenance). Do not move onto the next step until the mouse is unresponsive to external stimuli, such as a gentle tail pinch.

2 Using an electric trimmer, shave both flanks of the anaesthetised mouse, taking special care not to cause any skin damage that will disturb subsequent analysis.

3 Weigh the anaesthetised mouse and return it to its cage for it to regain consciousness.

4 Repeat steps 1 – 3 for each mouse to be studied. When using male mice, return each to an individual cage to prevent fighting after recovery and therefore skin damage and irritation; always use a cage that contains bedding from the home cage.

3.2 Injection of Evans Blue dye

1 For each mouse to be studied, prepare two 1 ml syringes with 30G needles in a sterile laminar flow cabinet. Load one syringe with 10 µl sterile pyrilamine maleate per gram of mouse body weight (as recorded in step 3.1.3); then load the second syringe with 100 µl sterile 1 % Evans Blue dye (w/v sterile PBS).

2 Administer pyrilamine maleate to the first mouse by intraperitoneal injection (Figure 1A). To do so, scruff the mouse and tilt it downwards to expose the lower abdomen. Insert the needle into the lower quadrant of the mouse,

avoiding the midline, where the bladder resides, and then inject the pyrilamine maleate solution.

3 Move the mouse to a 37 °C heat chamber for 10 minutes to promote vasodilation, and repeat steps 2 – 3 for the remaining mice.

4 Move the first mouse into a restrainer. Position the restrainer containing the mouse under a suitable light source and rub the tail with 70 % ethanol to enable tail vein visualisation.

5 Once the tail vein is readily observable, inject 100 µl Evans blue dye into the tail vein (Figure 1B). After removing the needle, immediately apply pressure to the injection site to prevent blood loss. After 10 seconds, return the mouse to its cage.

6 Repeat steps 4 and 5 for the remaining mice.

7 Allow Evans Blue to circulate for 30 – 60 minutes.

3.3 Induction of vascular leakage

1 For each mouse, prepare two 300 µl syringes with a 30 G needle in a sterile laminar flow hood.

2 Load one needle with 100 µl sterile PBS and the second needle with 100 µl sterile VEGF in PBS (2.5 ng/µl).

3 Anaesthetise the first mouse (3 % isoflurane for anaesthetic induction, 1.5 – 2 % for anaesthetic maintenance). Do not move to step 4 until the mouse is unresponsive to external stimuli such as a gentle tail pinch.

4 Administer 20 µl of VEGF solution into the dermis of the first flank of the mouse by intradermal injection (Figure 1C, see note 3).

5 Repeat the injection at a further two adjacent dermal sites, approximately 1 cm apart.

6 Record the location of each injection site on a piece of paper.

7 Turn the mouse to reveal the second flank.

8 Administer 20 µl PBS by intradermal injection.

9 Repeat the injection at a further two sites, approximately 1 cm apart. Record the locations of each injection site on a piece of paper.

10 Return the mouse to its cage and monitor it as it recovers from anaesthesia.

11 Repeat steps 2 – 11 for each mouse.

3.4 Quantification of vascular leakage

1 20 minutes after the intradermal injections, cull each mouse by cervical dislocation.

2 Place each dead mouse onto a cork/polystyrene board and pin their feet down.

3 Make an incision between the mouse abdomen and chest using blunt scissors, and then use forceps and a scalpel to peel the flank skin away from the mouse (Figure 1D).

4 Pin down the flank skin and use a scalpel to scrape away any fat connected to the skin.

5 Using forceps and a scalpel, excise the skin around the injection sites, where Evans Blue dye has accumulated. Records of injection sites will aid in this step, especially for the PBS control.

6 Place each skin sample into a labelled 1.5 ml reagent tube and dry in the open tube overnight at 55 °C.

7 In a fume cupboard, add 250 µl deionized formaldehyde to each tube and incubate with a closed lid overnight at 55 °C to extract the Evans Blue dye from the skin.

8 Centrifuge samples at 10,000 x g for 40 min.

9 In a fume cupboard, transfer 100 µl of supernatant (Evans Blue dye-containing formamide) from each tube to an individual well of a transparent 96 flat-well plate.

10 Measure the absorbance of each Evans Blue dye-containing formamide solution with a spectrophotometer at 620 nm using a reference wavelength of 740 nm.

11 For each mouse, average the absorbance readings for the triplicate injections (i.e. 3 x VEGF injections into the same flank, 3 x PBS injections into the same flank) and calculate the fold difference in absorbance for the VEGF-injected flank versus the PBS-injected flank of the same mouse.

4 Notes

1 Mice should be at least 8 weeks of age. Where possible, littermate mice should be used across experimental groups. Alternatively, mice in different experimental groups should be genetic background strain-, age-, weight- and gender-matched. Do not use more than 6 mice per experimental session to allow sufficient time for each step. To identify a significant difference in moderate to severe leakage across an experiment, 5 mice per experimental group are typically required.

2 Performing the tail vein injection is the most difficult step of this procedure, as it needs to be performed with similar efficiency in all the animals to ensure that

results between different animals can be reliably compared. Researchers should first clearly identify the tail vein they wish to inject. Visualising the length of the tail vein is aided by adequate light and rubbing 70 % ethanol onto the tail. Once the vein is clearly visible, researchers should angle the mouse so that the vein is facing upwards, and then extend the tail so that it runs parallel to the mouse body. Close to the tip of the tail, researchers should align the needle with the vein then slowly enter the vein towards the tail base and with the needle bevel facing upwards. If the needle has successfully entered the vein, the Evans Blue solution will inject with little resistance. If the researcher feels resistance when attempting to inject, or if Evans Blue dye accumulates at the injection site instead of entering the circulation, the researcher should withdraw the needle and attempt the injection again at a higher location on the tail (i.e. closer to the tail base).

3 To perform intradermal injections, the researcher should pull back the skin of the mouse so that the skin is tight at the injection site; it is important to not pinch skin near the injection site because this may promote unwanted leakage. Intradermal injections should be performed at approximately 15 ° to the skin. There should be a degree of resistance as the needle enters the dermis; if the needle slides quickly into the skin, the researcher may have passed the dermal layer and would be delivering the solution subcutaneously. A raised bump in the skin indicates a successful intradermal injection, whilst a flat injection site likely indicates that the injection load was delivered beneath the skin.

4 Cut similarly sized regions of skin from each injection site, irrespective of the apparent Evans Blue dye accumulation at each site. This helps to normalise for Evans Blue dye that has not leaked but still resides within the skin vessels. To facilitate equal sampling, a punch biopsy blade may be used.

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Figure 1 Main steps of the Miles assay for vascular leakage. (**A**) Intraperitoneal injection of pyrilamine maleate: scruff the mouse and tilt it downwards; insert the needle into the lower quadrant of the mouse abdomen, away from the midline. (**B**) Intravenous injection of Evans Blue dye: align the needle with the tail vein and insert; gently administer 100 µl Evans Blue dye. If the dye does not freely enter the circulation, cease the injection and attempt to inject at a second site further up the tail. (**C**) Intradermal injection of VEGF or vehicle solutions: tort the flank skin and insert the needle at a 15 degree angle; gently administer 20 µl of VEGF solution and withdraw the needle, observing a raised bump in the skin. Repeat the injection at two additional sites before turning the mouse and administering three intradermal injections of vehicle solution on the opposing flank. (**D**) After cervical dislocation, dissect the mouse skin to reveal Evans Blue accumulation at injection sites and proceed with skin sampling for Evans blue dye extraction and measurement.