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Improved semiautomatic method for morphometry of angiogenesis and lymphangiogenesis in corneal flatmounts

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ABSTRACT

Purpose of the study was to describe a novel semiautomatic, quantitative image analysis method based on threshold analysis for morphometry of corneal (lymph)angiogenesis and to test its validity, reliability and objectivity. Murine corneas were vascularized by using a suture-induced neovascularization assay. For immunohistochemistry, flatmounts of the vascularized corneas were stained with LYVE-1 as a specific lymphatic vascular endothelial marker and with CD31 as panendothelial marker. Morphometry of corneal hem and lymphangiogenesis was performed semi-automatically on digital images using image analysis software. Data were analyzed by a paired *t*-test, intraclass-correlation and systemic difference analysis compared to a manual method. The semiautomatic method based on threshold analysis was more valid in measuring the area covered by blood or lymphatic vessels. Both methods had a good reproducibility with respect to both vessel types (*blood vessels*: manual: 0.969, semiautomatic: 0.982; *lymphatic vessels*: manual: 0.951, semiautomatic: 0.966), whereas the systemic difference was significant for both groups measuring lymphatic vessels (manual: $p < 0.003$; semiautomatic: $p < 0.035$) and for the manual method measuring blood vessels (manual: $p < 0.0001$; semiautomatic: $p < 0.419$). The new semiautomatic morphometry method based on threshold analysis provides higher accuracy, is more valid than and at least as reproducible and objective as the manual outlining method. Therefore the semiautomatic method can be used to detect even small effects on hem and lymphangiogenesis in murine corneal flatmounts with greater precision.

1. Introduction

Angiogenesis and lymphangiogenesis become more and more important for pathological processes such as tumor growth (Cavallaro and Christofori, 2000) and graft rejection (Dana and Streilein, 1996; Cursiefen et al., 2004a,b). To analyze the molecular mechanism of these processes and to test novel drugs several animal models have been developed (for review see Auerbach et al., 2003). Some of these models use the cornea for two reasons: first, a normal healthy cornea is devoid of both blood and lymphatic vessels, but can be invaded by both vessel types. This process can therefore be stimulated and inhibited pharmacologically. Second, the transparency of the cornea and its normal avascularity (“angiogenic privilege”) permit excellent visualization of sprouting vessels with low background staining. Since lymphatic vessels are not visible using normal slit lamp magnification, blood and lymphatic vessels together can only be studied histologically. By specific staining for blood vessels (e.g., with CD31/von Willebrand factor) and lymphatic vessels (e.g., with LYVE-1, Podoplanin), hem and lymphangiogenesis can be quantified. Recent studies used different methods to measure the vascularized areas in animal corneas: (i) *semiquantitative method*: the cornea was divided into several quadrants and each sector was assessed with scores dependent on the number of vessels and the density of the vessel network (Cursiefen et al., 2001a,b; Lai et al., 2007) and (ii) *manual quantitative method by outlining of vascularized areas*: digital pictures of the cornea along with the limbus were taken and analyzed outlining the total corneal area using the innermost vessel of the limbal arcade as the border. In the latter instance, the neovascularized areas were quantified by manually circumscribing the blood vessel network or each single lymphatic vessel with the cursor. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the cornea covered by vessels was calculated (Wu et al., 2003; Cursiefen et al., 2004a,b, 2006; Yoon et al., 2006). (iii) *Here we describe a novel semiautomatic, quantitative method based on threshold analyses*: digital grey value threshold measurements are used to quantify the areas of neovascularization (Conrad et al., 1994; Bock et al., 2007a,b; Dietrich et al., 2007; Usui et al., 2007). In contrast to the quantitative manual outlining method, this new grey value based analysis method has

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two benefits: with the manual method it is hardly possible to distinguish between several complexity-levels of blood vessel networks like, e.g., many thin vessels with intervascular junctions or less but thicker vessels without intervascular junctions. The semiautomatic method measures only the areas, which are in fact covered by the single vessels. The second benefit is the faster and even more precise measurement of single vessels like lymphatic vessels. While with the manual method each vessel sprout has to be circumscribed, which is very time-consuming, the semiautomatic methods allow the detection of all sprouts in the region of interest at once, which is timesaving and even more accurate.

Aims of this study are (a) to describe in detail the used new algorithm for semiautomatic, threshold-based analysis of corneal hem and lymphangiogenesis and (b) to test the validity, reliability and objectivity of this new semiautomatic quantitative method based on threshold analysis in comparison to other methods.

2. Methods

2.1. Animals

For the suture-induced, inflammatory corneal neovascularization assay female BALB/c mice (aged 6–8 weeks) were used. For these experiments the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, were followed. The local animal care committee in accordance with the current version of the German law on the Protection of Animals approved all animal protocols.

2.2. Suture-induced, inflammatory corneal neovascularization assay

Prior to surgery, each animal was deeply anesthetized with an intramuscular injection of Ketanest®S (8 mg/kg) and Rompun (0.1 ml/kg). Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally with two stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal centre equidistant from limbus to obtain standardized angiogenic responses. Sutures were left in place for the duration of the experiment. Mice were sacrificed after 7 d.

2.3. Corneal whole mounts and morphological determination of hemangiogenesis and lymphangiogenesis

The corneas were excised, rinsed in PBS and fixed in acetone for 30 min as described previously (Cursiefen et al., 2004a,b). After three additional washing steps in PBS and blocking with 2% BSA in PBS for 2 h the corneas were stained overnight at 4 °C with rabbit anti-mouse LYVE-1 (1:500; a kind gift of D.G. Jackson, Oxford University, Oxford, United Kingdom). On day two the tissue was washed, blocked and stained with FITC-conjugated rat anti-CD31 (Acris Antibodies GmbH, Hiddenhausen, Germany) antibody overnight at 4 °C. After a last washing and blocking step on day three, a goat-anti-rabbit Cy3-conjugated secondary antibody was used. Isotype control was assured with an FITC-conjugated normal rat IgG2A for CD31/FITC and with a normal rabbit IgG (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) for LYVE-1 (Cursiefen et al., 2004a,b).

2.4. Image acquisition

Double stained whole mounts were analyzed with a fluorescence microscope (BX51, Olympus Optical Co., Hamburg, Germany) and digital grey value pictures were taken with a 12-bit monochrome CCD camera (F-View II, Soft Imaging System, Münster, Germany) at a resolution of 1376 × 1023 pixel. For the FITC stained blood vessels an *HQ-FITC selectiv.* filterset (Exciter: HQ 480/40; Emitter: HQ 527/30; AHF analysentechnik AG, Tübingen, Germany) was used. For the Cy3 stained lymphatic vessels the U-MWG2 mirror unit (Excitation filter: 510–550 nm; Emission filter: 590 nm; Dichromatic mirror: 570 nm; Olympus, Hamburg, Germany) was used. Each whole mount picture was assembled out of 9 pictures taken at 100× magnification.

2.5. Functional analysis

The areas covered with blood or lymphatic vessels were detected for the manual method with the image analysis programs NIH Image software ImageJ (http://rsb.info.nih.gov/nih-image; National Institutes of Health) and for the semiautomatic method with the image analysis program cell^F (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.6. Manual method of corneal image analysis

Using the NIH Image software ImageJ (http://rsb.info.nih.gov/nih-image; National Institutes of Health), total area of the cornea was measured manually by outlining the innermost vessel of the limbal arcade. Neovascularized areas were quantified manually by circumscribing the blood vessel network or each single lymphatic vessel with the cursor. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the cornea covered by vessels was calculated.

2.7. Semiautomatic method of corneal image analysis

A new method to quantitatively analyze the corneal area covered by vessels based on the image analysis program cell^F (Olympus Soft Imaging Solutions GmbH, Münster, Germany) was developed. The analysis bases on the detection of a grey value range, in which mainly blood or lymphatic vessels are presented. To avoid the detection of other objects and to enhance the exposure of the vessels, four software based filters were used consecutively (Figs. 1 and 2).

2.7.1. DCE filter

First, a DCE filter (Differential Contrast Enhancement) contrasts hardly distinguishable structures. Thereby the image becomes more detailed and is brought into sharper focus (Figs. 1 and 2b).

2.7.2. Erosion filter

Second, a morphological filter removes punctual noise. Hereby the filter identifies the lowest grey value in a defined pixel neighborhood and replaces the central pixel by this grey value (Figs. 1 and 2c).

2.7.3. Gradient

Now the vessels can be highlighted by a combined filter: the morphological filter *gradient* is a combination of the filter *erosion* and *dilatation* and an arithmetic subtraction: to erase final interferences and to carry off the outer rim of the vessels, the original image is initially eroded. Parallel, the original image is dilated, with small holes and irregularities being deleted from the vessel rims. The resulting, dilated image is subtracted from the eroded image. Altogether edges and rims are highlighted (Figs. 1 and 2d).