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Neovascularization in Alkali-Burned Rabbit Cornea

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Abstract

Objective: To study the neovascularization in regenerating and proliferating corneal cells following a standard alkali injury in rabbit eye.

Methods: Three and six weeks after the creation of an alkali burn in the center of the right cornea of six albino rabbits, the animals were killed and histological sections from the cornea of both eyes were stained, photographed and studied for a possible formation of a neovascularization. The photographs were examined using the Quantimet image analyzer (Leica) and statistical analysis of the data was performed.

Results: Sections of the injured cornea showed the formation of neovessels in the epithelial and superficial stromal layers. The neovascularization is present after 3 weeks of the corneal injury. After 6 weeks from the corneal alkali burn, neovessels are increased.

Conclusions: There is a growing body of evidence suggesting that vascular abnormalities may play a crucial role in several ocular diseases. To improve our knowledge of the vascular involvement in these conditions, there is a need for a non-invasive imaging modality capable of assessing microcirculation within ocular tissue beds both in vitro and in vivo. This study shows that ultra-high sensitive optical microangiography, associated with other experimental techniques, is an adequate technique to visualize the eye surface microcirculations and to quantify microvascular vessel density under both normal and physio-pathological conditions.

Keywords: Cornea; Injury; Neovascularization; Fluorescence; Capillaroscopy; Electron microscopy

Introduction

The characteristics of alkali-induced corneal lesions have been well defined in rabbits by histological, histochemical, autoradiographical and optical investigations [1-5]. In the past years our group has also contributed several studies on the rabbit cornea, describing the presence of contractile microfilaments in injured tissues [6,7]; the dynamics of catecholaminergic nerve fibers changes in normal and injured alkali-burned rabbit corneas [8,9]; the localization of dopaminergic receptors [10] and the distribution of nerve fibers with a selective affinity for the quinacrine dye in the rabbit cornea [11]. Moreover, we have performed experiments on human corneas addressing the presence of inorganic elements on hydrophilic lenses [12] and/or in the lachrymal film [13]. Finally, we have studied the microstructural changes occurring in the human corneal epithelium after application of contact lenses [14].

On the basis of these previous observations, the present exploratory study has been conducted on a limited number of animals with the aim to describe the way microvessels are formed and invade into the cornea after an alkali burn injury.

Materials and Methods

Six adult rabbits weighing 2-3 kg were used. All the rabbits were treated in agreements with the Helsinki Convention on the use of animals in medical research, approved by the Institutional Review Board [15].

Rabbits were anaesthetized by an intravenous injection of Nembutal (40 mg/kg weight) using Novesine as local anesthetic. A paper filter disc 3 mm in diameter with one drop of 0.5N sodium hydroxide solutions was placed in the center of the right cornea for 1 min to induce a reversible chemical injury of the cornea [16].

Corneal regeneration begins after few days from the injury, and continues for many days thereafter with development of fibrocellular connective tissue on the posterior surface of the injured cornea [4]. Three rabbits were sacrificed 3 weeks after the lesion took place, and the remaining three rabbits were killed 6 weeks after the lesion. Such time of sacrifice was selected in our experiments because by that time most of the healing process in an alkali wound cornea has taken place and initial conditions are almost restored. The normal (left) and injured (right) corneas were taken for further analyses.

Fluorescence microscopy

A small strip of ocular tissue 3 × 5 mm including conjunctiva, limbus and cornea was harvested in rabbits and posed on a glass. The little vessels of conjunctiva were injected with a solution 1% of

fluorescein isothiocyanate for the staining of microvessels. Thereafter, these injected samples were observed and photographed by means of a photomicroscope Zeiss PMQ II equipped with special objectives, filters and an apparatus for the observation of fluorescent samples.

The whole area of the injected capillaries was counted by quantitative analysis of images. Other details on this technique are reported in a previous publication [8].

Capillaroscopy

Ultra-high sensitive optical microangiography allows to visualize the ocular microcirculation and to quantify microvascular vessel density under normal and physio-pathological conditions both *in vivo* and *in vitro*. This system operating at a wavelength of 1.310 nm was used for *in vitro* imaging of microcirculation in rabbit corneas.

The whole rabbit cornea was harvested in normal control eyes (left eye: not injured) and in the alkali-burned eyes (right eye: injured). These samples were observed and counted by means of optical microangiography according to the techniques proposed by Qin and co-workers for the observation of the microcirculation of the human skin [17].

Electron microscopy

Small pieces of rabbit corneas were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 1 hour [18]. Dehydration and embedding were done according to routinary procedures. Ultra-thin cross-sections were cut with a Reichter ultra microtome and stained with uranyl acetate and lead citrate [19]. Sections through the whole thickness of the tissue (reaching from epithelium to endothelium) were examined by a Zeiss EM 109 microscope. Kodak TP 120 and Ilford Pan F films were used for photography.

Quantitative analysis of images

Quantitative analysis of staining intensity was performed by means of a Quantimet Analyzer (Leica™), provided with specific software including internal controls. Values coming from samples incubated without the dye were considered as "blank". The values reported in our experiments represent the staining intensity for each type of vessel and are expressed as Conventional Units (CU) ± standard error of the mean: further details on quantitative analysis of images and on definition of CU are reported in the Manual of the Quantimet Leica 2000 image analyzer [20].

Statistical analysis of data

The significativity of image quantitative analysis was based on basic statistical methods such as: mean values, maximum and minimum limits, variations, standard deviation (SD), standard error of mean (SEM) and probability index (p). The majority of these data were calculated, but non tabled (only mean values and SEM are tabled). All statistical results demonstrated a high significativity of the morphometrical data [21].

Results

Morphological results are shown in Figures 2, 3 and 4 whereas Table 1 and Figure 1 illustrate the quantitative analyses.

	Analysis at 3 weeks		Analysis at 6 weeks	
	Untreated eyes (n=3)	Treated eyes (n=3)	Untreated eyes (n=3)	Treated eyes (n=3)
Fluorescence	0	11.2 ± 1.3	0	13.4 ± 1.4
Capillaroscopy	0	8.1 ± 0.9	0	14.3 ± 1.5
n: Number of eyes examined				

Table 1: Quantitative data on fluorescent microscopy and capillaroscopy. Data are expressed as percentage of microvessels in 1 mm² of corneal area ± SEM.

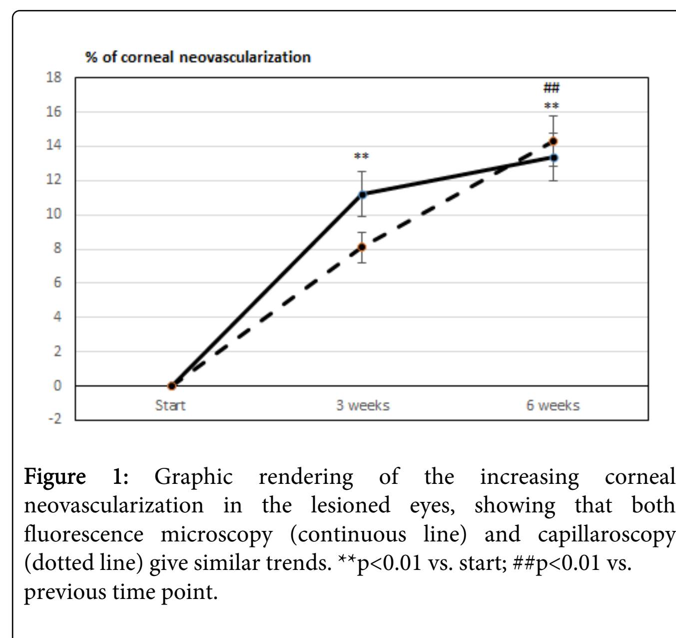

Figure 1: Graphic rendering of the increasing corneal neovascularization in the lesioned eyes, showing that both fluorescence microscopy (continuous line) and capillaroscopy (dotted line) give similar trends. **p<0.01 vs. start; ##p<0.01 vs. previous time point.

Figure 2 is an image of the cornea (visible at the top) limbus (visible at the center) and conjunctiva (visible at the bottom) 3 weeks after the central alkali-burn injury. Conjunctival vessels were injected with a 1% solution of fluorescein isothiocyanate. Magnification of the image is 25X. Corneal neovascularization can be clearly seen. In the right bottom corner of the image the fluorescent dye leaks from newly formed vessels.

Figure 3 is an image of corneal neovascularization 3 (A) and 6 weeks (B) after the central alkali-burn injury. The capillaroscopy was performed by means of the ultra-high sensitive optical microangiography. The magnification of this image is 100X. The control corneas (not injured) are not vascularized and appear completely normal, so the image is not shown. Injured corneas 3 weeks after chemical injury show presence of neovessel formation, with thin and numerous capillaries. Six weeks after the injury corneal neovascularization has increased and neo-formed capillaries appear thick and filled with erythrocytes.

Figure 2: Fluorescence microscopy of neo-formed microvessels at the border between cornea (top) and conjunctiva (bottom) after 3 weeks from a central alkali-burn injury. On the bottom right side the newly formed blood vessel is leaking fluorescein out from its wall. Magnification=25X.

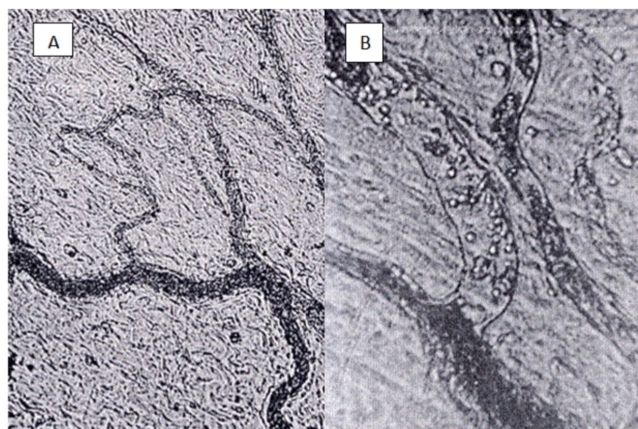

Figure 3: Capillaroscopy of neo-formed microvessels in the rabbit cornea after 3 weeks (A) and 6 weeks (B) from central alkali-burn injury. Neovessels are initially thin, while after 6 weeks they are dilated and flabby. Magnification=100X.

Figure 4 is a photograph taken by transmission electron microscopy of a small neo-formed micro-artery within the rabbit cornea 3 weeks after the alkali-burning. This vessel shows a wall formed by endothelial cells and two pericytes on the right side. Magnification of the image is 9800X. The whole diameter of the microvessel is <25 μm. Many red blood cells are present in the lumen of the microvessel.

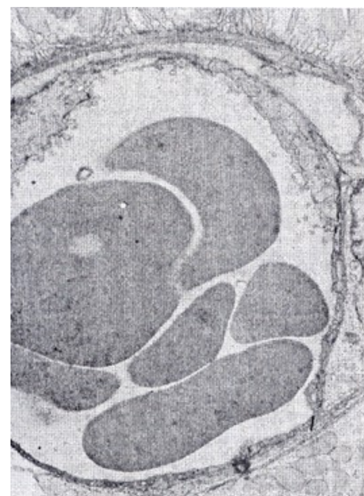

Figure 4: Microphotography obtained with transmission electron microscopy of a little neo-formed micro-artery within the rabbit cornea after 3 weeks from the alkali-burn. The vessel (diameter<25 μm) contains in the lumen many red cells. On the right side two pericytes can be observed. Magnification=9800X.

The quantitative analyses obtained using fluorescent microscopy or sensitive optical microangiographies (capillaroscopy) are reported in Table 1 and in graphic form in Figure 1. All quantitative values are expressed as percentage of the area occupied by the microvessels in 1 mm^2 of corneal surface ± SEM.

Control corneas (left eyes: not injured) show no microvessels (blank, set at quantitative value zero). On the contrary, corneas of the right eyes injured by a central alkali-burn show after 3 weeks a neovascularization that extends in 11.2% ± 1.3 (measured by fluorescein) and 8.1% ± 0.9 (measured by capillaroscopy) of the analyzed surface. Six weeks after the injury the values measured show a statistically significant increase to 13.4% ± 1.4 (measured by fluorescein) and 14.3% ± 1.5 (measured by capillaroscopy).

Discussion

The cornea is the clear, dome-shaped structure that covers the iris and is the first surface that light strikes on its way into the eye to the retina [22]. Normally, the cornea is very clear, with an even, smooth surface. It is clear because the layers of its stromal tissue are tightly aligned and because it is a relative dehydrated tissue [23]. The cells lining the back surface, the endothelium, are constantly pumping water out from the cornea into the anterior chamber filled by aqueous humor [24].

The cornea is an avascular tissue, meaning it has no blood vessels. Blood vessels growing in the corneal tissue would compromise its transparency, so the cornea must get its oxygen by other ways, through absorption from the tear film and the atmosphere [25,26]. Oxygen may also come up the edges of the cornea, through the tiny arteries of the conjunctiva: but once those vessels reach the area where the sclera becomes the clear cornea, they reverse direction and become veins [27].

When the cornea is deprived of oxygen, the endothelial pump loses efficiency and is unable to maintain the relatively dehydrated state, and therefore the cornea begins to swell. When the decrease in available oxygen becomes chronic, the cornea responds by allowing tiny new blood vessels to grow. This is corneal neovascularization [28,29].

An impressive number of specific agents has been found to induce neovascularization in animal corneas, but these observations cannot be automatically extended to human corneas, nor, indeed, to all animal species. Numerous agents have been found to be vasculogenic for the cornea in one or more animal species. The human cornea is much less susceptible to vascularization than the cornea of most laboratory animals [30].

The mechanism for vascularization of the cornea has been the subject of a wide discussion, without culminating in any one generally accepted hypothesis. Many suggestions have been so vague or lacking reliable support as to warrant no more than a simple listing. These include infection, injury, inflammation and changes in hydrogen ion concentration.

However, numerous factors are known that can induce neovascularization of the animal and/or human corneas, including corneal injuries and wound repair [31,32]. Robb and Kuwabara [1] performed studies on cellular components of corneal wound healing, while Kitano and Goldman described cytological and histochemical changes in corneal wound repair [2]. Varga and Feher described the wound healing after injuries on the center or in periphery of the cornea [3]. Francois and Feher studied corneal regeneration after burning [4]. Gipson and co-workers demonstrated that the corneal epithelium is capable of a full regeneration during wound repair in the rabbit [33].

Moreover, the morphology of corneal blood vessels has been well studied and described both in vivo and ex vivo, mainly in the last decade, by means of confocal microscopy [34-36] or other innovative techniques [37,38]. Our present results are in agreement with all these previous observations and add new evidence on the morphological development of corneal neovascularization.

Conclusions

The ultra-high sensitive optical microangiography is the best method for the studies on corneal neovascularization. This technique is capable of differentiating the microcirculation within the normal from that in the injured cornea. The optical images show blood vessel elongation and the dense network in the corneal lesion, the appearance of which is not observed within the normal cornea. Based on the results obtained from these experiments, the statistical analysis shows a higher blood vessel density present in the injured cornea with respect to the normal one. Therefore, it can be stated that optical microangiography is a valuable tool for imaging corneal microcirculation in a high speed, high sensitive and not invasive way. Therefore, such method may have a useful role in future clinical diagnosis and follow up of corneal neovascularization.

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