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Distribution of the regulatory peptide alarin in the eye of various species

Falk Schrödl [a,b,*,3], Andrea Trost [a,3], Clemens Strohmaier [a], Barbara Bogner [a], Christian Runge [a], Alexandra Kaser-Eichberger [a], Nicole Eberhard [c,1], Radmila Santic [c,2], Barbara Kofler [c], Herbert A. Reitsamer [a]

[a] Dept. of Ophthalmology, Paracelsus Medical University, SALK, Muellner Hauptstrasse 48, 5020 Salzburg, Austria
[b] Dept. of Anatomy, Paracelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria
[c] Laura-Bassi Centre of Expertise, THERAPEP, Dept. of Pediatrics, Paracelsus Medical University, SALK, Muellner Hauptstrasse 48, 5020 Salzburg, Austria

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ABSTRACT

Alarin is a recently discovered regulatory peptide with vasoconstrictive properties in murine skin. Control of vasoconstriction/-relaxation is essential for ocular blood flow and hence the eye's homeostasis, and regulatory peptides are involved in regulation of ocular blood flow. Here we describe the existence and distribution of alarin in the eye of human and potential experimental animals (rat, mouse). Eyes of rat, mouse, and human were prepared for immunohistochemistry against murine and human alarin, respectively. Additionally, double staining experiments for alarin and CD31 were performed in human choroidal flat-mount preparations. For documentation, confocal laser scanning microscopy was used while quantitative real-time-PCR was applied to confirm immunohistochemical data and to detect alarin mRNA expression in human retina and choroid. Alarin-like immunoreactivity (alarin-LI) was detected in corneal epi- and endothelium of human, mouse, and rat, as well as in the conjunctiva of mouse and rat. Alarin-LI was found in the iris of all the species investigated and, in humans, was concentrated around blood vessels. All three species showed distinctive alarin-LI in the non-pigmented epithelium of the ciliary body. In the retina of mouse and rat, maximum signals were detected in the outer nuclear and ganglion cell layer, whereas in humans a strong alarin-LI was found around retinal blood vessels and in intrinsic choroidal neurons (ICN). Quantitative RT-PCR in human confirmed alarin mRNA expression retina and choroid. The existence of alarin in cornea and conjunctiva might indicate a role in immune defense, while its presence in the non-pigmented ciliary epithelium favors an involvement in aqueous humor production. Alarin around blood vessels/in ICN might indicate an involvement in ocular blood flow regulation. Since alarin is found widely distributed in the eyes of species investigated, we were able to establish the basis for further functional experiments.

1. Introduction

To achieve proper homeostasis for optimal visual function, the eye is controlled by the autonomic nervous system (Neuhuber and Schrödl, 2011). This autonomic control is achieved via nerve fibers of sympathetic, parasympathetic as well as primary afferent origin, each using specific "modulators" to adequately set ocular blood flow or aqueous humor dynamics. These modulators include classical transmitters like catecholamines or acetylcholine for the sympathetic or parasympathetic pathways, respectively, however, during the last decades many other neuroactive substances have been introduced, such as the gas nitric oxide (Boeckxstaens et al., 1991), purinergic compounds (Burnstock, 2010), or regulatory peptides. Regulatory peptides represent a class of small protein-like molecules involved not only in neurosignalling or co-transmission of the classical neurotransmitters (Troger et al., 2007), but also in inflammatory processes and pain sensation (Raddant and Russo, 2011), immune defense (Souza-Moreira et al., 2011), osmoregulation (Kozniewska and Romaniuk, 2008), and behavior (Nixon et al., 2012) or memory function (Crawley, 2010).

In the eye, however, the interaction of the various neurotransmitters and regulatory peptides in ocular regulation is poorly understood.

Alarin is a recently discovered small peptide of 25 amino acids (Santic et al., 2006) and belongs to the galanin family of peptides

* Corresponding author. Dept. of Ophthalmology, SALK/PMU, Muellner Hauptstrasse 48, 5020 Salzburg, Austria. Tel.: +43 662 4482 57498; fax: +43 662 4482 3744.
E-mail address: falk.schroedl@pmu.ac.at (F. Schrödl).
[1] Present address: LKH Leoben, Vordernberger Straße 42, 8700 Leoben, Austria.
[2] Present address: AFFiRiS AG, Vienna Biocenter, 1030 Vienna, Austria.
[3] Authors contributed equally to this work.

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(Lang et al., 2007). It was described in human neuroblastic tumor as a splice variant lacking exon3 of the galanin-like peptide (GALP) mRNA (Santic et al., 2006), thereby also missing the galanin-receptor binding domain and thus possibly using different receptors for its action. Splice variants exist for other regulatory peptides, e.g. CGRP (Rosenfeld et al., 1983), or neuropeptide Y (Melas et al., 2012), which leads to a diversification of proteins resulting from a single gene and hence to a reduction of the underlying genetic code (Matlin et al., 2005).

Alarin is detectable in neurons of various CNS regions (Eberhard et al., 2012) as well as in epithelial cells and around blood vessels (Eberhard et al., 2012; Santic et al., 2007), but is also found in neuroblastic tumors (Santic et al., 2006). In animal experiments in mice and rats it shows endocrine functions stimulating appetite and reproductive hormone secretion (Boughton et al., 2010; Fraley et al., 2012; Van Der Kolk et al., 2010). Furthermore, alarin inhibits neurogenic inflammation by vasoconstriction in the murine skin (Santic et al., 2007).

Since the regulation of the vessel diameter is a key player in ocular blood flow, and therefore ocular homeostasis, alarin is of potential interest for experimental ophthalmology. Here, we investigate the existence of alarin in eyes of rat, mouse and human by morphological and molecular biological methods.

2. Methods

2.1. Tissue preparation

Eyes of rat (Brown Norway; $n = 4$), mouse (C57Bl/6; $n = 3$), and human ($n = 5$; of both sex; 47–84 years of age; post mortem time 5–12 h). Study with human tissue was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee being no clinical drug trial or epidemiological investigation. Furthermore, the study did not extend to examination of individual case records. The anonymity of the patients has been ensured. Human tissue samples were obtained from the cornea bank of the University Eye Clinic, Salzburg, Austria, or body donors of the Department of Anatomy I, University Erlangen-Nuremberg, Germany and were prepared for cryosections and subsequent immunohistochemistry.

Eyes were dissected free, opened along the ora serrata and fixed by immersion in phosphate buffered saline (PBS) containing 4% PFA (2 h at room temperature, RT). They were rinsed in PBS (24–48 h) and transferred into PBS containing 15% sucrose (24 h at 4 °C). Eyes were embedded in tissue embedding medium (NEG50, Fisher Scientific, Vienna, Austria) and frozen at −80 °C by using liquid nitrogen-cooled methylbutane and stored at −20 °C for further processing.

2.2. Immunohistochemistry

Eye cups were mounted in a cryostat (HM 550, Microm, Walldorf, Germany) and serial sections of 16–20 μm were collected on adhesion slides (Superfrost Plus; Thermo Scientific, Wien Austria) and air-dried for 1hr at RT. After a 5 min rinse in tris-buffered saline (TBS; Roth, Karlsruhe, Germany) slides were incubated for 1 h at RT in TBS containing 10% donkey serum (Sigma–Aldrich, Wien, Austria), 1% bovine serum albumin (BSA; Sigma–Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5 min rinse, slides were incubated with rabbit antisera (1:100 in TBS, containing 1% BSA and 0.5% Triton X-100, 12 h at RT) generated against human and murine alarin and in double staining experiments in human choroid additionally with mouse antibodies directed against human CD31 (1:50, Acris, Heidelberg, Germany). After a rinse in TBS (four times 5 min) binding sites of primary antibodies were visualized by Cy3- or Cy2-tagged antisera (1:1000; Invitrogen, Karlsruhe, Germany) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 h at RT) followed by another rinse in TBS (four times 5 min). Slides were embedded in TBS-glycerol (1:1 at pH 8.6).

Murine and human specimens were stained by using affinity-purified anti-alarin antisera. Because the homology between the human and the murine peptide is only 60%, two antisera, one directed against the murine alarin peptide (6–24) and one directed against the human homolog, were used. Because of the high homology of rat and murine alarin the anti-murine alarin antibody can also be used in rat tissues (Santic et al., 2007). For the generation of the antisera, synthetic alarin peptides from amino acids 6–24 were used to avoid cross-reactivity to the first 5 aa of the peptide that are identical with the first 5 aa of galanin-like peptide (GALP). The reliability of the antibodies used as well as the affinity purification have been described previously (Maguire and Davenport, 2005; Santic et al., 2006, 2007). Negative controls were performed by omission of the primary antibodies during incubation and resulted in no staining. For documentation of negative controls, all laser parameters were identical with those of the positive controls. For better visibility, controls were slightly adapted in brightness and contrast whenever necessary.

2.3. Documentation

In order to document single and double label immunohistochemistry, a confocal laser scanning unit (Axio ObserverZ1 attached to LSM710, Zeiss, Göttingen, Germany; ×20 dry or ×40 and ×60 oil immersion objektive lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss) was used. Sections were imaged using the appropriate filter settings for Cy3 (568 nm excitation, channel 1, coded red) and Cy2 (488 nm excitation, channel 2, coded green; used for detection of CD31 or background illumination). Colocalization of same structures in channel 1 and channel 2 resulted in yellow mixed color. All images presented here (Figs. 2–5) represent confoal images in single optical section mode.

2.4. Quantitative real-time-PCR (qPCR)

To confirm histological results in human tissue and to detect alarin mRNA expression in human choroid and retina, relative qPCR was performed on six additional donor eyes (cornea bank Salzburg; $n = 6$; of both sex; 54–78 years of age, post mortem time 10–22 h). After corneal preparation the retina and choroid were dissected immediately from the remaining eye cup, homogenized with the TissueRuptor (Qiagen, Hilden, Germany) and mRNA was isolated with the Rneasy® Mini Kit (Qiagen) according to the manufacture's instructions. Two μg of RNA were digested with the Dnase I Kit (Sigma–Aldrich, Vienna, Austria), three parts of the digested RNA were used for cDNA synthesis (iscript™cDNA synthesis Kit, BioRad,

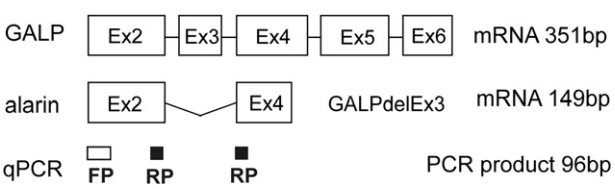

Fig. 1. Schematic overview of the coding sequence of alarin, representing a splice variant of galanin-like peptide (GALP), lacking exon3 and terminating at exon4. The primer for the qPCR was designed to cover the exon2–exon4 junction, specificly amplifying an 96 bp alarin PCR product (open box = forward primer, FP; black box = reverse primer, RP).