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Badania in vivo nad neowaskularyzacją naczyniówkową – model zwierzęcy

In vivo research of choroidal neovascularization – an animal model

Streszczenie

Wstęp. Neowaskularyzacja naczyniówkowa (choroidal neovascularization – CNV) to rozrost małych naczyń pochodzących z naczyń włosowatych naczyniówki, które poprzez błonę Brucha przedostają się do przestrzeni pod nabłonkiem barwnikowym siatkówki, jak i w obręb komórek nabłonka barwnikowego siatkówki i fotoreceptorów. Najczęściej spotykanym w praktyce klinicznej jest zwyrodnienie plamki związane z wiekiem (age-related macular degeneration – AMD). AMD jest najczęstszą przyczyną utraty widzenia w wieku powyżej 50 roku życia w krajach wysokorozwiniętych.

Cel. Ocena przydatności indukowanego laserem mysiego modelu neowaskularyzacji naczyniówkowej do badań nad neowaskularyzacją naczyniówkową.

Materiał i metody. Badania przeprowadzono na myszach linii C57BL6. Myszy poddawano fotokoagulacji laserowej, a następnie dwa tygodnie po niej wykonywano angiografię fluoresceinową.

Wyniki. Dwa tygodnie po laserowej fotokoagulacji w przebiegu angiografii fluoresceinowej myszy obserwowano w angiogramach wczesnych (1-3 min po iniekcji fluoresceiny) i późnych (6-8 min po podaniu fluoresceiny) faz obecność przecieku fluoresceiny z ognisk fotokoagulacji laserowej, odpowiadającym obszarom neowaskularyzacji naczyniówkowej. W miejscach po fotokoagulacji laserowej śledzono formowanie dużych i rozlanych obszarów przecieku, wykazujących wzrost wielkości i intensywności w trakcie badania.

Wnioski. Indukowany laserem mysi model neowaskularyzacji naczyniówkowej, odzwierciedla zmiany w przebiegu neowaskularyzacji naczyniówkowej u człowieka. Otrzymany in vivo model zwierzęcy może posłużyć dalszym badaniom nad neowaskularyzacją naczyniówkową, która jest odpowiedzialna za znaczne uszkodzenie siatkówki w przebiegu zwyrodnienia plamki związanego z wiekiem.

Słowa kluczowe: neowaskularyzacja naczyniówkowa, model zwierzęcy, zwyrodnienie plamki związane z wiekiem.

Abstract

Introduction. Choroidal neovascularization (CNV) is a vascular hyperplasia of vessels, originating from choroid capillary vessels which, through the Bruch's membrane, get into the area under the retinal pigment epithelium as well as the retinal pigment epithelium and the retinal photoreceptors' region. CNV is known to be the main reason for a severe visual loss in patients with age-related macular degeneration (AMD). AMD is the leading cause of blindness in people over 50 years of age in the western world.

Aim. Evaluation of the applicability of a laser-induced mouse model of CNV to investigate the choroidal neovascularization.

Material and methods. The research was carried out on C57/BL6 mice. The mice were subjected to laser photocoagulation and two weeks later fluorescein angiography was performed.

Results. Two weeks after the laser photocoagulation of the area of pathological fluorescein, a leakage, resembling CNV formation, appeared, after fluorescein injection, in the angiograms of the early (1-3 min) and the late (6-8 min) phase. In the place of the laser spots, a process of formation of large and diffused areas of leakage was observed. These lesions presented an increase in size and intensity during angiography.

Conclusions. The laser-induced mouse model of CNV resembles human CNV lesions. The obtained animal model of CNV could be used in further research on CNV which is responsible for the severe visual loss and the damage of retina in age-related macular degeneration.

Key words: choroidal neovascularization, animal model, age-related macular degeneration.

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INTRODUCTION

Choroidal neovascularization (CNV) is a vascular hyperplasia of vessels, originating from choroid capillary vessels which, through the Bruch's membrane, get into the area under the retinal pigment epithelium as well as the retinal pigment epithelium and the retinal photoreceptors' region. Newly developed curled and leaky capillaries can cause formation of subretinal hemorrhage under retinal pigment epithelium or in the neurosensory retina. In its natural course, CNV results in fibrotic scarring [1-5].

Age-related macular degeneration

CNV is known to be the main reason for a severe visual loss in patients with age-related macular degeneration (AMD). Age-related macular degeneration (AMD) is the leading cause of severe loss of vision in the elderly population over 50 years of age in the developed world [6]. The rise of CNV in AMD may result in a sudden loss of central vision.

According to Vindingt's report, the percentage of AMD affected patients increases substantially with age, from 2.3% in the age group of 60 to 64, up to 27.3% in the age group of 75 to 80 years of age [7]. There are two forms of AMD: the dry one and the wet one. The dry form is more frequent. It reveals drusen, pigment displacement or atrophy of pigment epithelium. About 10-20% of patients with AMD display symptoms of the wet type. Although the majority of the patients with AMD have a non-neovascular disease, it is the development of choroidal neovascularization (CNV), resulting in neovascular AMD with subretinal hemorrhage, retinal pigment epithelial cells detachment or cystic macular oedema, that often leads to severe visual loss. AMD – degenerative disease affecting macula is considered a great social problem [8].

Concept of CNV formation

Under normal conditions, endothelial cells are resistant to neovascular stimulated factors (angiogenesis). This is the result of keeping off the balance between pro-angiogenic and anti-angiogenic factors. The formation of CNV is unclear and can be a result of an unbalanced increase in pro-angiogenic activity or an unbalanced decrease in anti-angiogenic activity. The process of neovascularization is a complex interplay between numerous stimulators and inhibitors [5,9].

The processes as hypoxia, ischemia or inflammation can be an initial stimulus leading to the up-regulation of growth factors. The current view suggests that, in the development of CNV, a great role could be ascribed to a local inflammation [5,9].

At the moment the pathomechanisms of the formation of choroidal neovascularization are not well understood.

A laser-induced model of choroidal neovascularization

Previous attempts to generate an animal model of choroidal neovascularization were frustrating. To date, there is no generally accepted experimental in vivo model of choroidal neovascularization that fully recapitulates all the changes of AMD. Currently, the most established model of AMD uses laser-energy to rupture Bruch´s membrane and initiate choroidal neovascularization. [10-15]. However, the induction of choroidal neovascularization in this model is accompanied by an unspecific local inflammatory reaction.

AIM

Evaluation of applicability of a laser-induced mouse model of choroidal neovascularization to investigate the choroidal neovascularization.

MATERIAL AND METHODS

All the animal tests were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, the Animal Care and Use Committee, and the protocols were approved by the "Regierungspräsidium" North Rhine Westphalia. This study was conducted in the Heinrich-Heine University in Düsseldorf, Germany (Labor für Experimentelle Ophthalmologie; Universität in Düsseldorf; 40225 Düsseldorf; Moorenstrasse 5; Deutschland).

Animals

Female C57/BL6 (wild type, WT) mice (n = 10, 20 eyes) between 6 and 8 weeks were used. For all procedures anesthesia was achieved by intraperitoneal injection of ketamine (10%; Ratiopharm, Germany) xylazine (2%; Bayer, Germany) and the pupils were dilated with phenylephrine HCl (0.25%) and tropicamide (0.05%).

Laser photocoagulation

Choroidal neovascularization was generated by laser-induced rupture of the Bruch's membrane in the anesthetized mice. There were 5 argon laser spots made with a cover-slip used as a contact lens (120 mW intensity, 100 ms duration, 50 μm size; Argon Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany) [11].

Fluorescein angiography

Fluorescein angiography was performed two weeks after the laser photocoagulation, using a digital imaging system (Heidelberg Retina Angiograph II, Heidelberg Engineering, Heidelberg, Germany). A 30-D lens was attached to the objective of the camera to adapt the system for the mouse's eye. Angiography was performed in the anesthetized mice after intraperitoneal injection of 0.1 ml of 2.5% fluorescein sodium (Alcon, CITY, Germany). The early (1-3 min after injection) and the late phase (6-8 min after injection) images were analyzed.

The laser-induced lesions were graded according to the increase in fluorescein leakage between the early and the late phase and divided into four groups (Table 1). The areas of hyperfluorescence were measured with ImageJ (NIH, USA).

RESULTS

Two weeks after the laser photocoagulation, the areas of pathological fluorescein leakage, resembling CNV formation, were showed, after fluorescein injection, in angiograms of the early (1-3 min) and the late (6-8 min) phase. A formation of large and diffused areas of leakage was observed in the place of the laser spots. These lesions presented an increase in size and intensity during angiography (Fig. 2, Table 1).

Most of the evaluated animals (65%) had (+++) and (++++) grade lesions (leakage, severe or very severe dam-

age), and 35% of the mice presented (+) or (++) grade burns, corresponding to small or moderate damage (Fig. 1, 2).

TABLE 1. Semkova's classification.

(+) No leakage	Slight hyperfluorescence, without leakage or an increase in size and intensity. Weak damage.
(++) Leakage	Hyperfluorescence that increases in intensity, but not in size. Moderate damage.
(+++) Leakage	Hyperfluorescence that increases in intensity and in size. Severe damage.
(++++) Leakage	Confluent and increasing hyperfluorescence. Very severe damage; the retinas are almost completely occupied by the leakage; it is sometimes difficult to distinguish the different burns

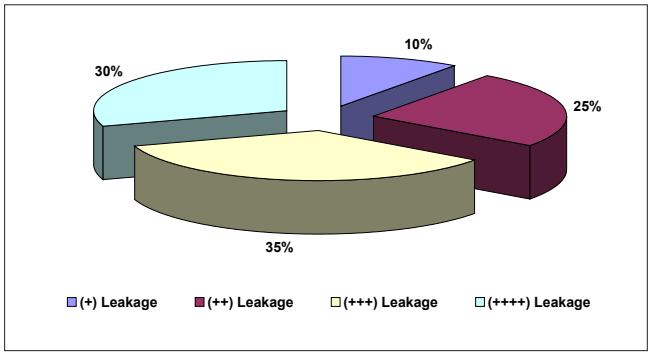

FIGURE 1. The arrangement of the intensity of fluorescence during fluorescein angiography in the C57/BL6 mice (Semkova's classification – Table 1).

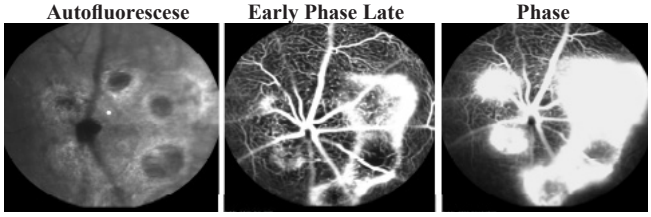

FIGURE 2. Pathological leakage, resembling CNV formation, two weeks after laser photocoagulation in the C57/BL6 mice. Representative angiograms from the early and the late phase, after fluorescein injection.

DISCUSSION

The discussion on CNV is still not resolved in the medical literature. It is known that the formation of pathological vessels plays a role in the development of many diseases. A precise interpretation of the pathogenetic mechanisms may be used in prevention and therapy. Therefore, it is fully understandable to trace all the processes responsible for the pathological vascularisation of the tissues. This experiment might help in the search for new data on pathogenesis of CNV.

The development of choroidal neovascularization can be linked to different diseases. Apart from choroidal neovascularization in AMD, the presence of subretinal neovascular membrane was observed in myopic patients, in post-traumatic eyes and during inflammatory diseases. Ruptures of the Bruch's membrane appear in all of these cases. For example, there is a major risk for formation of choroidal neovascularization in the myopic patients or the patients with pseudoxanthoma elasticum with thin retina. Similarly, local choroiditis (multi-focal choroidal inflammation or ocular histoplasmosis) with an increase of metalloproteinase expression can increase the probability of CNV development. It is correct to investigate Ryan's (1979) CNV laser-induced model of choroidal neovascularization [16].

In practice, the most frequently observed disease of CNV presents a wet form of AMD. It appears clear from the analysis of multicenter studies, that age-related macular degeneration is the main reason for visual impairment of individuals above 50 years of age, living in highly-developed countries [1,6,17-19]. In recent years, one may observe a growing number of individuals affected with AMD, which is connected with the aging process that affects societies and causes an incidence of various diseases (including AMD) of a pandemic scale. The evaluated incidence of AMD cases is 2-10% in people above 50 and over 13% in individuals over 85 [20]. It is estimated that the numbers will rise trebly in the next 25 years. Therefore, one may conclude that in the case of AMD we deal with a social disease.

AMD is a multi-factorial disease with a complicated pathomechanism. The pathophysiology of AMD is unclear and needs a more thorough investigation. Current studies are aiming at learning about the pathomechanism and the treatment of AMD. New methods of treatment, such as antiangiogenic therapy, allow to hope for an improvement of visual acuity of the AMD patients [21].

Although the laser-induced model of CNV may involve processes not relevant to AMD, it captures many of the important features of the human condition. Migration of choroidal vascular endothelial cells and newly formed vessels into the subretinal space through the defects in the Bruch's membrane, the accumulation of leukocytes adjacent to arborizing neovascular tufts and fibrovascular scarring, are the shared features of the experimental model and CNV secondary to AMD. In both cases, the newly formed CNV contains fenestrated endothelial cells and permeable inter-endothelial junctions [10,11].

Tobe et al. [14] described the laser-induced model of CNV, highlighting that CNV develops as fast as during one week and the lesions demonstrate no progress during one month. Edelman et al. [22] observed an increase in the laser-induced CNV mice model between the 3rd and 10th day after laser photocoagulation, and the areas of CNV achieved their maximal size and vasculature on the 10th day and did not transform for 30 days after the laser treatment.

Interestingly, experts suggest that infiltrating macrophages and a high expression of c cytokines, such as a tumor necrosis factor α (TNFα), seem to play a critical role in the development of laser-induced CNV [12,15,23-25]. Studies show a significant role of the TNFα (the major cytokine responsible for the macrophage-derived angiogenic activity) and the two receptors of TNFα: TNFRp55 and TNFRp75, in CNV development [15].

The fluorescein angiography is an important diagnostic test of CNV. The angiographs are characteristic and repeatable. A newly formed CNV typically demonstrates a fluorescein leakage during angiography in patients with AMD. Areas of CNV (hyperfluorescence) increase in size and intensity with time. By the use of fluorescein angiography, the patients with the wet form of AMD were described as

having classic neovascularization (bright, uniform hyperfluorescence in the early phase, intensifying with time) and a hidden one in the form of an exfoliation of the choroidal epithelium of retina (spotted, irregular hyperfluorescence from the early phases) or in the form of a late infiltration from an unspecified source (fluorescence at the level of choroidal epithelium of retina in late phases). In the animal model, the leakage of fluorescein from these immature, new vessels into the subretinal space appears angiographically similar to the early and late phases of leakage of classic CNV in AMD [10,11].

In this study, we analyzed, with the use of fluorescein angiography, lesions induced with an argon laser and a secondary vasculature tissue, two weeks after laser photocoagulation, in the peak of CNV expansion in a mouse [14,22]. Basing on a pattern of fluorescein angiography done in humans, there were two phases of angiography elaborated on mice: the early phase (1-3 min after fluorescein application) and the late phase (6-8 min after fluorescein application) [10]. The introduced time ranges are different from the consecutive stages of angiography done in humans, with regard to the shorter route to the mouse's eyeball to be covered by fluorescein [11,12,15]. An acute breakage of the retina-blood barrier, with large regions of fluorescein infiltration, were observed in the region of neovascularization foci in mice in the process of fluorescein angiography. The regions of early and late hyperfluorescence in mice are the visible reactions, in the form of neovascularization, to the used argon laser energy.

In the light of the results obtained in this study, one may say that the laser-induced mouse model of choroidal neovascularization is a perfect model of the changes arising in the tissues due to the development of choroidal neovascularization in humans. The obtained results point to new possibilities in CNV pathogenesis as well as an evaluation of new therapeutic options, crucial for the treatment of the problems resulting in choroidal vascularization.

CONCLUSION

The laser-induced mouse model of CNV resembles human CNV lesions. The animal model of CNV could be used to investigate CNV, which is responsible for severe visual loss and damage of retina in age-related macular degeneration.

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REFERENCES

1. Bressler NM. Age-related macular degeneration is the leading cause of blindness. JAMA 2004; 291(15):19000-1.
2. Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of VEGFR-1. J Biol Chem 2006; 281(6):3604–13.
3. Fauser S, Engelmann K, Krohne TU, Kirchcof B, Joussen AM. Pathogenese der choroidelen Neovascularisation; Opthalmologe 2003; 100(4):300-5.
4. Nowak JZ, Wiktorowska-Owczarek A. Neowaskularyzacja w tkankach oka: mechanizmy i rola czynników pro- i antyangiogennych. Klin Oczna 2004; 106(1-2):90–7.
5. Nowak JZ. Age-related macular degeneration (AMD): pathogenesis and therapy. Pharmacol Rep 2006; 58(3):353–63.
6. Bressler NM, Frost LA, Bressler SB, Murphy RP, Fine SL. Natural course of poorly defined choroidal neovascularization associated with macular degeneration. Arch Ophthalmol 1988; 106(11):1537-42.
7. Vindingt T. Macula – the eye in the eye. Acta Ophthalmol (Suppl.) 1995; 217:2-26.
8. Miller DW, Joussen AM, Holz FG. Die molekularen Mechanismen der neovaskulären AMD. Ophthalmologe 2003; 100(2):92–6.
9. Campochiaro PA. Ocular neovascularization and excessive vascular permeability. Expet Opin Biol Ther 2004; 4(9):1395–1402.
10. Semkova I, Fauser S, Lappas A, Smyth N, Kociok N, Kirchhof B, Paulsson M, Poulaki V, Joussen AM. Overexpression of FasL in retinal pigment epithelial cells reduces choroidal neovascularization. FASEB J 2006; 20(10):1689-91.
11. Semkova I, Peters S, Welsandt G, Janicki H, Jordan J, Schraermeyer U. Investigation of laser-induced choroidal neovascularization in the rat. Invest Ophthalmol Vis Sci 2003; 44(12):5349-54.
12. Shi X, Semkova I, Müther PS, Dell S, Kociok N, Joussen AM. Inhibition of TNF alpha reduces laser-induced choroidal neovascularization. Exp Eye Res 2006; 83(6):1325-34.
13. Rink L, Kirchner H. Recent progress in the tumor necrosis factor-alpha field. Int Arch Allergy Immunol 1996; 111(3):199-209.
14. Tobe T, Ortega S, Luna JD, Ozaki H, Okamoto N, Derevjanik NL, Vinores SA, Basilico C, Campochiaro PA. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am J Pathol 1998; 153(5):1641-46.
15. Jasielska M, Semkova I, Shi X, Schmidt K, Karagiannis D, Kokkinou D, Mackiewicz J, Kociok N, Joussen AM. Differential role of tumor necrosis factor (TNF)-α receptors in the development of choroidal neovascularization. Invest Ophthalmol Vis Sci 2010; 51(8):3874-83.
16. Ryan SJ. The development of an experimental model of subretinal neovascularization in disciform macular degeneration. Trans Am Ophthalmol Soc 1979;77:707–45.
17. Augood C, Fletcher A, Bentham G. Chakravarthy U, de Jong PT, Rahu M, Seland J, Soubrane G, Tomazzoli L, Topouzis F, Viogue J, Young I. Methods for population-based study of the prevalence of and risk factors for age-related maculopathy and macular degeneration in elderly European populations: the UREYE study. Ophthalmic Epidemiol 2004; 11(2):117-29.
18. Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P, Kempen J. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004; 122(4):564-72.
19. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP. Global data on visual impairment in the year 2002. Bull World Health Organ 2004; 82(11):844-51.
20. Ambati J, Anand A, Fernandez S, Sakurai E, Lynn BC, Kuziel WA, Rollins BJ, Ambati BK. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nat Med 2003; 9(11):1390-97.
21. Amin R, Puklin JE, Frank RN. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. Invest Ophthalmol Vis Sci 1994; 35(8):3178-88.
22. Edelman J.L, Castro M.R. Quantitative image analysis of laser-induced choroidal neovascularization in rat. Exp Eye Res 2000; 71(5):523–33.
23. Espinosa-Heidmann DG, Suner IJ, Hernandez EP, Monroy D, Csaky KG, Cousins SW. Macrophage depletion diminishes lesion size and severity in experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 2003; 44(8):3586-92.
24. Sakurai E, Anand A, Ambati BK, van Rooijen N, Ambati J. Macrophage depletion inhibits experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 2003; 44(8):3578-85.
25. Tsutsumi-Miyahara C, Sonoda KH, Eqashira K, Ishibashi M, Qiao H, Oshima T, Murata T, Miyazaki M, Charo I.F, Hamano S, Ishibashi T. The relative contributions of each subset of ocular infiltrated cells in experimental choroidal neovascularization. Br J Ophthalmol 2004; 88(9):1217-22.

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