Kinetics of integrin expression in the mouse model of proliferative retinopathy and success of secondary intervention with cyclic RGD peptides

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Abstract

Aims/hypothesis. Vitronectin-receptor-type integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) are thought to be involved in the selective ablation of tumorigenic and other pathologic angiogenesis. Specifically, it has been shown that ligation inhibition of the α_v -type integrins with cyclic penta-peptid peptide inhibits proliferative retinopathy by almost 80% in a hypoxia-induced mouse model.

Methods. On the basis of growth factor and integrin expression dynamics in this model, secondary intervention approaches with cyclic RGDfV peptide were investigated.

Results. α_v -integrin expression started immediately after induction of hypoxia (at postnatal day 12, p12) and persisted only during the initial period of neovascularization (until day p14). Vascular endothelial growth factor (VEGF) expression started at high values immediately after return of the mice into room air, and dropped rapidly to low values beyond day 13. In contrast, basic fibroblast growth factor (bFGF) was predominantly expressed during the

phase of maximum angiogenesis which was noted between day p17 and 19. Based on these findings, cyclic penta peptide was administered subcutaneously at varying doses (2–20 $\mu\text{g/kg/day}$) for 5 days beginning either at day p14 (early intervention) or at day p17 (late intervention). Early secondary intervention showed a dose-dependent reduction of new vessels with maximum inhibition of 57% (control 68.08 ± 3.21 nuclei/section compared with RGDfV-treated 29.35 ± 2.39 nuclei/section; $p < 0.0001$), whereas late secondary intervention had no effect.

Conclusion/hypothesis. These data indicate that angiogenesis-related α_v -integrin expression is VEGF-rather than bFGF-dependent, and the efficacy of cyclic penta-peptid (RGDfV)-treatment in proliferative retinopathy is only effective as long as the α_v -integrin target is prominently expressed. [Diabetologia (2002) 45: 262–267]

Keywords Endothelial proliferation, retinopathy, α_v -type integrins, microvessels, vascular endothelial growth factor, basic fibroblast growth factor.

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Abbreviations: ROP, Retinopathy of prematurity; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; RGDfV, cyclic penta-peptid

Angiogenesis during adulthood is limited to a few tissue sites such as the ovary and the uterus (during reproduction) and the skin (during wound healing) [1, 2]. However, despite the normal low turnover rate, adult endothelial cells are permanently able to form new blood vessels during pathologic angiogenesis which is involved in a variety of diseases such as tumor formation and metastasis, inflammatory skin and joint diseases [3], and in ocular diseases such as proliferative diabetic retinopathy [4]. These conditions require the transformation of the mature resting endothelial cell to an angiogenic endothelial pheno-

type. While physiologic angiogenesis is a self-limiting process, pathologic angiogenesis is a self-perpetuating process which requires active intervention. Angiogenesis from established quiescent vessels is a tightly regulated, complex sequence of events involving pericellular proteolysis, migration and proliferation of cells, final tube formation and recruitment of periendothelial cells [5]. Each step of this process offers therapeutic targets, but the complexity of the process and the multiplicity of factors involved at the induction level makes single target approaches difficult. This makes the inhibition of the downstream pathway of angiogenesis, which includes the adhesive interactions of angiogenic endothelial cells with components of the extracellular matrix, an alternative [6]. The approach is based on findings that endothelial cells of the angiogenic phenotype express specific adhesion receptors, predominantly of the vitronectin-type integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) [7], and that small peptide antagonists of this integrin system reduce by almost 80 % new vessels in the eyes of mice with experimental proliferative retinopathy [8]. Based on the unique linear integrin-binding motif RGD on several extracellular matrix ligands, stabilized low molecular weight cyclic structures containing a variety of different flanking regions have been constructed to serve as potential inhibitors of cell adhesion. Among these, the cyclic RGDfV-peptide was found to be particularly resistant against degradation, to show a more than 10000-fold higher affinity compared with natural integrin ligands as well as selectivity for α_v -integrins [9–13]. Due to these properties, relatively low doses of the peptide are therapeutically effective, while higher doses might not produce detectable side effects as shown earlier [8]. Despite its short-term nature, the mouse model mimics all essential steps of human proliferative retinopathy of various causes.

In the clinical setting, however, patients with proliferative ocular diseases such as diabetic retinopathy sometimes have pre-existing neovascularization, in particular when recommended screening intervals have not been considered. The extent to which active neovascularization in the retina is still susceptible to small peptide antagonists of the integrin system is still not clear. In order to obtain a rationale for secondary interventions, we studied the kinetics of angiogenesis in the retina by analysing the expression of α_v -integrins and VEGF, bFGF in the ROP mouse model. Based on these findings, we examined whether ligation inhibition of the integrin system by the cyclic RGDfV antagonist is still effective as an interventional approach. Our results indicate that the effect of secondary intervention is closely associated to the expression α_v -integrins and is not related to the extent of neovascularization.

Materials and methods

All experiments in this study were done according to the guidelines of the statement for animal experimentation issued by the Association for Research in Vision and Ophthalmology and were approved by the local board for animal care.

Animals. C57BL/6 J mice (Charles River, Sulzfeld, Germany) were used throughout the study.

Reagents. Cyclo-RGDfV peptide was obtained from Bachem Biochemica (Heidelberg, Germany). The polyclonal antibody against the mouse α_v -integrin subunit is described elsewhere [14] and was kindly provided by Dr. B. Imhof (University Hospital, Geneva, Switzerland). The antibody was raised against mouse $\alpha v\beta 3$ -integrin and also recognizes the $\alpha v\beta 5$ -integrin homologue.

Mouse model of hypoxia-induced retinal neovascularization (ROP model). Retinal fluorescein angiograms and quantitation of neovascular nuclei were done as described [15, 8]. Briefly, 7-day-old (p7) C57BL/6 J mice were exposed to 75 % oxygen for 5 days with their nursing mothers in an incubator (Stuart Scientific, Redhill, UK). At p12, the mice were returned to room air. Unexposed control mice were kept in room air under identical conditions including light cycle and temperature.

Time course of neovascularization in the ROP model. To determine the maximum response of neovascularization in the ROP model, the time course of neovascularization was done using 10 mice in each group to measure angiogenesis at days p12, p14, p17, p19, p22, p26, and p28 after the 5-day period of hyperoxia. We also used serial fluorescein angiographies of mice at days p11 (ie during the last day of hyperoxia exposure), p12, p13, and p 14 to confirm the onset of the neovascular process in comparison with previously published data.

Western blot analysis. To determine the temporal expression pattern of the α_v -type integrins in relation to two of its inducers, VEGF and bFGF, Western-blot analysis of retinal protein extracts of four retinæ from four different animals for each time point was carried out using the following antibodies: a polyclonal rabbit antibody directed against mouse $\alpha_v\beta_x$ -integrin (0.1 μ g/ml), a polyclonal rabbit antibody directed against the N-terminal domain of VEGF (0.1 μ g/ml; Santa Cruz, Ismaning, Germany), a polyclonal rabbit antibody directed against purified bovine brain bFGF with high specificity against mouse bFGF [16] (5 μ g/ml).

All procedures including incubation with secondary sheep anti-rabbit IgG are described elsewhere [17]. Control mice not exposed to high oxygen were killed on days 12, 13, 14, 15, 17, and 26 after birth, and retinal extracts of four retinæ from four different animals for each time point were exposed to the antibodies described above under identical conditions.

Systemic treatment with cyclic RGDfV peptide. Based on the data obtained from the time course of retinopathy in the ROP model (see Fig. 1), two treatments were designed: firstly, early secondary intervention of subcutaneous injections of either saline alone (vehicle) or cyclic RGDfV peptide (10, 20, 50, and 100 μ g) in 50 μ l. These were done two times a day for 5 days from day p14 to p19, ie during the period of the steepest increase of neovascularization. The other treatment was late secondary intervention initiated for 5 days from day p17 to p22, ie at the maximal neovascular response. Preliminary experiments revealed no differences in the outcome between 0.9 % saline and the RADfV control peptide.

Fig. 1. Time course of retinal neovascularization in the mouse ROP model. Following the 5 days hyperoxygenation period (days p7–p12), mice were returned to room air, and neovascular nuclei were consecutively measured in retinae at the indicated time points. Note that the maximal extent of angiogenesis was during day p17–p19

Statistics. All results were expressed as the means $\pm$ SEM of at least three independent determinations unless otherwise stated. Analysis of variance and the unpaired Student's *t* test was used for statistical analysis (GraphPad, Instat, San Diego, Calif., USA). A *p* value of less than 0.05 was considered to be statistically significant.

Results

Time course of retinal neovascularization. In order to determine the kinetics of new blood vessel formation in quantitative terms, retinal neovascular nuclei of mice, exposed for 5 days (p7–p12) to 75% oxygen were evaluated, beginning at the time of return to room air. Fluorescein angiograms revealed that the first neovascular tufts appeared at day p13 (not shown). The numbers of preretinal neovascular nuclei/section were 23 ± 2.1 at day p14, and peaked between day p17 and day p19 (p17: 77.2 ± 2.0 , p19: 81.5 ± 2.2 nuclei/section; *p* ns) with subsequent regression to 1.5 ± 0.1 at day p26. At day p28 retinal neovascularization had regressed completely in all animals examined (Fig. 1).

Time course of expression of α_v -integrins and growth factors. Retinal extracts from oxygen-exposed mice were analysed at different time points after re-exposure to normoxia for the presence of α_v -integrin, VEGF, and bFGF by Western blot analysis using specific antibodies. Figure 2 shows a strong expression of the integrins immediately upon return of the animals to room air. The integrin protein concentrations remained higher until day p14 after which they declined to baseline for the subsequent days (p15–p26). Con-

Fig. 2(A, B). Time course of synthesis of α_v -integrin, VEGF, and bFGF protein during retinal neovascularization. **A** Equal amounts of retinal extracts from four eyes at the time points indicated were subjected to Western blot analysis and developed with specific antibodies against α_v -type integrins, VEGF, and bFGF as described in the Materials and methods section. In parallel, analysis for beta actin was done. **B** Expression of VEGF, bFGF, and α_v -type-integrin in relation to beta actin in the normal developing mouse retina for comparison with (A). Retinal extracts from four eyes were pooled and subjected to blot analysis as described

sistent with ongoing physiologic angiogenesis in the developing retina, non-exposed normal mice had low but detectable concentrations of integrins discernible until day p17.

VEGF protein expression was strongest shortly after the mice had returned to room air (p12–p13), and VEGF decreased rapidly and reached baseline values at day p14, ie preceding the decrease of the α_v -type integrin (Fig. 2). Normal mice had very low concentrations of VEGF protein during days p12 to p17. At day p 26, VEGF expression was below the detection limit.

The strong protein expression level of bFGF was detectable between day p12 and p15. At day p17, during the maximum of neovascularization, bFGF expression was further increased (Fig. 2). At day p26, no bFGF was detected. In normal mouse eyes, bFGF was present between days p13 and p15.

Secondary interventions with cyclic RGDfV peptide. Following exposure to high oxygen, all retinae

Fig. 3(A, B). Therapeutic efficacy of secondary intervention with cyclic RGDFV peptide in the ROP model. **A** Dose-response of treatment with cyclic RGDFV peptide initiated at day p14 for 5 days (early intervention) compared with saline-treated mice. Quantitation of neovascular nuclei was done at day p19. **B** Dose-response of treatment with cyclic RGDFV peptide initiated at day p17 for 5 days (late intervention) compared with saline-treated animals. Morphometric analysis was done at day p22. All data are expressed as per cent of control $\pm$ SEM; * indicates statistical significance ($p < 0.05$) compared with sham-treated control; + indicates statistical significance vs $2 \cdot 20 \mu\text{g/day}$; # indicates statistical significance compared with $2 \cdot 50 \mu\text{g/day}$

showed a marked central avascular zone confirming considerable angiogenic stimulation due to vasoobliteration. Based on the time course of the angiogenic response in the ROP model, two secondary intervention treatments for a duration of 5 days each were designed. Early intervention with a dosage of $2 \cdot 2\text{--}20 \text{ mg/kg}$ body weight was initiated at day p14, when the steepest increase in retinal neovascularization had occurred. Despite the delayed treatment with cyclic RGDFV peptide, we found a linear dose-response relation of inhibition of retinal angiogenesis. While a threshold of inhibition was determined at a dose of $2 \cdot 20 \mu\text{g/day}$ (ie $2 \cdot 4 \text{ mg/kg}$) (mock-treated, 71.25 ± 2.08 nuclei/section vs RGDFV-treated, 56.40 ± 2.57 nuclei/section; $p < 0.0001$), maximum in-

hibition was observed with the highest dose ($2 \cdot 100 \mu\text{g/day}$) and was 56.9% (mock-treated, 68.08 ± 3.21 nuclei/section vs RGDFV-treated, 29.35 ± 2.39 nuclei/section; $p < 0.0001$) (Fig. 3A).

Late secondary intervention with the identical dosage and frequency of injections of the integrin antagonist was initiated at day p17, ie at the point of maximal neovascular response in the ROP model. As depicted in Figure 3B, there was no therapeutic effect of the peptide on the established neovascular process in the retina. Thus, beginning treatment of retinal proliferation at the time of its maximum is no longer effective.

Discussion

Our data show that retinal neovascularization in the ROP model begins 24 h after initiation of hypoxia as the angiogenic stimulus. Proliferative retinopathy degree peaks between day p17 and p19, and regresses completely around day p26. These data confirm previous assessments [13], but were needed because of the possible variability in the extent of retinal response.

According to our data, VEGF appears to be primarily involved in the initial proliferative response, while bFGF is upregulated during the maximum neovascular response. Interestingly, α_v -type integrin expression is strongly upregulated during the early phase of neovascularization and is completely absent during the period when new vessels become abundant in the retina. Consistent with the time course of integrin expression, a secondary intervention with cyclic RGDFV peptides is only effective early during the neovascularization period.

VEGF, a well-described hypoxia-induced growth factor [18, 19], is a major inducer of ocular neovascularization, mediating its effect by high-affinity VEGF receptors [20–22]. It has been shown in the mouse ROP model that VEGF mRNA expression in Müller and ganglion cells increased 12 h after hypoxia induction (ie the transition of the animals from the hyperoxic to the normoxic environment) approximately three-fold and persists until p17 when mRNA is still increased 1.5-fold above baseline expression, suggesting that VEGF could be important during the entire neovascularization process [23]. The reported time course of mRNA expression is inconsistent with our present data on VEGF protein expression probably because of the differences in sensitivities of the methods.

In contrast, the second growth factor, bFGF, studied in relation to ocular neovascularization in the ROP model is expressed throughout the entire angiogenesis process. bFGF is upregulated around the maximal neovascular response in the ROP model in the absence of VEGF, suggesting that bFGF is a pre-

dominant growth factor involved in the propagation of the neovascular response to ischaemia. Accordingly, despite the lack of a signal sequence, bFGF is increased in retinopathies with proliferative retinal diseases [24, 25]. The temporal but distinct expression and distribution of both growth factors in the ROP model contradicts a direct relation between bFGF and VEGF induction. Based on *in vitro* data it appears possible that bFGF can sensitize endothelial cells to VEGF by increasing the expression of the receptor tyrosine kinase flk-1 [26]. Preliminary data in the ROP model, however, indicate that flk-1 is downregulated in parallel with VEGF starting at day p14 (Hammes et al., unpublished observations).

Based on antibody blocking experiments, at least in the chick chorioallantoic membrane model or the rabbit cornea pocket assay, stimulation of angiogenesis by bFGF leads to the expression of $\alpha_v\beta_3$ -integrin, while the stimulation by VEGF induces the expression of $\alpha_v\beta_5$ -integrin [27]. Such a clear distinction between two different growth factor-related pathways of integrin induction were not observed in the ROP model because the expression of the entire pool of α_v -integrin protein appeared to correlate with VEGF rather than bFGF expression. The downregulation of the α_v -type integrins found after day p14 indicates the predominance of the VEGF-dependent pathway in the initiation of ischaemia induced proliferative retinopathy, whereas an α_v -integrin-independent but bFGF-dependent pathway appears to be operative during propagation of the process.

Our data are in accordance with other findings in a mouse tumor model. Previous studies showed that angiogenic endothelial cells are still sensitive to ligation inhibition when they have entered the cell cycle [6]. They administered cRGD peptide *i.v.* 24 h after the onset of angiogenesis and found an increasing number of apoptotic cells over the next 24 to 48 h as well as a substantial regression of tumours. These data implicate that a delay in the initiation of the treatment does not impair efficacy as long as integrins are expressed as is demonstrated in the present work. These data further support the notion that endothelial cell survival is dependent on adhesion through vitronectin-type integrins [28].

Overlapping functionality and compensation are two aspects of integrin function, recently brought to attention by work, which reported that mice with a null mutation of the α_v -integrin subunit die *in utero* from placental defects [29]. A total of 29% of these mice developed to term showing extensive angiogenesis, and their main defects were cerebral and intestinal haemorrhages, indicating organ-specificity of the α_v -integrin system and compensation by other integrins. The efficacy of ligation inhibition of the α_v -type integrin system in proliferative retinopathy can be reconciled with these data from knockout experiments because postnatal and pathological angiogene-

sis in specific organs such as the retina differ from developmental angiogenesis with regard to mechanisms of initiation and progression.

Taken together, our data indicate that ligation inhibition of the α_v -type integrin system as a secondary treatment is promising provided the respective targets are present in specific clinical settings. Although the application of these findings to treat human diseases might be problematic, it has been shown that α_v -type integrins are expressed in ongoing or even established neovascularization in the eye from patients with proliferative diabetic retinopathy [30], suggesting that the approach could be favourable because of the chronically present target integrin.

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