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Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan

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Abstract

The NG2 proteoglycan is expressed by nascent pericytes during the early stages of angiogenesis. To investigate the functional role of NG2 in neovascularization, we have compared pathological retinal and corneal angiogenesis in wild type and NG2 null mice. During ischemic retinal neovascularization, ectopic vessels protruding into the vitreous occur twice as frequently in wild type retinas as in NG2 null retinas. In the NG2 knock-out retina, proliferation of both pericytes and endothelial cells is significantly reduced, and the pericyte:endothelial cell ratio falls to 0.24 from the wild type value of 0.86. Similarly, bFGF-induced angiogenesis is reduced more than four-fold in the NG2 null cornea compared to that seen in the wild type retina. Significantly, NG2 antibody is effective in reducing angiogenesis in the wild type cornea, suggesting that the proteoglycan can be an effective target for anti-angiogenic therapy. These experiments therefore demonstrate both the functional importance of NG2 in pericyte development and the feasibility of using pericytes as anti-angiogenic targets.

Keywords

angiogenesis; cornea; endothelium; model; mural; neovascularization; NG2; pericyte; retina; targeting

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Abbreviations:

BrdU	bromodeoxyuridine
CD31	PECAM-1
CD105	endoglin
flk 1	VEGF receptor-2
NG2	nerve/glial antigen 2
PBS	phosphate-buffered saline
PDGF β-receptor	platelet-derived growth factor beta receptor

Introduction

Angiogenesis is an essential element of many pathological processes, including tumor growth and metastasis, psoriasis, acne rosacea, rheumatoid arthritis, proliferative diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration [1,2-4]. The development of anti-angiogenic therapies for treating these pathologies has therefore become an increasingly important goal of biomedical research. Most of these strategies have focused on targeting endothelial cells, which form the inner lining of the vascular tube and are by far the best understood component of neovasculature. However, the walls of typical angiogenic microvessels have a second cellular component: namely, pericytes (mural cells) which form an outer sheath around the endothelium [2,5,6]. Much less is known about these perivascular cells, as evidenced by the 115-fold difference in the number of publications on endothelial cells and pericytes, respectively (revealed by a recent search of the *PUBMED* database). The origin, function, and even reliable identification of pericytes have been elusive [5,7,8]. As a result, the benefits of using pericytes as an additional target for anti-angiogenic therapy are just beginning to be explored [9,10].

The effectiveness of using pericytes as anti-angiogenic targets would be expected to depend heavily on the importance of these cells in the development and function of microvessels: i.e. the more important their function, the greater the impact of targeting them. The functional importance of pericytes during angiogenesis is vividly illustrated by the phenotypes of mice in which pericyte development is disrupted. Ablation of PDGF-B or PDGF β -receptor, critical elements for the recruitment and development of pericytes, gives rise to mice that are pericyte-deficient. Depending on the timing and specificity of the ablations, microvessels in these animals, at the very least, have dramatically altered morphologies [11,12] and in some cases are subject to lethal microaneurysms [13]. Despite their importance, PDGF β -receptor and PDGF-B do not necessarily represent the only effective means of targeting pericytes. During the process of angiogenesis, extensive cross-talk occurs between pericytes and endothelial cells [2,14,15]. Accordingly, other cell surface and soluble components that mediate or modulate this cellular cross-talk are likely to be important candidates for targeting. One such pericyte component is the NG2 chondroitin sulfate proteoglycan, which is expressed on the surfaces of vascular mural cells during both normal and pathological angiogenesis [16-20].

The NG2 proteoglycan binds with high affinity to basic fibroblast growth factor (bFGF), platelet-derived growth factor AA (PDGF-AA), and the kringle domains of plasminogen and angiostatin [21,22]. In addition, NG2 appears to mediate signal transduction events that lead to increased cell spreading and motility [23-27]. This combination of properties, coupled with the high level of NG2 expression on nascent microvascular pericytes during developmental angiogenesis [19], has led us to investigate the functional role of the proteoglycan in neovascularization. Towards this end, we have utilized well-characterized retinal and corneal models to compare the details of pathological angiogenesis in wild type and NG2 null mice. We have previously demonstrated that NG2 expression is restricted to microvascular pericytes, rather than endothelial cells, in pathological ocular angiogenesis [18] and tumor angiogenesis [17]. The genetic ablation of NG2 can therefore be regarded as a specific “intrinsic” targeting of pericytes in pathological microvasculature. We have also used anti-NG2 antibodies for “extrinsic” targeting of pericyte-expressed NG2. Both types of studies demonstrate the functional importance of NG2 during pathological neovascularization, establishing the potential value of the proteoglycan as a pericyte-specific target for anti-angiogenic therapy.

Materials and methods

Experimental animals

NG2 null mice [28] were generated via a conventional homologous recombination approach [29,30]. The mice were back-crossed onto a C57Bl/6 genetic background for six generations, and NG2^{+/-} heterozygotes were mated to establish separate NG2 knockout (NG2^{-/-}) and wild type (NG2^{+/+}) colonies.

Animal models

All animal studies were performed in accordance with National Institutes of Health Office of Laboratory Animal Welfare (OLAW) guidelines, and were approved by the authors' institutional animal research committees.

Ischemia-induced retinal angiogenesis

Ischemic retinal angiogenesis was induced by withdrawal of neonatal mice from hyperoxia [31]. Litters of postnatal day 7 (P7) NG2 knockout and wild type mice were placed along with their nursing dams in an environmentally controlled chamber (75% oxygen-25% nitrogen atmosphere) for 5 days. At P12, the animals were returned to room air, and at P17 the mice were sacrificed and the eyes enucleated. In total, five mice of each genotype were utilized, allowing comparison of 10 wild type and 10 knockout eyes. The right and left eyes of each mouse were frozen in the same block and sectioned in a plane oriented sagittally to the optic nerve, so that each section represented complete slices of both eyeballs and retinas. Serial sections were cut through the entire thickness of the eyes, yielding between 85 and 132 sections per pair of eyes. Despite this range in the number of sections obtained, the variation was random, with no statistical difference between the number of sections derived from wild type or knockout eyes ($P=0.0952$, Mann-Whitney test). The total number of cryosections obtained from each group of mice was 1104.

Using systematic random sampling [32], we selected five sections per mouse to provide a representative sampling of both retinas. The sections were stained using the periodic acid-Schiff (PAS) method, with hematoxylin counter-staining as described [31]. This allowed the identification of the so-called neovascular tufts, or clusters of pathological angiogenic vessels protruding beyond the internal limiting membrane of the retina into the vitreous. Quantification of pathological angiogenesis was accomplished by counting the number of vascular cell nuclei in these tufts. We compiled the data according to the number of angiogenic nuclei per section (with each section representing a pair of eyes). We processed five sections per mouse, and therefore compared 25 wild type sections with 25 knockout sections.

In a separate experiment, mice received daily intraperitoneal injections of BrdU (80 μ g/g body weight) on postnatal days 14 through 18 after withdrawal from hyperoxia. This allowed subsequent identification of mitotic cells in the pathological vascular tufts present in the P18 retinas. Right and left eyes from each mouse (one wild type and one NG2 null mouse) were frozen in pairs into OCT blocks and sectioned. Two sets of right and left pairs were mounted on each slide. Systematic random sampling was then used to select five slides for each animal. Thus, each animal was represented by 20 sections (10 left eyes and 10 right eyes). Slides were immunostained for PDGF β -receptor and BrdU and counter-stained with hematoxylin. The percentages of mitotic pericytes and endothelial cells were determined after quantifying the number of BrdU-positive nuclei in each of the two immunohistochemically-defined cell types.

Dual hydron pellet corneal angiogenesis

The surgical procedure for inducing corneal angiogenesis in the mouse [33] was modified for this study to incorporate two pellets in the corneal pocket instead of just a single pellet. Slow-

release polyhydroxyethyl methacrylate (hydron) (Hydro Med Sciences, Cranbury, New Jersey) pellets ($0.4 \times 0.4 \times 0.2$ mm) were formulated to contain 45 μ g sucrose aluminum sulfate (sucralfate) (Sigma, St. Louis, Missouri) plus one of three experimental additives: 90 ng recombinant bFGF (Life Technologies, Carlsbad, California), 0.8 μ g affinity-purified rabbit anti-NG2 antibody [17-19], or PBS (control). Ten-week-old mice were anesthetized with Avertin (0.015-0.017 ml/g body weight), and under an operating microscope two pellets were surgically implanted into the corneal stroma of one eye at a distance of 0.7 mm from the corneal limbus. Ten NG2 wild-type mice received pairs of pellets containing bFGF and NG2 antibody. Another 10 NG2 wild type mice received pairs of pellets containing bFGF and PBS. Thirteen NG2 knockout mice received pairs of pellets containing bFGF and PBS. Over an 8 day-period after recovery from surgery, the mice were examined under a Leica GZ6 stereomicroscope (Leica, Allendale, New Jersey) to evaluate the progress of corneal angiogenesis in the operated eyes. On day 8, angiogenesis was quantified by determining the area of vascularization, as described previously [33,34].

Tissue processing, immunohistochemistry, and imaging

Tissues were fixed in 4% paraformaldehyde for 6 h, cryoprotected in 20% sucrose overnight, and frozen in OCT embedding compound (Miles, Inc., Elkhardt, Indiana). Cryostat sections (40 μ m) were air-dried onto Superfrost slides (Fisher Scientific, Pittsburgh, Pennsylvania). Immunohistochemical labeling was carried out as previously described [17-19]. Pericytes were identified by labeling with affinity-purified rabbit polyclonal antibodies against the NG2 proteoglycan or the PDGF β -receptor [13,17-19,35]. Both NG2 and PDGF β -receptor are regarded as specific markers for pericytes [36,37]. An affinity-purified rabbit antibody against the alpha subunit of hypoxia-inducible factor-1 (HIF1 α) was a generous gift from Dr. Robert Abraham (The Burnham Institute, La Jolla, California).

Since endothelial cells express different cell surface markers as a function of developmental age [38], we identified them using a cocktail of antibodies against endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) (Pharmingen, San Diego, California). This strategy has been previously utilized to maximize labeling of all vascular endothelial cells, both immature and mature [17,39].

Vascular cells in S (synthesis) phase of the cell cycle were identified by means of BrdU (Sigma, St. Louis, Missouri) incorporation and subsequent labeling with sheep anti-BrdU antibody (Fitzgerald Industries, Concord, Massachusetts) [40-42]. Briefly, frozen sections were digested with 0.005% pepsin (Sigma, St. Louis, Missouri) in 0.01 HCl for 30 min at 37 °C followed by treatment with 4 N HCl for 30 min at room temperature. Sections were then blocked by incubation in 5% goat serum in PBS for 30 min [43] prior to incubation with antibody. Fluorescence microscopic imaging of endothelial (CD31 + CD105 + flk1) [38,39], pericyte (NG2 or PDGF β -receptor) [13,18,19], and nuclear (BrdU) markers [43] was performed according to the published methods.

Statistical analysis

Prism 4.0 software (GraphPad, San Diego, California) was used for statistical analyses. Systematic random sampling of serial histological sections was carried out according to previously described methods [32].

Results

Ischemic angiogenesis is diminished in the NG2 null retina (intrinsic targeting)

The stereotyped laminar architecture of the retina makes it an ideal tissue for quantification of ischemic neovascularization[4,31]. In the hyperoxia model, the return from exposure to 75%

oxygen to a normal atmosphere represents relative hypoxia, resulting in the sprouting of new blood vessels from the primary vascular plexus at the inner face of the retina. Many of these new vessels protrude into the vitreous, where they are easily recognized as pathological angiogenic tufts composed of endothelial cells positive for endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) and pericytes positive for PDGF β -receptor. In the wild type mouse retina, the profusion of abnormal vascular protrusions beyond the inner limiting retinal membrane is readily apparent (outlined area in Figure 1b). By contrast, relatively few ectopic vessels are present in the NG2 null retina after parallel hypoxic induction (Figure 1a). The enlargement and morphological distortion of the hypoxic wild type retina are reproducible phenomena caused by edema due to leakage of fluid from the extensive pathological neovasculature. Edema is not apparent in the hypoxic NG2 null retina, presumably due to the relative scarcity of pathological vessels.

Figure 1c presents a quantitative comparison of ischemic neovascularization in the wild type and NG2 null retinas. Each of the data points represents the number of ectopic vascular nuclei (endothelial cells plus pericytes) counted in one of the 25 wild type and 25 knockout slides selected by systematic random sampling from the two sets of serial retinal sections. It is immediately apparent that wild type retinas have an increased tendency towards larger numbers of ectopic vascular nuclei. The averages for the entire data set are 119.8 nuclei per wild type section vs. 54.9 nuclei per NG2 null section (statistically significant by the Mann-Whitney test, $P = 0.0019$). Genetic ablation (intrinsic targeting) of NG2 therefore diminishes the angiogenic response of retinal vasculature to a hypoxic stimulus.

The results of a separate experiment designed to evaluate BrdU labeling suggest that cell proliferation offers at least a partial explanation for the observed difference between the responses of the wild type and NG2 null retinas to hypoxia. In this trial, pathological vascular tufts were again more numerous in wild type than in knockout retinas, confirming the results shown in Figure 1. In addition, BrdU-labeled nuclei were seen more frequently in wild type angiogenic sprouts than in NG2 null counterparts. Double-labeling of hematoxylin-stained sections for BrdU and the pericyte marker PDGF β -receptor allowed us to determine mitotic indices for pericytes in wild type and NG2 null angiogenic tufts. We have previously demonstrated the specific expression of PDGF β -receptor by pericytes in hypoxic retinal tufts [18]. This analysis revealed a large decrease in pericyte proliferation in the ischemic knockout retina. Figure 2a shows that 45.2% of pericytes in angiogenic tufts are labeled with BrdU in the ischemic wild type retina vs. 18.7 % of pericytes in the knockout retina (statistically significant, $P = 0.0068$ Mann-Whitney test). Counting BrdU-positive nuclei in PDGF β -receptor-negative cells also allowed us to compare mitotic indices for endothelial cells. Interestingly, 38.3% of endothelial cells are BrdU-positive in the wild type retina, vs. 22.8% in the NG2 null retina ($P = 0.0147$ Mann-Whitney test).

The validity of these results was confirmed in a separate set of experiments in which BrdU-positive nuclei were counted in conjunction with staining with the endothelial antibody cocktail to identify endothelial cells. The mitotic indices for both endothelial cells and pericytes were reduced in NG2 null retinas. Both types of labeling paradigms (endothelial cocktail and PDGF β -receptor) therefore demonstrate a reduction in proliferation of pericytes and endothelial cells in the NG2 null retina. Reduced proliferation of these cell populations is likely to be an important factor in the sub-normal angiogenic response of the NG2 null retina to hypoxia.

We have previously demonstrated in wild type mice that the extensive investment of ischemic retinal vessels by NG2-positive, PDGF β -receptor-positive pericytes is comparable to the high pericyte:endothelial cell (P/E) ratio normally seen in the central nervous system [18,19]. An additional important distinction between pathological vessels in the wild type and NG2 null retinas is the relative scarcity of pericytes relative to endothelial cells in the knockout

neovasculature. Determination of the respective numbers of pericyte and endothelial cell nuclei associated with the angiogenic tufts allows us to determine the pericyte to endothelial cell investment ratio in these clusters of vessels (Figure 2b). In wild type neovasculature the P/E investment ratio is 0.86 (i.e. close to one pericyte per endothelial cell), while in the knockout retina the P/E value falls to 0.24 (only one pericyte for every four endothelial cells) ($P = 0.0011$ Mann-Whitney test). The observation that pericyte proliferation is more adversely affected than endothelial cell proliferation in knockout retinas may partially account for this difference in P/E investment ratios.

It has been shown that an early step in the angiogenic response of the retinal vasculature to withdrawal from hyperoxia is up-regulation of the HIF-1 transcription factor. HIF-1 plays a critical role in the induction of VEGF expression and subsequent steps in the angiogenic process [44]. Immunostaining for the HIF-1 α subunit was used to evaluate this initial response of wild type and NG2 null retinas to hypoxia. Very low HIF-1 α levels were observed in control retinas from P13 wild type and knockout mice. In contrast, 16 h after removal of experimental P13 pups from 75% oxygen, HIF-1 α was up-regulated in similar fashion in the inner layers of both the wild type and NG2 null retinas (data not shown). Differences in pathological retinal neovascularization between the two genotypes therefore are not due to the initial response of retinal cells to hypoxia, but to subsequent neovascularization events downstream of HIF-1 α expression.

Corneal angiogenesis is reduced by both intrinsic and extrinsic targeting of NG2

Implantation of a bFGF-containing pellet along with a control PBS-containing pellet induces a robust angiogenic response in the wild type mouse cornea (Figure 3a). We have shown that these corneal microvessels are richly invested by NG2-positive, PDGF β -receptor-positive pericytes [18]. If the second pellet contains anti-NG2 antibody instead of PBS, the angiogenic response to bFGF is substantially reduced (Figure 3b). A diminished response to bFGF is also observed in the NG2 null cornea (Figure 3c). These qualitative observations were quantified by measuring the extent of the vascularized areas in each of the three experimental situations (Figure 3d). The mean area of corneal neovascularization was 0.3863 mm² in the control group of wild type mice ($n = 10$), compared with 0.087 mm² in the NG2 knockout animals ($n = 13$). In the presence of an NG2 antibody-containing pellet, corneal vascularization in wild type mice was reduced to 0.1445 mm² ($n = 10$). Both of these differences were statistically significant ($P = 0.0006$ for wild type mice vs. knockout mice, and 0.0039 for control wild type mice vs. antibody treated wild type mice). Thus both intrinsic (genetic ablation) and extrinsic (antibody blocking) targeting of NG2 result in diminished corneal angiogenesis.

Discussion

Since the cellular processes underlying neovascular sprout formation remain incompletely understood [45,46], increased attention to pericytes and their interaction with endothelial cells will be required not only to attain a better understanding of neovascularization in general, but also to realize the full potential of anti-angiogenic therapy. The critical contribution of pericytes during angiogenesis has been well established by observation of the pathological phenotypes of mice in which pericyte development is blocked [11,13,47]. The functional importance of pericytes has been attributed largely to their ability to stabilize and provide structural support to pre-existing endothelial tubes. They are thought to accomplish this by controlling endothelial cell proliferation and motility, and by contributing to the establishment of a permeability barrier and the regulation of blood flow [36,48-53]. However, it is now becoming clear that pericytes can play a much earlier role in microvascular development than previously realized. The use of NG2 and other markers for nascent pericytes has revealed the participation of these cells in the earliest stages of angiogenesis [6,36,16-20,53-56]. Pericytes may even be important for the

stimulation and guidance of nascent vascular tubes. Strategies for targeting pericytes may therefore be able to affect not only existing vessels, but also the formation of new vessels.

Our current studies show that intrinsic targeting of NG2 (by genetic ablation) leads to decreased ischemic angiogenesis in the mouse retina in response to hypoxia. The wild type mouse retina contains more than twice as many pathological vascular tufts as the retina of the NG2 null mouse. Since HIF-1 α induction is similar in wild type and knockout retinas, we know that the defect in the null mouse lies not in the initial response of retinal cells to hypoxia, but probably in later stages of vascular cell responsiveness to HIF-1-induced factors such as VEGF. This seems reasonable in light of the fact that NG2 is not expressed by cells of the retina *per se*, but instead by pericytes in the microvasculature [17-19].

A major factor in the sub-normal angiogenic response of the NG2 null retina appears to be reduced vascular cell proliferation. Only 41% as many mitotic pericytes are present in the ischemic vasculature of the NG2 null retina as in the wild type retina. These data represent the first direct *in vivo* evidence in support of a role for NG2 in cell proliferation. The ability of NG2 to sequester growth factors such as bFGF and PDGF-AA and possibly assist in presentation of these factors to their respective signaling receptors could represent one mechanism by which the proteoglycan promotes cell proliferation [21,28].

Interestingly, the absence of NG2 and the decreased number of mitotic pericytes is accompanied by a 1.7-fold decrease in the number of mitotic endothelial cells, suggestive of a stimulatory effect of pericytes on endothelial cell proliferation. This idea is somewhat at odds with previous reports that pericytes can inhibit endothelial cell proliferation in cell culture models [48] and that the absence of pericytes is accompanied by endothelial cell hyperplasia *in vivo* [11]. However, the pericyte/endothelial cell relationship is a complex, dynamic one that is likely to vary depending on the specific model under investigation. An excellent example of this is provided by a recent study of the proliferative retinopathy that results from endothelial cell-specific ablation of PDGF-B [47]. The general conclusion from this work was that reduction of pericyte density below 50% of normal, invariably led to the development of proliferative retinopathy. Nevertheless, localized instances were also encountered in the same investigation [47] in which increased pericyte density promoted the formation of chaotic, endothelial cell-rich vasculature, demonstrating that under certain conditions pericytes can have pro-angiogenic properties. The ability to use pericytes as effective anti-angiogenic targets also is suggestive of the pro-angiogenic nature of these cells [9,10].

Our current data support a pro-angiogenic role for pericytes in the formation of ischemic retinal microvessels. Our results with wild type mice show that endothelial cells are richly invested by NG2-positive, PDGF β -receptor-positive pericytes in this pathological vasculature (see also [18]). Coupled with our documentation of the early participation of NG2-positive pericytes during neovascularization [17], these observations suggest the possibility that pericyte-derived factors or NG2-dependent sequestration of growth factors might act to promote the proliferation of endothelial cells. Alternatively, the ability of NG2 to neutralize the growth-inhibitory effects of angiostatin [22,57] may promote endothelial cell proliferation in the wild type mouse, an effect that would be absent in the NG2 null mouse.

In addition to the quantitative reduction of ischemic angiogenesis in the NG2 knockout mouse, capillaries in the null mouse also have an altered cellular composition. The pericyte:endothelial cell investment ratio in ischemic vessels of the wild type retina is 0.86, or almost one pericyte per endothelial cell. This high investment ratio is characteristic of capillaries in the central nervous system in general, and the retina in particular, possibly contributing to the integrity of the blood-brain barrier and the high metabolic needs of neural tissues [5,36]. This investment ratio falls to 0.24 in the ischemic neovasculature of the NG2 null retina. The relative changes

in pericyte and endothelial cell proliferation in the NG2 knockout mouse would not appear to account for the magnitude of this decrease. Thus other factors that we have not yet investigated, such as decreased cell motility or increased apoptosis, may contribute to the large decrease in pericyte number relative to that of endothelial cells. While a specific role for NG2 in apoptosis has not been explored, there are numerous indications of NG2 involvement in cytoskeletal reorganization and cell motility [23-28]. In future work it will therefore be important for us to investigate the impact of NG2 on these processes in the context of neovascularization.

Intrinsic targeting of NG2 by genetic ablation leads to an even more pronounced decrease in bFGF-induced corneal angiogenesis. Neovasculature covers a 4.4-fold greater surface area in the wild type cornea than in the NG2 null cornea, once again supporting the idea that NG2 plays a role in pericyte development and/or function, and in the development of new vasculature. Interestingly, extrinsic targeting of NG2 through the use of a neutralizing antibody also produces a significant decrease in corneal angiogenesis (2.7-fold). While our data do not allow us to determine which aspects of pericyte function are blocked by the antibody, previous studies have shown that anti-NG2 antibodies are capable of blocking growth factor-induced cell proliferation [58] and both growth factor-induced and extracellular matrix-induced cell motility [27,59] in cell culture models.

Our demonstration of the functional importance of NG2 during pathological ocular angiogenesis logically raises the question of the proteoglycan's function during normal developmental neovascularization. How can we rationalize the observation that the NG2 knockout mouse possesses functional vasculature? More than one answer is possible. First, pathological angiogenesis may differ in some respects from normal angiogenesis. During pathological angiogenesis, the vasculature may be responding to combinations of signals that are not normally experienced during development or else are occurring out of their normal sequence (multiple factors released by tumor cells would be a good example). The role of NG2 may be magnified under these abnormal circumstances. Additional experiments with wild type and NG2 knockout mice are planned in order to examine NG2-dependent aspects of angiogenesis in other types of pathological models such as tumor progression and wound healing. Second, both of our pathological angiogenesis models have utilized postnatal animals, whereas the bulk of developmental neovascularization takes place during embryogenesis. It seems possible that embryonic development involves a higher degree of plasticity than events that occur postnatally. In other words, the ability to compensate for the loss of NG2 may be greater during embryogenesis. In response to postnatal challenges, such compensatory mechanisms may not be available, thus facilitating our ability to detect the contribution of NG2. Examination of normal angiogenic events that occur postnatally (for example in normal retinal development) may therefore reveal the effects of NG2 ablation. It has required detailed and careful experimentation to detect changes in pathological retinal and corneal angiogenesis in the NG2 null mouse. The same type of painstaking analysis may be required to detect subtle deficiencies in developmental neovascularization in the knockout mouse. Such studies remain to be undertaken, but in light of our current results would appear to offer great promise.

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Figure 1.

Ischemic retinal neovascularization. Sections of NG2 null (a) and wild type (b) retinas from the ischemia protocol were examined at P17 after PAS/hematoxylin staining. In the wild type retina there is a profusion of vascular tufts protruding past the internal limiting membrane into the vitreous (area enclosed by dashed line). These ectopic vessels are much less common in the NG2 null retina (arrow indicating neovasculation). The wild type retina is also characterized by swelling and distortion from edema caused by leakage from the extensive pathological vasculature. Bars in A and B = 50 μ m. (c) Retinal sections from wild type and NG2 knockout mice subjected to the ischemia protocol were analyzed for vascular cell nuclei associated with angiogenic tufts protruding into the vitreous. The number of ectopic vascular nuclei per section (5 mice $\times$ 5 sections per mouse = 25 sections) is shown for both genotypes to illustrate the distribution of the data. Wild type mice show a clear trend toward increased ischemic retinal angiogenesis. The average numbers of nuclei per section are shown by the solid bars through each data set (119.8 for wild types vs 54.9 for knockouts. $P = 0.0019$).

Figure 2.

Pericyte/endothelial cell mitotic indices and investment ratios. (a) By combining hematoxylin counter-staining with double staining for PDGF β -receptor and BrdU, we were able to calculate the mitotic index for pericytes and endothelial cells associated with vascular tufts. The number of BrdU-positive pericyte nuclei divided by the total number of hematoxylin-stained pericyte nuclei $\times 100$ = the percentage of mitotic pericytes. Thus, 45.2% of pericytes are BrdU-positive in the wild type retina (WT-PC) vs. only 18.7% in the knockout retina (KO-PC) ($P = 0.0068$). Total and BrdU-positive nuclei were also counted in PDGF β -receptor negative cells to provide endothelial cell data. In the wild type retina 38.3% of endothelial cells are BrdU-positive (WT-EC), compared to 22.8% in the NG2 null retina (KO-EC) ($P = 0.0147$). (b) The data used to construct (a) were also used to determine the pericyte/endothelial cell investment ratio in ischemic vascular tufts (total number of pericyte nuclei/total number of endothelial cell nuclei). For wild type retinas this ratio was almost one pericyte per endothelial cell (0.86), while in NG2 null retinas the ratio fell to 0.24 ($P = 0.0011$). The high ratio of pericytes to endothelial cells in the wild type retina is characteristic of microvasculature in the central nervous system.

Figure 3.

Corneal angiogenesis. Corneal angiogenesis was compared in wild type corneas implanted with a bFGF-containing pellet and a PBS-containing pellet (a), wild type corneas implanted with a bFGF-containing pellet and an NG2 antibody-containing pellet (b), and NG2 null corneas implanted with a bFGF-containing pellet and a PBS-containing pellet (c). Pellets are labeled with the letter P. Invasion of the cornea by new blood vessels in response to bFGF (arrows in a) is greatly reduced in the knockout mouse and by the presence of antibody against NG2 in the wild type mouse. (d) The angiogenic responses shown in (a-c) were quantified by measurement of the area (in mm²) occupied by new vasculature. Compared to bFGF-induced vascularization of the wild type mouse cornea, vascularization of the NG2 null cornea is reduced by a factor of 4.4 ($P = 0.0006$). Vascularization of the wild type cornea is reduced by a factor of 2.7 by inclusion of anti-NG2 antibody to inhibit invading pericytes ($P = 0.0039$).