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TARGETING NEOVASCULAR PERICYTES IN NEUROFIBROMATOSIS TYPE 1

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Abstract

Apart from tumor-driven neovascularization, a less-appreciated consequence of neurofibromatosis type 1 (NF1) is the hyperproliferation of vascular mural cells (pericytes). This study aims at establishing a role for pericytes in NF1, and determining whether interference with the function of a key pericyte component (NG2 proteoglycan) inhibits NF1 tumor neovascularization. Neovascularization in NF1 was studied in *Nf1*^{+/+} (control), *Nf1*^{+/-}, and *Nf1*^{-/-} embryos at E-10, ischemia-induced retinal angiogenesis model in 24 eyes in *Nf1*^{+/-}, *Nf1*^{+/+} mice, and in malignant peripheral nerve sheath tumors derived from NF1 patients (ST88-14, NMS-2PC) orthotopically-grown in nude mice (Crl: nu/nu). The anti-angiogenic effect of intracorneal polymer pellets containing anti-NG2 neutralizing antibody was quantified in the nude-mouse corneal angiogenesis model in which angiogenesis was induced by xenografting NMS-2PC tumor into the corneal stroma of 22 eyes. By using confocal microscopy, immunohistochemistry, and BrdU proliferation assay, the pericyte/endothelium ratios and proliferation rates were measured. Activated pericytes were present at the leading tip of the angiogenic sprouts. Pericytes showed continuous investment of endothelium in both NMS-2PC and ST88-14 MPNST tumor xenografts. Mean corneal angiogenesis induced by NMS-2PC tumor grafts in NG2-antibody treated eyes was 1.491mm², and 3.186 mm² in isotype-matched non-immune globulin treated eyes (control) (p=0.0002). Ischemia-induced retinal angiogenesis was 193.8 vascular nuclei in angiogenic retinal tufts in *Nf1*^{+/-} mice compared to 89.23 in *Nf1*^{+/+} mice (control) (p<0.0001). Mean pericyte/endothelium investment ratios were 1.015, 1.380, and 2.084 in control, *Nf1*^{+/-}, and *Nf1*^{-/-} embryos, respectively. Pericytes were 23% (control), 49% (*Nf1*^{+/-}), and 69% (*Nf1*^{-/-}) BrdU-positive. Endothelial cells from the same embryos were 29% (control), 47% (*Nf1*^{+/-}), and 62% (*Nf1*^{-/-}) BrdU-positive. Angiogenesis is accelerated in NF1 due to hyperproliferation of pericytes and endothelial cells. Mitotically activated NG2-positive pericytes, and endothelial cells may serve as potential therapeutic targets in NF1.

Keywords

angiogenesis; endothelium; pericyte; mural cell; neurofibromatosis; NF 1; NG2; type 1

ABBREVIATIONS

BrdU, bromodeoxyuridine; CD31, PECAM-1; CD105, endoglin; flk 1, VEGF receptor-2; MPNST, malignant peripheral nerve sheath tumor; NG2, nerve; glial antigen 2; NF1, neurofibromatosis type 1; *Nf1*, neurofibromatosis type1 gene; PAS, periodic acid-Schiff method; PBS, phosphate-buffered saline; PDGF β -receptor, platelet-derived growth factor beta receptor

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is the most common cancer-predisposing genetic syndrome, with an incidence of 1 in 3500–4000 live births. The most common malignant tumor and the leading cause of mortality in NF1 is the malignant peripheral nerve sheath tumor (MPNST). The prognosis for patients with MPNST is poor. Only 30% of the MPNST patients remain disease free for 5 years even after the most aggressive therapeutic modalities. The progression of these neoplasms depends on their ability to recruit a new vascular supply from the host tissue through angiogenesis (1) (2). In this respect, it is critical to note that the angiogenic vessel wall is composed of two cell types: endothelial cells which form the vessel lumen and vascular pericytes which ensheath the endothelium (3) (4) (5) (6) (7). While endothelial cells in neurofibromatosis have been studied extensively (8) (9) (10), much less is known about the pericyte in neurofibromatosis type 1. This investigation aims at elucidating the spatial relationship between pericytes and endothelium in neurofibromatosis type 1 associated MPNST, determining whether therapeutic interference with the function of a key pericyte component (NG2 proteoglycan) inhibits NF1 tumor angiogenesis, and examining the angiogenic consequences of *Nf1* deficiency.

MATERIALS AND METHODS

All animal studies were performed in accordance with National Institutes of Health Office of Laboratory Animal Welfare (OLAW) guidelines, and were approved by the La Jolla Institute For Molecular Medicine animal research committee.

Orthotopic malignant peripheral nerve sheath tumor (MPNST) xenografts in mice were established by using human tumor cell lines derived from NF1 patients. The ST 88-14 and NMS-2PC malignant peripheral nerve sheath tumor cell lines were kind gifts from Dr. Abhijit Guha at University of Toronto, Canada, and Dr. Akira Ogose at Niagata University, Japan. Malignant peripheral nerve sheath tumor (MPNST) cell lines (ST88-14 or NMS-2PC) were injected along the sciatic nerves of six-week-old athymic mice (CrI:nu/nu). Briefly, following anesthesia with intraperitoneal avertin injection (0.017 ml/g body weight), a custom-made 32 gauge, 3/8 inch, Hamilton needle with a bevel angle of 12 degrees attached to a no.701 syringe (Hamilton, Reno, Nevada) is advanced intramuscularly forming an angle of 20 degrees with the skin. A 10 microliter volume of cell suspension containing 5×10^5 cells was injected along the right sciatic nerve. The needle was withdrawn 30 seconds after injection.

The cornea is a thin (400 microns), transparent, avascular tissue in which the growth of all new angiogenic vessels generated in response to implanting tumor xenografts can be quantified in a straightforward manner using a stereomicroscope. In a modification of two established *in vivo* techniques (11) (12), pellets containing anti-NG2 antibody were tested for their ability to inhibit corneal angiogenesis induced by NF1-derived NMS 2C tumor xenografts. These tests were performed to reveal the extent to which NG2 blockage can slow the angiogenesis that occurs in response to NMS-2PC tumors implanted in six-week-old outbred athymic mice (CrI:nu/nu).

The surgical procedure for inducing corneal angiogenesis in the mouse (12) is modified in this investigation to accommodate both tumor (NMS-2PC) xenografts and hydron pellets containing either rabbit anti-NG2, or isotype-matched non-immune globulin (control) by making a wider keratotomy incision and a deeper micropocket. Hydron pellets (0.4x0.4x0.2 mm) containing the NG2 antibody or control non-immune globulin, and tumor fragment (0.3x0.3x0.3 mm) were implanted in the corneal pocket in 22 eyes. Slow-release polyhydroxyethyl methacrylate (hydron) (Hydro Med Sciences, Cranbury, NJ) pellets are formulated to contain 45ug sucrose aluminum sulfate (sucralfate) (Sigma, St.Louis, MO) plus

one of two experimental additives: 0.8ug affinity-purified rabbit anti-NG2 antibody (13) (5) (6) (7), or 0.8 ug non-immune globulin. Six-week-old mice were anesthetized with Avertin (0.015–0.017 ml/g body weight), and under an operating microscope, one pellet and tumor fragment were surgically implanted into the corneal stroma of one eye at a distance of 0.7 mm from the corneo-scleral limbus. On day 8, angiogenesis was quantified by determining the area of vascularization, as described previously (12) (14).

C57BL/6-*Nf1^{tm1Fcr}* breeders, in which the *Nf1* gene was targeted (15), were obtained from the National Cancer Institute, Mouse Models of Human Cancers Consortium catalog number 01XF3 (Frederick, MD). *Nf1*^{+/-} mice had been back-crossed at least 10 generations onto the C57BL/6 background at the time of these experiments. For genotyping, DNA was isolated, and the targeted allele was determined by PCR as described previously (15). Ischemic retinal angiogenesis was induced by withdrawal of neonatal mice from hyperoxia (16). Litters of postnatal day 7 (P7) *Nf1*^{+/-} and *Nf1*^{+/+} 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, 6 mice of each genotype were utilized, allowing comparison of 12 wild type (*Nf1*^{+/-}) and 12 *Nf1*^{+/+} (control group) 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. Using systematic random sampling (17), 5 sections per mouse were selected 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 (16). This allowed the identification of 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. The data were compiled according to the number of angiogenic nuclei per section (with each section representing a pair of eyes). A total of 5 sections per mouse were processed permitting comparison of 30 *Nf1*^{+/-} sections with 30 *Nf1*^{+/+} sections (control).

Since mice homozygous for the *Nf1^{tm1Fcr}* targeted mutation die during late embryonic development (E13) due to severe heart malformation (double-outlet right ventricle) we retrieved and investigated younger embryos. Wild-type E10.5 *Nf1*^{+/+} (2), *Nf1*^{+/-} (3), and *Nf1*^{-/-} (4) embryos were obtained from a pregnant *Nf1*^{+/-} female after mating with a *Nf1*^{+/-} male. The morning that the vaginal plug was detected was considered as 0.5 dpc. Four hours prior to sacrifice, pregnant mice were injected intraperitoneally with bromodeoxyuridine (BrdU) (Sigma, St Louis, MO) at a dose of 80 jg/g body weight. Embryos were dissected and rinsed in PBS. A small fragment of left lower extremity was excised from each embryo by using micro-scissors (F.S.T., Foster City, CA) for DNA purification and PCR genotyping.

Following fixation of all tissues in 4% paraformaldehyde, cryopreservation in 20% sucrose in PBS at 4°C, embedding in O.C.T. compound (Sakura Inc., Torrance, CA), and snap-freezing, frozen histologic sections (40 µm) were cut using a cryostat.

Endothelial cells have been shown to express different cell surface markers as a function of developmental age (18). We therefore identified endothelial cells using a cocktail of antibodies against endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) (PharMingen, San Diego, CA) (19) (13) (5), a strategy that was utilized previously to maximize labelling of all vascular endothelial cells, both immature and mature. Pericytes were identified by labelling with affinity-purified rabbit polyclonal antibodies against the NG2 proteoglycan or the PDGF β-receptor (13) (5) (6) (7). Both NG2 and PDGF β-receptor are regarded as specific markers for pericytes (20) (4) (21). Proliferative pericyte and endothelial cell populations were

identified immunohistochemically using anti-BrdU antibody (Fitzgerald Industries, Concord, MA) (22) (23) (24). Briefly, frozen sections were digested with 0.005% pepsin (Sigma, St. Louis, MO) in 0.01 HCl for 30 minutes at 37°C followed by treatment with 4N HCl for 30 minutes at room temperature. Sections were then blocked by incubation in 5% goat serum in PBS for 30 minutes (25) prior to incubation with antibody. Slides were mounted with DAPI (Vectashield 1200, Burlingame, CA).

Confocal microscopic imaging of combined endothelial (CD31+CD105+flk1) (19) (18) and pericyte markers (NG2 or PDGF β -receptor) was performed as described previously (26) (7) (6). Multiple staining for endothelium, pericyte, BRDU, and nucleus (DAPI) were performed to quantify the percentage of proliferating endothelium and pericytes to all endothelium or pericytes. Briefly, optical sections were obtained from the specimens using the Fluoview 1000 confocal microscope (Olympus USA, Melville, NY). Serial optical sections (1 μ m each) across the entire thickness (40 μ m) of the histological specimens were overlaid (Z-stack) to provide reconstructions of entire vessels. This allowed unambiguous identification of the spatial relationship between pericytes and endothelial cells in the vessel wall. In order to estimate the frequency of vessels in any given tissue that contained endothelium-free segments (pericyte tubes), systematic random sampling (17) was performed to obtain 10 histologic sections for each tissue type to represent the corresponding frozen tissue block.

Prism 4.0 software (GraphPad, San Diego, CA) was used for statistical analyses. Systematic random sampling of serial histological sections was carried out according to previously described methods (17).

RESULTS

In five weeks, NMS-2PC and ST88-14 cell injections led to palpable tumor masses around sciatic nerves (**Figure A**). Activated pericytes were present at the leading tip of the angiogenic sprouts (**Figure B**). Pericytes showed continuous investment of endothelium (**Figure B-D**) in both NMS-2PC and ST88-14 MPNST tumor xenografts.

Mean angiogenesis induced by NMS-2PC tumor fragments was smaller (1.491 mm²) (**Figure E**) in corneas implanted with NG2-antibody-containing hydron pellets compared to those treated with hydron pellets containing isotype-matched non-immune globulin (3.186 mm²) (control) (**Figure F**). **Figure G** depicts the distribution of magnitude of tumor (NMS-2PC) induced angiogenesis in the cornea ($p=0.0002$, Mann Whitney test) in the treatment and control groups.

The magnitude of mean ischemia-induced retinal angiogenesis was 193.8 vascular nuclei in angiogenic retinal tufts in *Nf1*^{+/-} mice (**Figure I**), compared to 89.23 in *Nf1*^{+/+} mice (control) (**Figure H-J**) ($p<0.0001$, Mann Whitney test).

As revealed in BrdU assays of E 10 embryos, both pericytes and endothelial cells showed accelerated proliferative responses in embryonic angiogenesis. Pericytes were 23% (control), 49% (*Nf1*^{+/-}), and 69% (*Nf1*^{-/-}) BrdU-positive. Endothelial cells from the same embryos were 29% (control), 47% (*Nf1*^{+/-}), and 62% (*Nf1*^{-/-}) BrdU-positive. Mean pericyte/endothelium investment ratios were 1.015, 1.380, and 2.084 in *Nf1*^{+/+} wild type, *Nf1*^{+/-} heterozygote, and *Nf1*^{-/-} homozygote embryos, respectively.

DISCUSSION

In addition to its role in the onset of neoplasia, mutation of neurofibromin also leads to alterations in macrovascular development due to its expression by endothelial and smooth muscle cells (27) (28). Patients with mutations in *Nf1* have been reported to have macrovascular

defects in neural crest derivatives. Increased smooth muscle cell proliferation in the macrovascular intima and media may account for vascular hyperplasia in NF1 (28). These reports support the notion that tumor suppressor gene mutations result in non-tumor phenotypes in NF1.

Consistent with several reports that demonstrate accelerated proliferative responses in multiple cell types because of loss of one *Nf1* allele (29) (30) (31) (32), loss of a single *Nf1* was sufficient to increase proliferation of pericytes and endothelial cells. This correlated with the substantial increase in angiogenic response of retina to ischemia. In the present study, we also show that loss of one or two *Nf1* alleles results in increased pericyte and endothelial proliferation in E10 embryos. Neurofibromin is reported to be expressed by endothelium (8). Thus, it is reasonable to speculate that loss of neurofibromin expression in endothelial cells may alter the cross-talk mechanisms between endothelial cells and pericytes and provides stimulatory signals for both cell types to proliferate. Microvascular pericytes of the head, neck and central nervous system originate from neural crest derivatives (33) (34). In that regard, our results using the retinal angiogenesis model yields clues about the accelerated neovascular response in one of the neural-crest derivatives, namely the pericytes.

As previously reported, targeting strategies taking both pericyte and endothelial cell types into account maximize the efficacy of the anti-angiogenic treatment (35) (36) (13). Our results using corneal hydon pellets targeting NG2 proteoglycan on MPNST pericytes decreased angiogenesis by more than 50% indicating a crucial role for pericytes in angiogenesis.

Therefore, elucidation of the role of neurofibromin in endothelial and pericyte migration, proliferation and signaling may lead to novel approaches in the treatment of the accelerated angiogenesis seen in patients with NF1-associated tumors. Further studies are warranted to delineate the attachment migration, cross-talk and proliferation properties of pericytes and endothelial cells isolated from NF1 mice in response to angiogenic growth factors.

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Figure A.

Orthotopic NMS-2PC malignant peripheral nerve sheath tumor (MPNST) reaching a palpable size 5 weeks after grafting along the sciatic nerve (SN) in Crl:nu/nu mouse. Scale bar, 300 μ m.

Figure B-D.

Frozen sections taken from ST88-14 orthotopic MPNST xenograft, 5 weeks after grafting along the sciatic nerve in Crl:nu/nu mouse. Pericytes are identified by staining for NG2 (Figure B). NG2-positive pericytes continuously-investing the endothelium in angiogenic sprouts (arrows in Figure B). Endothelium is identified by combined immunohistochemical staining for CD31, CD105, and flk-1 in Figure C. Figure D is a merged image of figures B and C, and shows endothelium continuously invested by pericytes in angiogenic microvasculature. Scale bars indicate 10 μ m.

Figure E-G.

Mean angiogenesis induced by NMS-2PC tumor fragments was smaller (1.491 mm^2) in corneas implanted with NG2-antibody-containing hydron pellets (Figure E) compared to those treated with hydron pellets containing isotype-matched non-immune globulin (3.186 mm^2) (control) (Figure F). Arrows indicate individual vessels penetrating the cornea. T denotes NMS-2PC tumor fragment, P denotes hydron pellet. Figure G depicts the distribution of magnitude of NMS-2PC induced angiogenesis in the cornea in the treatment and control groups ($p=0.0002$, Mann Whitney test).

Figure H-J.

The magnitude of mean ischemia-induced retinal angiogenesis was 193.8 vascular nuclei in angiogenic retinal tufts in *Nf1*^{+/-} mice (Figure I), compared to 89.23 in *Nf1*^{+/+} mice (control) (Figure H). Angiogenic tufts are labeled with dotted lines in Figures H and I. Figure J depicts the distribution and number of vascular nuclei in angiogenic tufts in ischemia-induced retinal angiogenesis model in histologic sections obtained from control group (*Nf1*^{+/+}) and *Nf1*^{+/-} ($p < 0.0001$, Mann Whitney test).