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Original article

Neurofibromin is a novel regulator of Ras-induced reactive oxygen species production in mice and humans

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ABSTRACT

Neurofibromatosis type 1 (NF1) predisposes individuals to early and debilitating cardiovascular disease. Loss of function mutations in the *NF1* tumor suppressor gene, which encodes the protein neurofibromin, leads to accelerated $p21^{Ras}$ activity and phosphorylation of multiple downstream kinases, including Erk and Akt. *Nf1* heterozygous ($Nf1^{+/-}$) mice develop a robust neointima that mimics human disease. Monocytes/macrophages play a central role in NF1 arterial stenosis as *Nf1* mutations in myeloid cells alone are sufficient to reproduce the enhanced neointima observed in $Nf1^{+/-}$ mice. Though the molecular mechanisms underlying NF1 arterial stenosis remain elusive, macrophages are important producers of reactive oxygen species (ROS) and Ras activity directly regulates ROS production. Here, we use compound mutant and lineage-restricted mice to demonstrate that $Nf1^{+/-}$ macrophages produce excessive ROS, which enhance $Nf1^{+/-}$ smooth muscle cell proliferation *in vitro* and *in vivo*. Further, use of a specific NADPH oxidase-2 inhibitor to limit ROS production prevents neointima formation in $Nf1^{+/-}$ mice. Finally, mononuclear cells from asymptomatic NF1 patients have increased oxidative DNA damage, an indicator of chronic exposure to oxidative stress. These data provide genetic and pharmacologic evidence that excessive exposure to oxidant species underlie NF1 arterial stenosis and provide a platform for designing novels therapies and interventions.

1. Introduction

Neurofibromatosis type 1 (NF1) is a common genetic disorder resulting from germline mutations in the *NF1* tumor suppressor gene and affects over 2 million people worldwide [1]. Neurofibromin, the protein product of *NF1*, functions as a catalyst for the slow, intrinsic hydrolysis of active $p21^{Ras}$ (Ras) [1]. Thus, loss of neurofibromin expression increases Ras-dependent kinase activity in response to growth factor stimulation of receptor tyrosine kinases. The downstream Ras kinases Erk and Akt turn on multiple molecular switches to promote a pro-survival phenotype in neurofibromin-deficient cells.

Persons with NF1 have a strong predisposition for cardiovascular disease, which often presents in adolescence and early adulthood [2–4]. Upwards of 8% of NF1 patients will develop hypertension, arterial stenosis, aortic aneurysms, or moyamoya,

Abbreviations: EEL, external elastic lamina; IEL, internal elastic lamina; I/M ratio, intima/media ratio; MNC, peripheral blood mononuclear cells; NF1, neurofibromatosis type 1; $Nf1^{+/-}$, heterozygous for the *Nf1* allele; $Nf1^{+/-}$;$p47^{-/-}$, heterozygous for the *Nf1* allele and homozygous deletion of $p47^{phox}$; $Nf1^{flox/+}$;$LysM^{cre}$, heterozygous for the *Nf1* allele in myeloid cells alone; $Nf1^{flox/flox}$;$LysM^{cre}$, homozygous for the *Nf1* allele in myeloid cells alone; $Nf^{flox/+}$;$gp91^{flox/flox}$;$LysM^{cre}$, heterozygous for the *Nf1* allele and homozygous deletion of $gp91^{phox}$ in myeloid cells alone; NOX2, NADPH oxidase 2; PMA, phorbol myristate acid; Ras, $p21^{Ras}$ pathway; ROS, reactive oxygen species; SMC, smooth muscle cell; SOD, superoxide dismutase; WT, wild type

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though universal screening has not been adopted and may underestimate the true prevalence of disease [4–6]. The distribution of NF1 vasculopathy within a single patient is often patchy and affects multiple vessels [4]. The varied presentation of arterial lesions suggests that NF1 patients may require a "second hit" mutation in the normal *NF1* allele or, more likely, a local insult in the vessel wall leading to dysregulation of normal repair mechanisms. Constitutional homozygosity for *NF1* mutations is embryonic lethal in humans and mice; therefore, inherited mutations in a single *NF1* allele are likely sufficient for the increased disease prevalence and provide a platform for investigation [7].

We have developed a mouse model of NF1 arterial stenosis using *Nf1* heterozygous ($Nf1^{+/-}$) mice that phenotypically resembles human NF1 arterial lesions [8–12]. Following carotid artery injury, $Nf1^{+/-}$ mice develop a robust neointima when compared with WT mice, which is characterized by α-SMA positive smooth muscle cells (SMC) and a predominance of bone marrow-derived macrophages within the neointima [10–12]. Disruption of PDGF-Ras-Erk signaling inhibits $Nf1^{+/-}$ SMC proliferation and prevents neointima formation in $Nf1^{+/-}$ mice[9]; however, SMC-specific *Nf1* heterozygosity failed to replicate the enhanced neointima observed in $Nf1^{+/-}$ mice and provides evidence that other cell populations are required to initiate neointima formation in $Nf1^{+/-}$ mice [8,10]. In support of this hypothesis, WT mice reconstituted with $Nf1^{+/-}$ bone marrow developed a pronounced neointima following carotid artery ligation while $Nf1^{+/-}$ mice reconstituted with WT bone marrow developed a modest neointima similar in size to WT lesions [8]. Further, we recently showed that loss of a single *Nf1* gene copy in myeloid cells is sufficient to reproduce the exaggerated arterial lesions observed in $Nf1^{+/-}$ mice [11]. These experiments in lineage-restricted and chimeric mice provide strong evidence that neurofibromin-deficient monocytes and macrophages are critical mediators of $Nf1^{+/-}$ arterial stenosis. However, the mechanisms through which $Nf1^{+/-}$ monocytes and macrophages directly influence $Nf1^{+/-}$ SMC proliferation and arterial stenosis is completely unknown.

Emerging evidence suggests that Ras kinases directly regulate reactive oxygen species (ROS) production and, in turn, ROS may modulate Ras activity [13–15]. Constitutive activation of Ras in hematopoietic progenitor and cancer cells dramatically increases ROS production via activation of the NADPH oxidase complex [16–19]. Also, *Drosophila* harboring mutations in the *Nf1* gene exhibited shortened lifespan and increased production of and vulnerability to ROS [20,21], while overexpression of neurofibromin prolonged lifespan and reduced ROS production [22]. More recently, neurofibromin deficiency or Ras activation significantly increased oligodendrocyte ROS production and disrupted endothelial tight junctions, which was restored by daily administration of the antioxidant N-acetyl cysteine [23]. These findings are intriguing since neurofibromin occupies a unique position in the regulation of kinases that activate ROS production and enhance SMC proliferation [24–26]. For example, the Ras-dependent kinases Akt and Erk directly phosphorylate the $p47^{phox}$ subunit of NADPH oxidase 2 (NOX2) and facilitate Rac2-dependent recruitment of $p67^{phox}$ to the transmembrane component of NOX2 to increase superoxide production in phagocytes [27–29]. Overproduction of ROS in infiltrating leukocytes via NOX2 therefore may augment SMC function and participate in the pathogenesis of arterial lesions in NF1 patients. Therefore, we hypothesize that loss of neurofibromin in monocytes/macrophages enhances ROS production via NOX2 activation and amplifies $Nf1^{+/-}$ SMC proliferation leading to occlusive arterial disease. As a corollary to our experimental murine work, we seek to identify whether NF1 patients experience chronic oxidative stress.

2. Materials and methods

2.1. Animals

Protocols were approved by Laboratory Animal Services at Augusta University and Indiana University. $Nf1^{+/-}$ mice were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed 13 generations into the C57BL/6J strain. $p47^{phox}$ (4742) knockout mice ($p47^{-/-}$) were purchased from The Jackson Laboratory and maintained on C57BL/6 strain. $Nf1^{+/-}$ mice were intercrossed with $p47^{-/-}$ mice to produce $Nf1^{+/-}$;$p47^{-/-}$ mice. $Nf1^{flox/flox}$ mice were obtained from Luis Parada (University of Texas Southwestern Medical Center, Dallas, TX) and maintained on C57BL/6 background. $gp91^{flox/flox}$ mice were obtained from Abay Shah (King's College, London, UK). *LysMcre* (4781) mice were purchased from The Jackson Laboratory and maintained on C57BL/6 background. $Nf1^{flox/flox}$ mice were crossed with $gp91^{flox/flox}$ and *LysMcre* mice to generate $Nf^{flox/+}$;$gp91^{flox/flox}$;*LysMcre* mice (heterozygous loss of *Nf1* and homozygous loss of $gp91^{phox}$ in myeloid cells only). LysM is expressed in neutrophils and macrophages. *Cre*-mediated recombination was confirmed by PCR as previously described [11]. Inbreeding of $Nf1^{flox/flox}$ mice with *LysMcre* mice yielded $Nf1^{flox/+}$;*LysMcre* (heterozygous loss of *Nf1* in myeloid cells alone) and $Nf1^{flox/+}$ (WT) controls. Male mice, between 12 and 15 weeks of age, were used for experiments.

2.2. Carotid artery ligation

Carotid artery injury was induced by ligation of the right common carotid artery as previously described [11]. Briefly, mice were anesthetized by inhalation of an isoflurane (2%)/oxygen (98%) mixture. Under a dissecting scope, the right carotid artery was exposed through a midline neck incision and ligated proximal to the bifurcation using a 6-0 silk suture. The contralateral carotid artery was sham ligated as a control. Mice were administered 15 μg of buprenorphine (IP) following the procedure and recovered for 28 days. Whole ligated and control arteries were harvested from experimental mice for analysis as previously described.

2.3. Morphometric analysis

Van Gieson-stained arterial cross Sections 400, 800, and 1200 μm proximal to the ligation were analyzed for neointima formation using Image J (NIH, Bethesda, MD). Lumen area, area inside the internal elastic lamina (IEL), and area inside the external elastic lamina (EEL) were measured for each cross section. To account for potential thrombus formation, arteries containing significant thrombus (> 50% lumen occlusion) at 400 μm proximal to the ligation were excluded from analysis. The number of excluded arteries was not different between experimental groups. Representative photomicrographs for each figure are taken from arterial cross sections between 600 and 1200 μm proximal to the bifurcation. Intima area was calculated by subtracting the lumen area from the IEL area, and the media area was calculated by subtracting the IEL area from the EEL area. Intima/media (I/M) ratio was calculated as intima area divided by media area.

2.4. Arterial ROS detection in vivo

Carotid arteries from $Nf1^{+/-}$ and WT mice were injured as described above. Forty-eight hours after injury, dihydroethidium (20 mg/kg) was provided via IP injection. After an additional 24-h recovery period, mice were sacrificed and whole control and injured carotid arteries were perfused with heparinized saline and flash frozen in OCT compound. Arterial cross sections (20 μm)