Reduced Apoptosis and Increased Lesion Development in the Flow-Restricted Carotid Artery of p75NTR-Null Mutant Mice

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Abstract—Apoptosis of neointimal smooth muscle cells is a well-recognized component of the pathogenesis of vascular lesions. In recent studies, we have identified the neurotrophin receptor, p75NTR, as a mediator of apoptosis of neointimal smooth muscle cells. Neurotrophin ligands and p75NTR are selectively expressed in areas of atherosclerotic lesions with increased smooth muscle cell apoptosis and the neurotrophins are potent apoptotic agents for p75NTR-expressing smooth muscle cells in vitro. In the present study, we directly assess the role of p75NTR in lesion development in the flow-restricted carotid artery, a model of murine vascular injury. Ligation of the left carotid artery resulted in a 3- to 4-fold increase in lesion development in p75NTR-null mutant mice as compared with wild-type mice. The increase in lesion size was associated with a 70% decrease in apoptosis of neointimal smooth muscle cells, as assessed by in situ TUNEL analysis. These data suggest that under conditions of flow restriction, p75NTR activation impairs lesion formation by promoting smooth muscle cell apoptosis. These results further implicate p75NTR as an important regulator of smooth muscle cell apoptosis and lesion development after vascular injury. (Circ Res. 2002;91:494-500.)

Key Words: p75 neurotrophin receptor■ neurotrophins■ smooth muscle cells■ apoptosis■ vascular lesions

The events leading to the development of neointimal lesions after vascular injury have been well studied. The accumulation of smooth muscle cells in the intimal space as a result of their migratory and proliferative activities is a critical event in atherogenesis and restenosis. It is now recognized, however, that apoptosis also contributes to the pathogenesis of vascular disease. It has been postulated that the development of atherosclerotic and restenotic lesions is regulated by the competing forces of migration and proliferation balanced by plaque remodeling through apoptotic cell death. The molecular mechanisms that regulate neointimal smooth muscle cell apoptosis, however, are incompletely understood, and few factors initiating apoptosis of smooth muscle cells have been identified.

Recent studies from our laboratory identified the p75 neurotrophin receptor (p75NTR) as a potential regulator of smooth muscle cell apoptosis in neointimal lesions. The neurotrophins bind to 2 classes of receptor, p75NTR, a member of the tumor necrosis factor (TNF) receptor superfamily, and the trk family of receptor tyrosine kinases. Although p75NTR can enhance the affinity of interaction of the neurotrophins to the trk receptors, ligand-induced activation of p75NTR can induce apoptosis of certain classes of neuronal and glial cells, under conditions where trk activation is reduced or absent. Moreover, p75NTR can promote cell death in the nervous system both during embryonic development and after neuronal injury.

In vascular injury, p75NTR is upregulated and its expression correlates both temporally and spatially with apoptosis of neointimal smooth muscle cells. For example, after balloon de-endothelialization of the rat thoracic aorta, p75NTR is temporally expressed only in the later stages of lesion development, when apoptosis is prominent. In human atherosclerotic lesions, p75NTR spatially localizes to regions of the neointima that demonstrate ongoing smooth muscle cell death. The expression of p75NTR is restricted to neointimal smooth muscle cells and is not observed in medial smooth muscle cells. The ligands for p75NTR, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are also coordinately upregulated in these neointimal lesions. Finally, neurotrophins, at physiological concentrations, induce apoptosis of p75NTR-expressing smooth muscle cells in vitro. Collectively, these data support a role for neurotrophin-induced activation of p75NTR as a mediator of smooth muscle cell apoptosis and lesion development after vascular injury.

The aim of the present study was to address the question: does p75NTR regulate lesion development after vascular injury? The model used was that of complete ligation of the left carotid artery in mice, in which complete ligation of a carotid artery near its bifurcation reduces lumenal area as a result of both neointimal lesion formation and negative remodeling. Although a direct comparison of the mecha-
nisms regulating neointimal lesion development and remodeling in the ligated carotid artery to those occurring in atherosclerosis and restenosis are controversial, this model of acute murine vascular injury has proven useful for studying genes important for neointimal lesion development and vascular remodeling.15,16 Using this model, we now demonstrate that ligation of the carotid artery of p75NTR-null mutant mice results in a significant increase in lesion size when compared with wild-type mice. This increase is accompanied by a decrease in the apoptotic activity of neointimal smooth muscle cells. These studies support the hypothesis that p75NTR is an important regulator of neointimal lesion development and vascular remodeling in response to vascular injury.

Materials and Methods

Animals
All animal studies were approved by the Institutional Animal Care and Use Committee. P75NTR knockout mice on a C57B1/J6 background were purchased from Jackson Laboratories (Bar Harbor, Maine). The mice were bred with wild-type C57B1/J6 to obtain heterozygous animals for breeding and heterozygous crosses were utilized in the study. Ligation of the left carotid artery was performed as previously described.13 All analyses were performed within 1.5 mm of the ligature (See diagram, Figures 1 and 2). A detailed description of the methodology and data analysis is provided in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Morphometric Analysis
Sections at 500-μm increments starting from the ligature and extending 1.5 mm toward the aorta were stained with hematoxylin and eosin. Digital microscopic images were analyzed using image analysis software for Apple Macintosh computers (NIH Image 1.62). The circumferences of the lumen, the inner elastic lamina (IEL), and the external elastic lamina (EEL) were determined by tracing. The measurements were used to calculate the lumenal area, intimal area, and medial area as previously described.14 Neointimal thickness was expressed as the ratio of the area of the neointima to the area of the media.

Immunohistochemistry and TUNEL Analysis
Immunohistochemical analysis and TUNEL assays for detection of apoptotic nuclei were performed on parallel sections as previously described.3 Detailed descriptions are provided in the online data supplement.

Statistical Analysis
Statistical differences in lesion size, medial area, luminal area, and the circumference of wild-type and p75NTR-/- mice were determined by two factor analysis of variance (ANOVA). Statistical differences in the means of cellular density, BrdU, CD45, and TUNEL reactivity between wild-type and p75NTR-/- animals were determined by a Student t test. Statistical significance was determined at a value of P≤0.05.

Results
Expression of p75NTR in the Neointimal Lesions of Flow-Restricted Carotid Arteries
Previous studies demonstrated that both p75NTR and its ligands, the neurotrophins, NGF and BDNF,12 are expressed in vascular lesions in various models of vascular injury. To assess the expression of this ligand/receptor system in the ligated carotid artery model of murine vascular injury, immunohistochemical analysis was performed on frozen sections from lesions that developed in the left carotid arteries 2 to 4 weeks after ligation (Figure 1). The small neointimal lesions that developed after 2 weeks demonstrated limited immunoreactive p75NTR (Figure 1C). Increased p75NTR expression, however, was readily apparent in the larger lesions that developed 4 weeks after ligation (Figure 1E). The predominant cell type in the lesions at 4 weeks after ligation were smooth muscle cells, as assessed by immunoreactive smooth muscle cell α-actin (Figure 1H). p75NTR expression was...
Enhanced Lesion Formation in p75<sup>NTR</sup>-/- Mice 2 and 4 Weeks After Ligation

The coexpression of p75<sup>NTR</sup> and its ligands in the neointimal lesions of the ligated carotid artery suggest that neurotrophins could be exerting an autocrine/paracrine action to affect lesion development and vascular remodeling in this model of acute vascular injury. To test this hypothesis, lesion development and vascular remodeling after ligation of the carotid artery of C57Bl/6J wild-type mice was compared with that of the ligated carotid arteries of p75<sup>NTR</sup>-null mutant mice on the same genetic background. Lesion development was initially assessed 4 weeks after ligation, a time when the lesion is fully established.<sup>13,14</sup> In wild-type mice, 4 weeks after ligation, a neointimal lesion, 2 to 4 cell layers thick, was observed in the area 500 μm to 1 mm proximal to the ligation (Figures 2C and 2E), similar to what has been reported by other laboratories.<sup>16</sup> This resulted in an intimal/medial ratio ranging from 0.4 to 0.1 (500 μm to 1 mm from ligation, respectively) (Figure 3A). The decrease in neointimal area moving away from the ligature is consistent with what has been reported by other laboratories.<sup>13,14</sup> Importantly, in the p75<sup>NTR</sup>-null mutant mouse, ligation of the left carotid artery caused a significant increase in neointimal lesion development compared with wild-type mice (Figures 2D and 2F). The increase was exemplified by a 2- to 4-fold increase in the intimal/medial ratio at 500 μm and 1.0 mm proximal to the ligation (Figure 3A). The increase was mostly due to a significant increase in the intimal area, which increased 2- to 3-fold in the p75<sup>NTR</sup>-/- mice (Figure 3A). A small, but statistically significant, decrease in the medial area of p75<sup>NTR</sup>-/- mice also contributed to the increase in the intimal/medial ratio (Figure 3A). This decrease appeared to be due to a small, but statistically insignificant, decrease in the circumference of the EEL, with no change in the IEL (online Table in the online data supplement available at http://www.circresaha.org). The increase in neointimal lesion development in p75<sup>NTR</sup>-deficient mice was accompanied by a significant decrease in luminal area (online Table). Because there was no significant difference in the circumference of the EEL or the IEL of the ligated carotid artery of p75<sup>NTR</sup>-null mutant mice compared with wild type, particularly at the 1-mm mark, the decrease in luminal area could mostly be attributed to increased lesion development and not an increase in negative, or inward remodeling. In addition, there was no significant difference in the deposition of extracellular matrix in the neointimal lesions of wild-type and p75<sup>NTR</sup>-deficient mice, because the cellular density in the lesions did not differ between the two groups (65.8±5.8 versus 65.7±4.2 cells/10<sup>4</sup> μm<sup>2</sup>; p75<sup>NTR</sup>+/+ versus p75<sup>NTR</sup>-/-). As occurred in wild-type mice, there was a gradient of lesion development in the ligated carotid arteries of p75<sup>NTR</sup>-deficient mice (Figure 2 and 3A) with greater...
lesions occurring closer to the ligation. 13,14 These results indicate that in the flow-restricted carotid artery, at 4 weeks after ligation, p75 NTR activation acts to negatively regulate lesion development, such that in the case of p75 NTR deficiency, lesion size is increased.

Lesion development was also examined 2 weeks after ligation (Figure 3B), a time when the lesion is continuing to develop. In both wild-type and p75 NTR-null mutant mice, lesion development was highly variable down the length of the vessel (Figure 3B). Thus, although there was an increase in mean lesion size in the ligated carotid artery of p75NTR/H11001 mice 2 weeks after ligation, consistent with the observations after 4 weeks ligation, the increase was not statistically significant compared with wild-type mice (Figure 3B). The variability observed at this time point may reflect the dynamic state of lesion development after only 2 weeks of ligation, where the competing forces resulting in cell accumulation and cell loss may be at various levels in different mice even of the same strain.

Infiltration of Inflammatory Cells in the Flow-Restricted Carotid Artery Is Not Altered in p75NTR-Null Mutant Mice

The accumulation of leukocytes into the ligated vessel wall in the early stages after cessation of blood flow is thought to contribute to lesion development in this murine model of vascular injury. 15,16 To address whether the increase in lesion size in the ligated carotid artery of p75NTR-deficient mice was due to an increase in leukocyte accumulation, CD45 immunohistochemistry was performed on carotid arteries from wild-type and p75 NTR-deficient mice 1 week after ligation (Figure 4). In the carotid arteries of both wild-type and p75NTR−/− mice, leukocytes adherent to the vessel wall (arrowheads, Figure 4) could be observed within 1 week of ligation, similar to what has been previously reported. 15,16 Little to no leukocytes were observed in the adventitia of either wild-type or p75NTR-deficient mice at this time point. The average number of leukocytes adhering to the lumenal surface of the vessel wall was quantified and no significant difference was observed between wild-type (10.3 ± 2.6 CD45-positive cells) and p75NTR-null mutant mice (13.1 ± 5.9 CD45-positive cells). These results demonstrate that the increase in lesion development observed in the flow-restricted carotid artery of p75NTR−/− mice was not due to alterations in the early recruitment of leukocytes to the injured vascular wall.

Decreased Apoptosis in the Neointimal Lesions of Flow-Restricted Carotid Arteries From p75NTR−/− Mice

Previous studies established that ligand-induced activation of p75 NTR causes apoptosis of vascular smooth muscle cells and that p75 NTR localizes to areas of increased smooth muscle cell apoptosis in vascular lesions. 3 Because both the ligand and receptor are expressed in developing (2 weeks ligation) and

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**Figure 3.** Morphometric analysis of neointimal lesions in the flow-restricted carotid arteries of p75NTR+/+ and p75NTR−/− mice 4 weeks (A) and 2 weeks (B) after ligation. Digital microscopic images of hematoxylin and eosin stained sections were obtained and analyzed using image software analysis for Apple Macintosh computers (NIH Image 1.62). A, p75NTR+/+, n = 9; p75NTR−/−, n = 8. B, p75NTR+/+, n = 6; p75NTR−/−, n = 7.

**Figure 4.** Immunohistochemical analysis for expression of CD45-positive cells in left carotid artery 1 week after ligation. After antigen retrieval, sections of left carotid arteries from p75NTR+/+ (A and C, n = 4) and p75NTR−/− (B and D, n = 4) mice were incubated with an anti-CD45 antibody. Insets, A and B, Control sections incubated with rat IgG. Boxed areas in A and B are shown at higher magnification in C and D, respectively. L indicates lumen; M, media; and A, adventitia. Arrowheads are pointing to CD45-positive cells adherent to the lumenal surface. A and B, 45×; C and D, 90×.
both developing and established lesions, which may ultimately contribute to the increase in lesion development observed in these mice. In addition, BrdU immunohistochemical analysis of carotid arteries 1, 2, and 4 weeks after ligation was performed, to determine if p75NTR deficiency altered cellular proliferation in the ligated carotid artery. No significant difference between in BrdU incorporation was noted in p75NTR+/+ or p75NTR−/− mice (online Figure in the online data supplement available at http://www.circressha.org). This indicates that the proliferative index in the flow-restricted carotid artery was not affected by the absence of p75NTR. These results support the hypothesis that p75NTR-induced apoptosis regulates neointimal lesion development in response to interrupted blood flow.

**Discussion**

It is now recognized that apoptosis contributes to the pathogenesis of vascular disease, via several distinct potential mechanisms. These include decreasing cellularity and thinning of the fibrous cap and activation of tissue factor, which may increase the thrombogenicity of atherosclerotic lesions. Also, impaired apoptotic signaling may contribute to the development of restenotic lesions after balloon angioplasty or atherectomy of diseased arteries. Despite evidence of apoptosis in vascular lesions, however, few factors that initiate apoptosis of neointimal smooth muscle cells in vivo have been identified to date.

Previous studies identified p75NTR as a potential mediator of neointimal smooth muscle cell apoptosis. The present study provides the first in vivo evidence that p75NTR deficiency is associated with reduced apoptosis of neointimal smooth muscle cells and increased lesion development in a murine model of vascular injury. First, in the ligated carotid artery, p75NTR and the neurotrophins were expressed by neointimal smooth muscle cells in the lesions that formed 2 and 4 weeks after ligation, similar to what has been observed in other models of vascular injury. Second, lesion development was augmented in the ligated carotid artery of the p75NTR−/− mice as compared with p75NTR+/+ mice. Moreover, the increase was accompanied by a decrease in the density of apoptotic smooth muscle cells in the neointimal lesions of the flow-restricted carotid arteries of p75NTR−/− mice when compared with wild-type mice. These data support the hypothesis that p75NTR-induced apoptosis of neointimal smooth muscle cells limits lesion development in the ligated carotid artery.

Apoptosis of vascular smooth muscle cells has been observed in response to decreases in blood flow both during neonatal vascular remodeling and experimental induced ligation. Increased apoptosis is thought to counter the proliferative response and limit neointimal formation in response to vascular injury. Thus, when apoptosis is reduced and the proliferative response is left unchecked, neointimal size increases. Conversely, increased apoptosis should decrease lesion development in response to vascular injury. This was demonstrated in studies where reduced expression of the antiapoptotic protein, bcl-xL, in rabbit atherosclerotic lesions increased apoptosis of neointimal cells, which resulted in 50% decrease in lesion size. These studies support a role for

![Figure 5. Quantitative analysis of the TUNEL-positive cells in neointimal lesions from p75NTR+/+ and p75NTR−/− mice after carotid artery ligation. A and B, TUNEL-positive cells (arrowheads) in lesions of flow-restricted carotid artery of p75NTR+/+ (A) and p75NTR−/− (B) mice 4 weeks after ligation. Arrow is pointing to inner elastic membrane. C, Quantitative analysis of TUNEL-positive cells in neointimal lesions of p75NTR+/+ and p75NTR−/− mice at 2 (p75NTR+/+ n=6; p75NTR−/− n=7) and 4 weeks (p75NTR+/+ n=9; p75NTR−/− n=8) after ligation. Data were quantified by averaging the number of TUNEL-positive cells in the lesion in 4 to 8 sections within 2 mm of the ligature. Data are presented as the number of TUNEL-positive cells/10,000 μm² (intimal area).](image-url)
apoptosis of neointimal cells as a negative regulator of lesion
development in response to vascular injury.

However, a decrease in apoptosis in vascular lesions does
not necessarily decrease the lesion size. In studies aimed to
address the role of apoptosis in lesion development, overexpres-
sion of the death-domain containing adapter protein
FADD did not reduce the size or cellularity of the lesions that
developed after balloon de-endothelialization of the rat car-
rotid artery. In those experiments, however, increased
neointimal cell apoptosis after overexpression of FADD was
also associated with increased macrophage accumulation due
to FADD-induced expression of monocyte chemoattractant
protein-1 and interleukin-8. This is consistent with the
hypothesis that exaggerated apoptosis may induce or prolong
an inflammatory response to injury to induce further neoin-
timal lesion development or prevent regression. This is one
potential explanation for the rapid increase in medial smooth
muscle cell apoptosis observed after denudation of the rat car-
rotid artery, which may act to increase later neointimal
lesion formation by provoking a greater wound-healing re-
sponse. It is unclear if a similar degree of apoptosis is
occurring in the flow cessation model of vascular injury,
which could be considered a much more passive form of
injury. Thus, the potential for the high levels of apoptosis
necessary to induce or prolong an inflammatory response
may be relatively less in this model. Moreover, p75NTR
deficiency was not associated with a greater inflammatory
response in the ligated artery, suggesting that this is not a
mechanism for the increased lesion development observed in
these animals.

Although p75NTR deficiency resulted in a significant de-
crease in apoptosis at 2 and 4 weeks after ligation, as assessed
by in situ TUNEL assays, a significant increase in lesion
development was only observed after 4 weeks of ligation.
The development of a neointimal lesion is a dynamic process and
reflects the pathological processes of cell accumulation due to
increased proliferation and migration into the intima, coun-
tered by increased apoptosis to reduce lesion progression.
Thus, at 2 weeks after ligation, when the lesion is still
developing and cells are still accumulating due to increased
proliferation, a decrease in apoptosis would have a limited
effect. At 4 weeks after ligation, when proliferation of
neointimal smooth muscle cells has decreased, the lesion
may begin to undergo regression through apoptosis. Thus,
at this stage, a decrease in apoptosis would lead to less lesion
regression, resulting in a significant increase in lesion size.

In addition to p75NTR, FAS, another member of the tumor
necrosis factor receptor superfamily, has also been identified
as a potential mediator of neointimal smooth muscle cell
apoptosis. In the ligated carotid artery, FAS deficiency is
associated with an increase in lesion formation in the ligated
carotid artery, most likely due to an increase in both T
lymphocytes and macrophage infiltration in the flow-
restricted artery of the FAS ligand–deficient mouse in the
early stages after ligation. FAS is also expressed by neoin-
timal smooth muscle cells in atherosclerotic and restenotic
lesions and FAS activation can induce apoptosis of cytokine-
primed smooth muscle cells. However, FAS ligand is only
expressed by macrophages within lesions, particularly in
areas of plaque rupture, suggesting that apoptosis of FAS-
expressing smooth muscle cells is dependent on their colo-
calization with FAS ligand–expressing macrophages. Indeed,
coculture systems suggest that macrophage-induced
apoptosis of vascular smooth muscle cells is dependent on
direct cell-to-cell contact and is mediated, in part, by FAS/
FAS ligand interactions. In the present model system, leukocytes are generally not present 4 weeks after ligation.
Thus, FAS-induced apoptosis could not account for the
apoptosis observed at this time. The results of the present and
prior studies indicate that neointimal smooth muscle cells
coexpress both neurotrophins and p75NTR. Thus, in contrast to
the FAS/FAS ligand system, neurotrophins can potentially act
in an autocrine/paracrine fashion to regulate apoptosis in the
injured vascular wall.

The flow-restricted carotid artery has proven useful to
identify genes important for neointima formation and vascu-
lar remodeling in the presence of an intact endothelium. The
premise of the model is based on the observation that
reduced blood flow increases intimal lesion formation in
smooth muscle cell vascular grafts and in balloon-injured
vessels. Studies in other laboratories demonstrated both
neointimal formation and decreases in vessel diameter
through inward remodeling. The degree to which these
responses contribute to the remodeling of the flow-restricted
carotid arteries varies between different strains of mice. In the
C57BL/6J strain of mice, ligation of the carotid artery is associated with a decrease in the lumen area, which is due predominantly to a decrease in vessel diameter, ie, inward remodeling. A small neointima does
develop, however, in the ligated carotid artery of C57BL/
6J and in the present study, a similar degree of neointimal
formation in the C57BL/6J mice was observed. Although the
model does not accurately reflect the pathophysiological events associated with atherosclerosis and restenosis, in that
there is a lack of intraluminal injury, neointimal lesions in
humans are known to develop at sites of altered hemodynam-
ics associated with low shear stress. Moreover, recent evi-
dence indicates that the mechanisms that regulate lesion
development in the flow-restricted carotid artery are similar
to those regulating lesion development in other models of
murine vascular injury. For example, P-selectin–deficient mice are resistant to the development of intimal hyperplasia in
both the flow-restricted carotid artery and in a model of
transluminal endothelial injury of the femoral artery. Thus,
results obtained in this model of vascular injury may be
applicable to other models.

The results of the present and previous studies point to
the neurotrophins as critical regulators of the development
and remodeling of plaque. The biological activity of the
neurotrophins is dependent on the class of receptor that is
expressed. Trk receptor activation initiates signaling path-
ways leading to cell survival or chemotaxis. p75NTR also
has a dual function: (1) when coexpressed with Trk, p75NTR
enhances the affinity of neurotrophin binding, generating a
high-affinity site consisting of Trk:p75NTR complexes; and
(2) neurotrophin-induced activation of p75NTR can initiate
apoptosis when the p75NTR is expressed alone. This raises
the question of how neurotrophins could activate both che-
motactic and apoptotic actions concomitantly in a vascular plaque? This question has recently been addressed by studies that describe the secretion of both an active 30-kDa proform as well as the 13.5-kDa mature neurotrophins, the form previously considered to be the biologically active form.26 The proneurotrophins were found to selectively bind p75<sup>NTR</sup>, but not Trk, and were 10 to 20 times more effective in inducing p75<sup>NTR</sup>-mediated apoptosis of vascular smooth muscle cells.26 These observations suggest that the proform is a selective and effective ligand for the proapoptotic p75<sup>NTR</sup> receptor. The antibodies used in the present study to assess neurotrophin expression in the vascular lesions recognized both the mature and pro forms of NGF and BDNF26 (results not shown), and thus, could not distinguish between them in the lesions. There are currently no known antibodies that can preferentially recognize the proforms of the neurotrophins. Future studies to distinguish between expression of pro and mature neurotrophins will aid in the understanding of the complex biological activities of neurotrophins in plaque development and remodeling.

The biological activity of neurotrophin-induced activation of p75<sup>NTR</sup> in the vasculature, coupled with recent studies in the nervous system, indicate that the apoptotic activity of p75<sup>NTR</sup> may be most important as an adaptive response to injury. In the nervous system, p75<sup>NTR</sup> is expressed by hippocampal neurons undergoing apoptosis after seizures30 and by both oligodendrocytes and microglia in areas undergoing apoptosis in lesions from patients with multiple sclerosis.9 The expression of p75<sup>NTR</sup> is also increased after injury to motor neurons27 and the sciatic nerve, particularly in Schwann cells.28 In the p75<sup>NTR</sup>−/− mouse, axonal survival is improved when compared with p75<sup>NTR</sup>+/+ mice.27 Moreover, in the injured sciatic nerve, the density of apoptotic nuclei was reduced in p75<sup>NTR</sup>−/− mice, similar to our present data in the flow-restricted carotid artery. Thus, these data support a role of p75<sup>NTR</sup> as a regulator of apoptosis in response to injury.

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References

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Reduced apoptosis and increased lesion development in the flow-restricted carotid artery of p75<sup>NTR</sup> null mutant mice. Rosemary Kraemer, Ph.D.

MATERIALS AND METHODS

Animals

Briefly, the animals were anesthetized with an intraperitoneal injection of ketamine and xylazine. The left common carotid artery was isolated through a small midline incision in the neck. The artery was completely ligated immediately proximal to the carotid bifurcation and the animals were allowed to recover. After 3 days, 1, 2 or 4 weeks, the animals were reanesthetized and perfused with 3% paraformaldehyde in phosphate buffered saline. Prior to sacrifice (18 hours and 1 hr.), the mice were injected with Bromodeoxy-uridine (BrdU; Roche Biochemicals, Indianapolis, IN; 2 mg in PBS by subcutaneous injection). The left common carotid artery was excised from the ligature to the aorta and embedded in paraffin, and 10 μ serial sections were obtained. In some experiments, mice were perfused only with PBS, afterwhich, the left carotid arteries were cryopreserved in 30% sucrose/OCT (1:1) and 10 μ frozen sections were obtained. In some experiments, the right common carotid artery was taken as a control (3-day ligation only).

Immunohistochemistry

Immunoreactive p75<sup>NTR</sup> was detected by incubating methanol-fixed frozen sections with anti-p75<sup>NTR</sup> antibody (goat polyclonal, C-20; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with a biotinylated rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA). Immunoreactive NGF or BDNF were detected on methanol-fixed frozen sections with either an anti-NGF antibody (rabbit polyclonal, αMC51 Cederlane Laboratories,
Hornby, Ontario, Canada) or an anti-BDNF antibody (chicken polyclonal; R&D Systems, Inc., Minneapolis, MN) followed by incubation with an appropriate biotinylated secondary antibody. The presence of inflammatory cells was assessed by immunohistochemical analysis for the expression of the common leukocyte antigen, CD45, using a rat monoclonal antibody (clone 30-F11, BD Pharmingen, San Diego, CA), followed by incubation with a biotinylated rabbit anti-rat IgG (mouse absorbed; Vector Laboratories). Prior to incubation with the anti-CD45 antibody, antigen retrieval was performed using the Retrievagen A solution (BD Pharmingen). Immunoreactive proteins were detected using an avidin-biotin-based horse radish peroxidase kit using Vector VIP® as a chromogenic substrate (Vector Laboratories). The sections were counterstained with hematoxylin.

Smooth muscle cell α-actin and BrdU were detected using the animal research kit from Dako Corporation (Carpinteria, CA). Briefly, either monoclonal anti-smooth muscle cell α-actin antibody (clone 1A4, Dako Corporation), anti-BrdU (clone Bu20a, Dako Corporation) or murine IgG (Santa Cruz Biotechnology) were incubated with anti-mouse biotinylated Fab2′ fragments. After 15 minutes, the reaction was terminated by incubation with blocking reagent. Immunohistochemical analysis then proceeded as per the kit instructions, and immunoreactive proteins were detected as above.

TUNEL assays for detection of apoptotic nuclei were performed on paraffin-embedded sections using a kit from Roche Biochemicals. Following incubation with the TUNEL assay solution, the sections were analyzed under a fluorescent microscope.

Data Analysis

Cell density in the lesion was determined by averaging the number of nuclei in the neointima stained with hematoxylin. The data was normalized to the number of cells/10,000 μ^2.
(intimal area). Cell density was determined in 3 to 6 sections over 1.5 mm in lesions that developed 4 weeks following ligation.

The average number of CD45+ cells/cross section was determined in 10-12 sections over 2.0 mm and is expressed as the number of CD45+ cells/cross section. The average number of BrdU (+) cells in either the media (1 week ligation) or the entire intima (2 and 4 week ligation) were counted in 6-8 sections over 1.5 mm and the data expressed as the number of BrdU (+) cells per medial cross section or BrdU (+) cells/20,000 \( \mu^2 \) (intimal area), respectively. The intimal area of the sections used for BrdU analysis was determined by morphometric analysis of the digital images as described in the methods section of the paper.

The average number of TUNEL (+) cells in the entire intima was counted and the data expressed as the number of TUNEL (+) cells/10,000 \( \mu^2 \). 6-8 sections over 1.5 mm were examined/animal. The intimal area of the sections used for the TUNEL assays was determined by morphometric analysis of digital phase images as described in the methods section of the paper.

**FIGURE LEGENDS**

**FIGURE 1. Quantitative analysis of BrdU (+) cells in the ligated carotid artery of \( p75^{NTR} \) (+/+) and (-/-) mice. Panel A.** BrdU (+) cells in the medial wall 1 week after ligation of the left carotid artery. Data was quantitated by averaging the number of BrdU (+) cells in the medial wall in 7-10 sections over 2.0 mm. \( p75^{NTR} (+/+), n=4; \) \( p75^{NTR} (-/-), n=4 \). **Panel B.** BrdU (+) cells in neointimal lesions of \( p75^{NTR} (+/+ \) and (-/-) mice 2 (\( p75^{NTR} (+/+), n=6; \) \( p75^{NTR} (-/-), n=7 \) and 4 weeks (\( p75^{NTR} (+/+), n=3; \) \( p75^{NTR} (-/-), n=4 \)) following ligation. Data was quantitated by averaging the number of BrdU (+) cells in the lesions in 7-10 sections over 2.0 mm. Data is presented as the number of BrdU (+) cells/20,000 \( \mu^2 \) (neointimal area). The area of the neointimal lesion in the sections used for BrdU immunoreactivity was determined using digital phase images, as described in the methods.
**TABLES**

Table 1. Circumferences of EEL and IEL and lumen area of wild type and p75<sup>NTR</sup> null mutant mice 4 weeks post-ligation.

<table>
<thead>
<tr>
<th></th>
<th>EEL**</th>
<th>IEL</th>
<th>Lumen Area*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μ)</td>
<td>(μ)</td>
<td>(x1000 μ²)</td>
</tr>
<tr>
<td><strong>Distance from Ligation (mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt; +/-</td>
<td>860±16</td>
<td>800±15</td>
<td>818±32</td>
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<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt; -/-</td>
<td>901±40</td>
<td>759±19</td>
<td>713±25</td>
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<tr>
<td></td>
<td>590±19</td>
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</tr>
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<td>680±41</td>
<td>524±14</td>
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<tr>
<td>Distance from Ligation (mm)</td>
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<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
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</tr>
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<td>12±2</td>
<td>17±2</td>
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<tr>
<td></td>
<td>12±2</td>
<td>12±2</td>
<td>15±2</td>
</tr>
</tbody>
</table>

**. p=0.09, p75<sup>NTR</sup> +/- vs. P75<sup>NTR</sup> -/-, ANOVA  *p=0.05, p75<sup>NTR</sup> +/- vs. P75<sup>NTR</sup> -/-, ANOVA

**REFERENCES**

Figure 1.

A. 

BrdU + cells/medial cross section

1 Week Ligation

+/+  -/-

p75NTR

B. 

BrdU + cells/20,000 um^2

2 Week Ligation

4 Week Ligation

p75NTR +/+  -/-