Direct evidence for the importance of p130 in injury response and arterial remodeling following carotid artery ligation

Jürgen R. Sindermann, Jessica Smith, Christiane Köbert, Gabriele Plenz, Adriane Skaletz-Rorowski, James L. Solomon, Li Fan, Keith L. March

Abstract

Objective: Remodeling of arterial morphology in atherosclerosis, hypertension, and restenosis following angioplasty involves controlled alterations in total vascular circumference which critically modulate sequelae of changes in vessel wall mass. Despite the clinical relevance of this process little is known about the pathophysiology, especially the correlation between smooth muscle cell proliferation and remodeling. Methods: Carotid artery ligation was applied to mice with targeted disruption of the p130 gene (p130/2/2). Mice were allowed to recover for 3 weeks after ligation and then perfusion fixed for histologic and morphometric analysis. Results: P130/2/2 mice were indistinguishable from control littermates concerning size and weight. As for the aorta, carotid arteries and femoral arteries, no significant differences were found between the groups with regard to vessel size and cellular density of the vessel wall of non-instrumented vessels. In contrast, following carotid artery ligation we found p130/2/2 mice (n = 8) to develop a significant increase in vessel wall area compared to controls (n = 9). Mean values ranged from 3.07 ± 0.20 × 10^6 mm^2 for p130/2/2 mice versus 2.26 ± 0.13 × 10^6 mm^2 for p130/2/2 mice and 2.57 ± 0.26 × 10^6 mm^2 for controls (p = 0.02) along the lesion studied. This increase in vessel wall area was primarily due to a sevenfold mean increase in neointima in p130/2/2 mice yielding mean values of 0.43 ± 0.18 × 10^6 mm^2. Remarkably, despite vessel wall increase, the lumen area was not statistically different for both groups. Conclusions: The data indicate that the loss of the cell cycle inhibitor p130 leads to an enhanced injury response, implicating a central role of p130 in cell cycle control during response to injury in the vessel wall. The enhanced injury response in the context of p130/2/2 preserves the ability to perform perfect remodeling, thus the remodeling capacity is preserved even in the context of this injury model.

Keywords: Arteries; Gene expression; Remodeling; Restenosis; Smooth muscle

1. Introduction

Remodeling of arterial morphology in atherosclerosis, hypertension, and restenosis following angioplasty involves controlled alterations in total vascular circumference which critically modulate the implications and sequelae of changes in vessel wall mass. Although much work has focused on understanding the regulation of vascular smooth muscle proliferation and synthetic activity, relatively little is known concerning the mechanisms that control the three-dimensional arrangement of smooth muscle cells in the arterial wall. The modulation of vascular wall geometry in response to physiologic and pathophysiologic stimuli are collectively referred to as vascular remodeling, which is a dynamic process involving both the cellular and non-cellular components of the vessel. Remodeling plays a pivotal role in the injury model.

Time for primary review 28 days.
response mechanisms of atherosclerosis as well as after vessel trauma, like that found with balloon angioplasty. Several categories of geometric responses can be distinguished. Outward or adaptive remodeling may occur at the site of the atherosclerotic plaque, where it is understood as a reaction of the vessel wall to compensate for the luminal decrease caused by the abnormal tissue mass [1–3]. Outward remodeling can also be found after vessel trauma, where it is associated with an aneurysmal shape of the vessel wall, implying that an increase of luminal area is achieved by a thinning of the vessel wall. Inward remodeling can be found in adaptation to a diminished flow requirement, while an inward, maladaptive remodeling may occur after vessel trauma such as that found with angioplasty [4]. This behavior results in the reduction of luminal area due to shrinkage of the vessel circumference which is superimposed on vessel wall thickening. Another definition of remodeling is given by Schwartz et al. [5], who distinguish perfect remodeling from favorable, unfavorable or no remodeling. While the artery expands to perfectly accommodate neointima during perfect remodeling, only partial expansion or constriction is found during favorable or unfavorable remodeling. Despite the apparent importance of this process in determining clinical outcomes, relatively little is known about its key underlying regulatory mechanisms, which comprise mechanical forces as well as the proliferative activity of smooth muscle cells (SMCs) governed by the balance of mitogenic stimuli and the activity of cell cycle inhibitors.

The proteins of the pRb family have been shown to play a pivotal role in regulating cell cycle progression and cell cycle reentry not only in SMCs [6–9]. These proteins, pRb, p130 and p107, function in a cell cycle dependent manner to regulate the activity of numerous important cellular transcription factors such as the E2F family, which in turn regulate the expression of genes important for cell cycle progression and cellular differentiation. These are important features for the regulation of smooth muscle cell cycle reentry which is an integral mechanism for the thickening of the vessel wall due to a traumatic stimulus. Cytostatic gene therapy with a constitutively active form of the retinoblastoma gene product and p130 gene transfer revealed a significant decrease in SMC proliferation and neointima formation in a rat carotid and porcine femoral artery model of restenosis [10,11]. This suggested an approach studying the influence of the p130 gene, a pRb family member formerly referred to as pRb2, on vascular remodeling following ligation of the common carotid artery in a p130 knockout mouse model.

2. Methods

2.1. Animals

Mice with targeted disruption of the p130 gene were kindly provided by T. Jacks from the Massachusetts Institute of Technology and have already been characterized in detail as published elsewhere [12]. Briefly, the p130 gene targeting vector was introduced into strain 129/Sv D3 embryonic stem cells, before the targeted embryonic stem cell clones were injected into C57BL/6 blastocysts. The mice were finally bred into a DBA background (Jackson Laboratories, ME, USA). In order to obtain p130+/− and p130+/+ offspring, heterozygous p130+/- animals were mated. The animals included in this study were between 3 and 5 months of age and selected as littermates comprising p130−/− mice and +/+ controls. Genotypic status was assessed by polymerase chain reaction (PCR). Briefly, pups derived from the mating cages were screened for the presence of the neogene by PCR amplification using a set of three primers as published [12] allowing to distinguish between the neogene and the naturally existing p130 gene as to amplify a readily distinguishable DNA fragment. Reaction products were analyzed by electrophoresis through a 1% agarose gel and detected by ethidium bromide staining.

2.2. Mouse carotid ligation model

Carotid artery ligation model was performed as a modification of the model published by Kumar et al. [13]. All manipulations were performed according to NIH and Institutional Animal Care and Use Guidelines. Briefly, the animals were anesthetized with 2.5% Avertin (0.015 ml/g body weight i.p.). The right common carotid artery was exposed through a small midline incision in the neck and ligated by a 6-0 propylene suture about 2 mm proximal from the carotid bifurcation. The animals were allowed to recover for 3 weeks and were then sacrificed by an overdose of halothane and perfusion fixed. For perfusion fixation, the chest was opened immediately after death, and the heart punctured with a 25 gauge cannula for the infusion of phosphate-buffered saline (PBS) under physiologic pressure. The blood was drained through an incision of the inferior vena cava. After 5 min, PBS was substituted by 10% buffered formaldehyde and the mice perfused for an additional 5 min prior to dissection and overnight post-fixation with 10% buffered formaldehyde.

2.3. Histological analysis and morphometry

Tissues were dehydrated through various concentrations of ethanol and embedded in paraffin using standard methods. Sections were rehydrated and visualized with Verhoeff–van Gieson stain or H&E stain. Morphometric analysis was performed using NIH Image software by measuring the circumference of the external elastic lamina (EEL), internal elastic lamina (IEL), and the luminal border. Areas were calculated from circumference measurements assuming a circular structure under in vivo conditions. All morphometric measurements were per-
formed on Verhoff–van Gieson stains. H&E stains were used for assessing cellular densities. For the right femoral artery and the non-instrumented left common carotid artery morphometric values were obtained from five sections, for the thoracic aorta values were obtained from three sections and calculated as a mean for each animal.

For morphometric measurements of the ligated carotid arteries (n=9 for p130−/− mice versus n=10 for p130+/+ mice) sections immediately distal from the ligation site were chosen in order to exclude the sections narrowed by the suture itself. By taking every third section, a total of 12 sections per animal were analysed distal from the ligation site spanning a distance of about 250 μm. Data are given as means±standard error of mean (S.E.M.) of each position of the sections and for each group. Two animals were excluded from morphometric analysis because of thrombosis of the ligated carotid artery or values beyond mean plus three standard deviations, respectively.

Cellular cross-sectional density was determined from hematoxylin stained sections by counting at least five area defined representative microscopic fields (per animal) of the ligated common carotid arteries (n=10 for p130−/− and n=9 for p130+/+ mice) and the non-ligated common carotid arteries (n=5 mice each group), and relating the cell numbers to the areas, respectively.

For immunohistochemistry, tissues were blocked with PBS containing 5% goat serum and 20 mg/ml bovine serum albumin, and treated with 2% H2O2 for 20 min to inactivate endogenous peroxidases. Staining for proliferative cell nuclear antigen (PCNA) (Dako, Carpinteria, CA, USA) was performed on a representative mid-section of each ligated carotid artery of n=11 p130−/− and n=9 p130+/+ mice and employed a mouse monoclonal antibody diluted 1:300 in blocking solution. Primary antibody was labeled using Vectastain ABC kit (Vector, Burlingame, CA, USA) and color development was finally performed by using DAB (Sigma, St. Louis, MO, USA). Sections were briefly counterstained with hematoxylin for the visualization of all nuclei. For all immunohistochemical stainings a negative control was included which was treated identically but received no primary antibody. This allowed to evaluate the specificity of the primary antibody.

2.4. Statistical analysis

For comparison of morphometric values of non-instrumented vessels and the cellular densities Student’s t-test for independent samples was used. Statistical analysis of ligated common carotid arteries was performed using repeated measures analysis of variance (RM ANOVA) where cuts within a vessel were treated as a repeated measure and the two types of mice as the grouping factor. In case of missing values of a certain section due to the preparation procedure data have been imputed using the average of the values on either side. Values are given as means±S.E.M.

3. Results

3.1. Morphometric vessel analysis of p130−/− mice

P130−/− mice were virtually indistinguishable from control wild-type siblings (+/+), concerning size and body weight with values of 26.62±1.74 g body weight compared to 22.50±1.28 g body weight for controls (not significant: n.s.). When dissecting the mice no macroscopic irregularities were found along the vascular tree and the heart. Mean heart weight was 192.00±11.58 mg for p130−/− mice compared to 183.33±8.43 mg for controls (n.s.). We evaluated the arterial vessel geometry and analysed lumen and vessel wall areas of the thoracic aorta, the left common carotid artery, and the right femoral artery. Morphometric analysis revealed all vessels studied to be indistinguishable for both, p130−/− mice and their controls. Values for lumen areas and media areas for p130−/− mice and controls are given in Table 1. In correspondence with indistinguishable luminal areas and vessel wall areas no spontaneous neointima formation or spotted thickening of the vessel wall and no differences in elastic laminae (both groups: aorta: 5–6 elastic layers, carotid: 3–4 elastic layers) were found. Fig. 1 exemplarily shows Verhoff–van Gieson stains of the thoracic aorta and the femoral artery as well as H&E stains of the common carotid artery of p130−/− mice and p130+/+ controls. In addition to morphometric analysis SMCs were counted on H&E stained cross sections of the carotid arteries in order to calculate the cell density of the vessel wall. In correspondence with the other parameters, no significant difference was found between the two groups. For p130−/− mice the cell density was 3916±141 cells/mm² compared to 4145±200 cells/mm² for control littermates (n.s.).

3.2. Influence of carotid artery ligation on vessel remodeling in p130−/− mice

In order to test the influence of the p130 gene on vessel remodeling following a traumatic and flow changing stimulus we performed ligation of the right common carotid artery. Three weeks after ligation the animals were sacrificed and prepared for morphometric analysis by perfusion fixation under physiologic pressure. Morphometric analysis revealed the lumen of ligated carotid arteries of both, p130−/− mice (n=8) and controls (n=9), to decrease by about threefold compared to the non-instrumented contralateral carotids. Furthermore, the lumen area of the ligated carotid arteries was found to be indistinguishable for p130−/− mice and controls. Mean values for lumen area ranged from 2.64×10^{-2}±0.77×10^{-2} to 3.34×10^{-2}±0.81×10^{-2} mm² for p130−/− mice versus 2.55×10^{-2}±0.36×10^{-2} to 3.08×10^{-2}±0.34×10^{-2} mm² for controls (Fig. 2A).

In contrast, the total vessel wall cross sectional area...
Increase in medial SMC density with values of 4779±2286 cells/mm² for controls, the neointima of p130−/− mice showed a weak trend towards an increase in neointima of controls. These features are illustrated in Fig. 2C and cell cycle inhibitors exerting their effects on the cell cycle and factors [6,7]. Morphometric analysis revealed normal vessel geometry of non-instrumented vessels such as the aorta, femoral and carotid arteries. When applying carotid artery ligation, p130−/− mice developed a significantly increased vessel wall area which was primarily due to a substantially increased neointimal area caused by a hyperplastic response of p130−/− mice still consisted of about twice as many SMCs as the neointima of controls.

4. Discussion

This study presents a p130 knockout mouse model demonstrating increase in vessel wall area primarily due to neointima formation associated with perfect arterial remodeling induced by the ligation of the common carotid artery. p130 is a member of the pRb family, a family of cell cycle inhibitors exerting their effects on the cell cycle at least by controlling the activity of E2F transcription factors [6,7]. Morphometric analysis revealed normal vessel geometry of non-instrumented vessels such as the aorta, femoral and carotid arteries. When applying carotid artery ligation, p130−/− mice developed a significantly increased vessel wall area which was primarily due to a substantially increased neointimal area caused by a hyperplastic response of the vasculature. Furthermore, this neointima revealed higher cell density than the media but less cell density than control neointima suggesting relatively bigger SMCs in p130−/− neointima or more deposition of extracellular matrix than in control neointima. However, hyperplastic response of p130−/− mice was not mirrored by the portion of PCNA-positive cells 3 weeks after ligation which probably indicates that hyperproliferative reaction is switched on during a time
Fig. 1. Morphology of non-instrumented vessels of p130 \(-/-\) mice and +/+ control animals. This panel exemplarily displays Verhoeff-van Gieson stains of the thoracic aorta and right femoral artery as well as H&E stains of the left common carotid artery of p130 \(-/-\) mice and controls. All vessels were obtained from perfusion fixed animals. For perfusion fixation, the chest was opened immediately after death, and the heart punctured with a 25 gauge cannula for the infusion of PBS under physiologic pressure. The blood was drained through an incision of the inferior vena cava. After 5 min, PBS was substituted by 10% buffered formaldehyde and the mice perfused for an additional 5 min prior to dissection and overnight post-fixation with 10% buffered formaldehyde. Tissues were dehydrated through various concentrations of ethanol and embedded in paraffin using standard methods. Sections were rehydrated and visualized with Verhoeff-van Gieson stain or H&E stain. (Bars: low magnification: 200 \(\mu\)m; high magnification: 25 \(\mu\)m).

frame shortly after ligation. Remarkably, the increase in vessel wall area did not result in relative luminal narrowing, in fact the lumen was preserved. Nevertheless, the lumen of ligated carotid arteries was smaller than that of contralateral non-instrumented carotids for both, p130 \(-/-\) and controls. This was an anticipated response to chronically reduced blood flow [14] caused by the ligation but might also be due to diminished flow during the perfusion fixation process. As a result, in p130 \(-/-\) mice perfect adaptive remodeling was superimposed on any remodeling response induced by the ligation. The data indicate that loss of p130 leads to an enhanced injury response thus implicating p130 in cell cycle control during response to injury in the adult vessel. The mice are indeed able to undergo perfect vessel remodeling in response to the p130 \(-/-\) enhanced injury-response, thus the remodeling capacity is preserved even in the context of this injury model.

The occurrence of neointimal and vessel wall expansion is an anticipated consequence of SMC proliferation. A central role in SMC proliferation is attributable to mitogenic factors as well as to factors governing the cell cycle. The master check point in cell cycling is the restriction check point at the G1 phase at which cells are committed to enter S phase. The ultimate substrate in this pathway comprising key regulators such as cyclins and
cyclin dependent kinases are the proteins of the pRb family, namely pRb itself, p130 and p107 [7,15–18]. All of these factors have to be activated and inactivated in a cell cycle dependent fashion to enable the cells to progress through the cell cycle and finally perform cell division and proliferation. An important finding was the increase in p130 levels as cells reach a quiescent state after serum starvation [19]. This suggested an important role of p130 in controlling cell cycle arrest in the G0 phase. It further highlights the question whether inactivation p130 would play a central role during increased SMC proliferation in the context of neointima formation and vessel wall thickening following arterial injury.

Several in vitro and in vivo models have been established illuminating the role of various factors causing SMC proliferation and neointima formation after mitogenic stimulation or after arterial injury [20]. Studies on cytostatic gene therapy overexpressing pRb or p130 [10,11] in animal vascular injury models revealed a significant reduction in neointima formation due to decreased SMC proliferation. The adenovirus-mediated localized arterial transduction of p130 at the time of vascular injury reduced neointimal hyperplasia and prevented restenosis by blocking SMC proliferation along with influencing the capacity to bind and sequester transcription factor regulators E2Fs. However, the lack of p130 and its biological function for vascular geometry under basal conditions and injury repair have not been studied to date. Furthermore, a considerable amount of studies has put emphasis on influencing neointima formation rather than studying vascular remodeling, a process that appears to be of utmost relevance for the clinical outcome post interventions and during atherosclerosis.

The proliferation of SMCs resulting in thickening of the vessel wall is not the cause of lumen reduction per se. The important issue is the vessel geometry, superimposing increase in vessel wall mass over lumen area in an adaptive or maladaptive fashion, or under another definition in a perfect, favorable or unfavorable fashion. The mechanisms which regulate the disposition of tissue mass and thereby determine the lumen size and wall thickness for any particular degree of proliferation have not been defined to date. Transgenic mice overexpressing human growth hormone established a model which revealed the response is disrupted. While in our model elastic lamellae

Fig. 2. Morphometric analysis of the ligated right common carotid arteries of p130 −/− mice and controls. Panel A displays cross sectional luminal areas of the ligated common carotid arteries 3 weeks following ligation with a 6-0 suture. Corresponding values of the vessel wall area are shown in panel B while panel C reveals values of the tunica media and neointima for both groups, p130 −/− mice and controls. Morphometric analysis was performed using NIH Image software by measuring the circumference of the external elastic lamina, the internal elastic lamina and the luminal border. Areas were calculated from circumference measurements assuming a circular structure of the vessels under in vivo conditions. The animals were perfusion fixed as described in the legend to Fig 1. By taking every third section, a total of 12 sections per animal were analysed distal from the ligation site spanning a distance of about 250 μm. Values are expressed as mm² and are given as means±S.E.M. derived from p130 −/− mice (n=8) and controls (n=9).
Fig. 3. Morphology of ligated common carotid arteries of p130 $-/-$ (panels A, C and E) and control mice (panels B, D and F) displaying H&E stains (panels A and B) and Verhoff-van Gieson stains (panels C–F). The panels exemplarily display cross sections of the common carotid arteries of perfusion fixed animals as described in the legend to Fig. 1. Tissues were dehydrated through various concentrations of ethanol and embedded in paraffin using standard methods. Bar: 50 μm. (N: neointima; M: media).

were overtly indistinguishable for p130 $-/-$ and controls, studies on mice with homologous deletion of the elastin gene revealed that the loss of one allele is associated with an increase in elastic lamellae and smooth muscle [22]; while mice completely lacking elastin died of smooth muscle cell proliferation with eventual vascular occlusion [23], suggesting that the absence of elastin rendered the vessel unable to maintain an adaptive relationship between wall mass increase and vessel circumference.

Many recent studies have pointed out a dominant role of vessel remodeling for luminal loss or gain, depending on the ratio between lumen area and vessel wall area [24,25]. Adaptive remodeling appears to be an early physiological reaction to compensate for lumen loss in atherosclerosis [25,26]. Following angioplasty, the impact of remodeling increases while the relative contribution of neointimal formation to late lumen loss decreases over time [27]. However, several clinical studies have established a remarkable inter-individual variability in the extent and direction of remodeling [28]. Constrictive remodeling has been partially related to the presence of specific environmental factors such as smoking [29,30] and diabetes [31]; but it is clear that genetic factors as well may help determine the balance between constrictive and adaptive or unfavorable and perfect remodeling in each case.

Our data clearly indicate that induced smooth muscle proliferation and neointima formation is not necessarily accompanied by progressive vascular occlusion. In fact, increased proliferative activity of SMCs resulting in vessel wall increase is capable of preserving the lumen. Our data further point out that the loss of the cell cycle inhibitor p130 leads to an enhanced injury response, thus implicating a central role of p130 in cell cycle control during response to injury in the adult vessel wall. The enhanced injury response in the context of p130 $-/-$ preserves the ability to perform perfect remodeling, thus the remodeling capacity is preserved even in the context of this injury model.

Acknowledgements

We acknowledge the support of Tyler Jacks for providing p130 knockout mice. This work has been supported in part by a Merit Review from the Veterans Administration (K.L.M.) and grant R01-HL57411 from the NIH/US
Public Health Service (K.L.M.) as well as the Cryptic Medical Research Foundation (J.R.S.).

References


Fig. 4. Vessel wall cell densities of p130−/− mice and controls under basal conditions and following carotid artery ligation. Animals were perfusion fixed as described in the legend to Fig. 1. Cellular cross-sectional density was determined from hematoxylin stained sections by counting at least five area defined representative microscopic fields (per animal) of the non-ligated common carotid arteries and the ligated common carotid arteries and relating the cell numbers to the areas respectively. Data are given as mean numbers of cells/mm² ±S.E.M.