Atheroma development in apolipoprotein E-null mice is not affected by partial inactivation of PTEN

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TABLE OF CONTENTS
1. Abstract
2. Introduction
3. Materials and methods
   3.1. Mice, genotyping and diet
   3.2. Serum cholesterol, quantification of atherosclerosis, and immunohistochemistry
   3.3. Statistical analysis
   3.4. Western blot analysis
4. Results
   4.1. Aortic expression of PTEN and quantification of atherosclerosis in fat-fed apoE-/- and Pten+/-apoE-/- mice
   4.2. Immunohistochemical characterization of atherosclerotic lesions
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

PTEN is a dual-specificity phosphatase that has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation and migration, two key events in the ethiopathogenesis of atherosclerosis. Adenovirus-mediated PTEN overexpression inhibited the formation of vascular obstructive lesions induced by mechanical injury of the vessel wall. In this study, we investigated whether PTEN protects against atheroma formation in apolipoprotein E-null mice (apoE-/-), a widely used animal model characterized by the development of hypercholesterolemia and atherosclerosis. We examined atheroma development in the aorta of apoE-/- mice with an intact Pten gene and apoE-/- mice lacking one allele of Pten (Pten+/-apoE-/-) that were challenged for six weeks with an atherogenic diet. Compared with apoE-/- controls, Western blot analysis of arterial cell lysates from Pten+/-apoE-/- mice revealed a decrease in PTEN expression. This correlated with increased phosphorylation of AKT, thus demonstrating that Pten inactivation in Pten+/-apoE-/- mice has functional consequences. However, the extent of atherosclerosis was undistinguishable in both groups of fat-fed mice. Likewise, the atheroma of Pten+/-apoE-/- and apoE-/- mice displayed similar VSMC content, cellularity and rates of proliferation and apoptosis. Thus, in spite of the cytostatic and antimigratory activities of PTEN, and in contrast to previous studies demonstrating that Pten is haplo-insufficient for tumor suppression, our results demonstrate that atherosclerosis in hypercholesterolemic mice is not aggravated by partial inactivation of Pten.

2. INTRODUCTION

Atherosclerosis (from the Greek words athero: gruel or paste, and sclerosis: hardness) is a complex multifactorial disease of medium and large arteries that involves distinct cell types and molecular events, including both adaptive and innate immune mechanisms (1-6). Endothelial dysfunction induced by a variety of atherogenic stimuli promotes the adhesion and transendothelial migration of blood circulating leukocytes, which accumulate within the subendothelial space to form the so-called fatty streak, an early atheromatous lesion which contains mostly highly proliferative macrophages that avidly uptake lipoproteins to become lipid-laden foam cells (1,6). At homeostasis, VSMCs are primarily located in the arterial tunica media in a non-proliferative state. However, activated leukocytes in growing atheromas produce a plethora of inflammatory chemokines and cytokines that promote VSMC proliferation and migration from the tunica media towards the neointimal lesion, thus further contributing to plaque development (7,8). It has been demonstrated that hyperplastic growth and migration of adventitial myofibroblasts also contributes to neointima formation (9). In addition to cellular components, atheromatous lesions contain cholesterol and other fatty materials, and increased content of specific extracellular matrix components. Plaque rupture or erosion at advanced disease stages can lead to acute occlusion due to thrombus formation, resulting in myocardial infarction or stroke.
PTEN and diet-induced atherosclerosis

Animal and human studies have identified signaling networks and factors that play a key role in the regulation of VSMC proliferation and migration in vitro and in vivo (7,8). The tumor suppressor PTEN is a dual-specificity lipid and protein phosphatase that negatively regulates the PI3K/AKT and FAK signaling pathways (10-12). In addition to its role as tumor suppressor, mounting evidence strongly implicates PTEN in cardiovascular physiology and disease (13). PTEN can modulate cardiac myocyte hypertrophy and survival (14). Moreover, the physiology and disease (13) . PTEN can modulate cardiac evidence strongly implicates PTEN in cardiovascular 12). In addition to its role as tumor suppressor, mounting regulations of VSMC proliferation and migration in vitro signaling networks and factors that play a key role in the PTEN and diet-induced atherosclerosis PTEN (20). Similarly, adenovirus-mediated overexpression of a dominant negative PTEN mutant enhanced VEGF-mediated cell survival, mitogenesis and migration, and these processes were strongly inhibited by overexpression of wild-type PTEN (20). Similarly, adenovirus-mediated expression of PTEN inhibited both basal and PDGF-mediated proliferation, migration and survival in VSMCs (18). More recently, it has been shown that PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in VSMCs (21). In addition, adenovirus-mediated intraarterial delivery of PTEN inhibits neointimal hyperplasia and percent of stenosis in a rat model of balloon angioplasty (22). Finally, morpholino-mediated loss of endogenous PTEN induced a serum-independent growth phenotype in cultured serum-dependent VSMCs, and decreased activity of PTEN was associated with high in vivo VSMC growth rates (23).

More recent investigations have also correlated increased levels of PTEN with decreased lesion development or VSMCs proliferation. Cholesterol-fed rabbits treated with propylthiouracil (PTU) (a drug with hypothyroid effect) showed a marked reduction in VSMC/macrophage ratio in atherosclerotic plaque, and addition of PTU to cultured rat VSMCs led to increased PTEN expression and reduced cell proliferation (24). It has been also suggested that PPARgamma-mediated transcriptional activation of PTEN by Rosiglitazone and Lovastatin might contribute to the therapeutic effects of these drugs (25).

In view of the above results, we hypothesized that PTEN inactivation would enhance atheroma progression. To examine this possibility, we took advantage of the availability of the atherosclerosis-prone apoE-/ mouse (26,27), a widely used animal model that has allowed major advances in understanding the molecular basis of atherosclerosis (28). These mice spontaneously develop hypercholesterolemia and complex atherosclerotic lesions resembling to those observed in humans, a process that can be accelerated upon exposure to a high-fat cholesterol-rich diet. Since full inactivation of Pten causes embryonic lethality (29), the present study was designed to assess the effect of inactivating one allele of Pten on atherosclerosis in apoE-null mice. As expected, Pten+/−-apoE-/− mice exhibit reduction expression of PTEN in aortic tissue and this correlated with augmented AKT phosphorylation compared to apoE/- mice. However, atheroma size, VSMC content, cellularity and rates of proliferation and apoptosis were similar in both groups of mice.

3. MATERIALS AND METHODS

3.1. Mice, genotyping and diet apoE−/− mice (C57BL/6J, Charles River) and Pten+/− mice (29) (mixed 129/C57BL/6 genetic background) were mated and the double heterozygous F1 offspring were crossed with apoE−/− mice. The F2 offspring was genotyped by PCR analysis to identify Pten+/−/apoE−/− and apoE−/− mice and brother-sister mating of mice of these genotypes was performed to obtain the two experimental groups (apoE−/− and Pten+/−/apoE−/− mice). After weaning, mice were maintained on a low-fat standard diet (2.8% fat, Panlab, Barcelona, Spain). At 2 months of age, mice received for 6 weeks an atherogenic diet containing 15.8% fat, 1.25% cholesterol and 0.5% sodium cholate (S4892-S010, Ssniff, Germany).

3.2. Serum cholesterol, quantification of atherosclerosis, and immunohistochemistry Blood was withdrawn before, and after the high-fat diet to measure plasma cholesterol levels using enzymatic procedures (Sigma, St. Louis, Missouri). To determine the extent of atherosclerosis in the aortic arch region, fat-fed mice were killed and their aorta was fixed in situ with 4% paraformaldehyde. Tissue was extracted and fixation continued for approximately 24h. Specimens were paraffin-embedded and mounted in a Microm microtome (Heidelberg, Germany) to quantify atherosclerosis essentially as previously described (30). Briefly, once the 3 valve cusps were reached, sections throughout the first ~2-mm of the ascending aorta were discarded. Then, ~25 consecutive sections (4 µm thickness) were taken from 2-3 regions of the aortic arch separated by ~60 µm. Three cross-sections from each region were stained with hematoxylin/eosin. Images were captured with a Sony DKC-CM30 camera (Tokyo, Japan) mounted on a Zeiss Axiolab stereomicroscope and the area occupied by atherosclerotic lesions (intima) and the area of the media was determined by computer-assisted quantitative morphometry to determine the intima-to-media ratio using Sigma Scan Pro v5.0 (Jandel Scientific, San Rafael, California). Blood cholesterol and atheroma size were measured by a researcher who was blinded to genotype. The extent of atherosclerosis for each animal was calculated by averaging the values obtained in 2-3 independent aortic arch regions. Differences in lesion area between males and females were not significant, so data from both sexes were included in the analyses.

To quantify lesion cellularity, the number of cells per mm² of plaque was determined by examining hematoxylin-stained arterial cross-sections. VSMCs were identified with alkaline phosphatase-conjugated anti-smooth muscle alpha-actin (SMalpha-actin) antibody (1/20, a-5691, Sigma). Alkaline phosphatase activity was detected with Fast Red (Sigma). VSMC content in atherosclerotic...
PTEN and diet-induced atherosclerosis

Figure 1. Body weight and plasma cholesterol level in mice fed control chow or a cholesterol-rich diet. Data are shown as mean ± SEM of the indicated number of animals. Differences among groups were evaluated using ANOVA and Fisher’s PLSD post hoc test. There were no differences in the body weight between both genotypes. Regardless of the genotype for Pten, 2 and 6 weeks of fat feeding produced a statistically significant increase in plasma cholesterol versus prediet level (*, p<0.0001). No differences were observed between 2 and 6 weeks of fat feeding.

lesions was determined morphometrically by dividing the SMalpha-actin-positive area by total plaque area. Apoptosis was measured using the ApopTag Peroxidase in situ Apoptosis Detection Kit according to the recommendations of the manufacturer (Serologicals Corporation, Norcross, GA). The enzymatic addition of deoxynucleotides to nicked ends of DNA was stopped with a Stop/Wash buffer, and the slides were incubated with the anti-digoxigenin conjugate. After incubation, slides were washed and then colour was developed by addition of the peroxidase substrate. Cell proliferation within atherosclerotic lesions was quantified using a monoclonal antibody against the proliferation marker Ki67 (clon SP6, Master Diagnostics). Before immunostaining, slides were boiled with 10 mM citrate buffer for 10 min for antigen retrieval and endogenous peroxidase was blocked with 0.3% H2O2. Detection was performed using a biotin-conjugated anti-rabbit secondary antibody and ABC kit system (Vectastin) using DAB as peroxidase substrate (Vector laboratories). Slides were counterstained with hematoxylin as before and immunoreactive cells per mm² of atheroma were counted.

3.3. Statistical analysis
Results are reported as mean ± SEM. In experiments with 2 groups, differences were evaluated using a 2-tail, unpaired Student t-test. Analyses involving more than 2 groups were done using ANOVA and Fisher’s post-hoc test (Statview, SAS institute, Cary, North Carolina).

3.4. Western blot analysis
Snap-frozen arteries from three fat-fed mice of each genotype were pooled and lysed in ice-cold 50mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150mM NaCl, 1mM DTT and protease inhibitor Complete Mini cocktail (Roche, Mannheim, Germany) using an Ultraturrax T25 basic (IKA Labortechnik, Staufen, Germany). Western blot analysis was performed using the following primary antibodies: rabbit polyclonal anti-PTEN (1/500, NeoMarkers RB-072-PO), rabbit polyclonal anti-phospho-Ser473-AKT (1/250, Cell Signalling 9271S), goat polyclonal anti-AKT (1/1000, Santa Cruz sc-1619) and mouse monoclonal anti-tubulin (1/100, Santa Cruz sc-3035). Immunocomplexes were detected using an ECL detection kit according to the recommendations of the manufacturer (Amersham Biosciences). The relative intensity of protein bands was determined by densitometry.

4. RESULTS

4.1. Aortic expression of PTEN and quantification of atherosclerosis in fat-fed apoE-/- and Pten+/-apoE-/- mice
We intercrossed apoE-null mice and mice deficient for one allele of Pten to generate apoE-null mice with an intact Pten gene (apoE-/-) and with one allele of Pten disrupted (Pten+/+apoE-/-). After weaning, mice were maintained on a low-fat standard diet. At two months of age, blood was collected and mice were switched to a high-fat cholesterol-rich diet for six weeks. As shown in Figure 1A, body weight before the onset of the atherogenic diet and throughout fat-feeding was undistinguishable when comparing apoE-/- and Pten+/+apoE-/- mice. Likewise, fat-feeding caused similar level of hypercholesterolemia in both groups of
PTEN and diet-induced atherosclerosis

Figure 2. Expression of PTEN and AKT in the aorta of fat-fed apoE-/ and Pten+/−apoE-/ mice. Aortic lysates were subjected to Western blot analysis using the indicated antibodies. For AKT expression, we used antibodies directed against total AKT or AKT phosphorylated in Ser473. Relative protein abundance was estimated by densitometric analysis of two independent blots. Aortic PTEN expression in Pten+/−apoE-/ mice was reduced to 0.51 ± 0.02 versus the level in apoE-/ (set as 1, normalized by tubulin content). By contrary, the level of phosphorylated AKT in the aorta of Pten+/−apoE-/ mice was increased to 1.48 ± 0.06 as compared to the level in apoE-/ (set as 1, normalized by total AKT).

Importantly, the level of AKT phosphorylation, a parameter that is negatively regulated by PTEN (32), was increased in the aorta of Pten+/−apoE-/ mice (Figure 2), demonstrating that diminished PTEN expression in these mice has functional consequences.

We next examined the extent of diet-induced atherosclerosis in aortic tissue. Consistent with numerous studies in apoE-/ mice, atherosclerosis prevailed within the aortic arch in both groups of mice. Thus, we quantified by computer-assisted planimetry the area of atheroma in aortic arch cross-sections stained with hematoxylin/eosin (Figure 3). This analysis disclosed no statistical differences in the intima-to-media ratio when comparing apoE-/ and Pten+/−apoE-/ mice (0.70 ± 0.09 and 0.64 ± 0.07, respectively; n=11, p>0.05). Collectively, these results demonstrate that reduced PTEN expression does not affect aortic atherosclerosis in fat-fed hypercholesterolemic apoE-/ mice.

4.2. Immunohistochemical characterization of atherosclerotic lesions

We next carried out immunohistochemical analysis to characterize the atheroma in fat-fed mice. Both the total number of cells per mm² of atheroma (Figure 4A) and the area of atheroma occupied by VSMCs, as determined by SMalpha-actin immunoreactivity (Figure 4B), were statistically undistinguishable when comparing apoE-/ and Pten+/−apoE-/ mice (cellularity: 6168±637 versus 6971±806 cells per mm² atheroma, respectively, p>0.05; VSMC content: 6±3 versus 4.7±0.9 SMalpha-actin-positive cells/mm² atheroma, respectively, p>0.05). Similarly, the percentage of proliferating cells, as determined by Ki67 immunoreactivity (Figure 4C) and apoptotic cells, as determined by the Apoptag kit (Figure 4D), were comparable in the atheroma of both groups of
PTEN and diet-induced atherosclerosis

Figure 3. Reduced PTEN expression in the artery wall of Pten+/−apoE−/− does not affect the size of aortic atherosclerotic plaques. Mice with the indicated genotypes were challenged with a high-fat diet for six weeks. The graph represents the intima-to-media ratio in cross-sections of the aortic arch, which did not show statistical differences between the two experimental groups (Student’s t-test, p>0.05). Representative examples of cross-sections stained with hematoxylin and eosin are shown at the bottom. The edge of the atherosclerotic plaque is drawn with a discontinuous line.

Figure 4. Immunohistochemical analysis of atherosclerotic lesions. Analysis was performed in cross-sections from the aortic arch and all parameters were quantified in the atheroma: lesion cellularity in hematoxylin-stained specimens (A), VSMC content as determined by SMalpha-actin immunoreactivity (B), proliferative cells as determined by Ki67 immunoreactivity (C), and apoptotic cells as revealed using the Apoptag kit (D). In all cases, differences between apoE−/− and Pten+/−apoE−/− mice were not statistically significant (Student’s t-test, p>0.05).
Previous studies have conclusively demonstrated that PTEN inhibits VSMC proliferation and migration in vitro and reduces neointimal thickening in the rat carotid artery model of balloon angioplasty (18,19,21,22). To our knowledge, however, the role of PTEN on atheroma development has not been investigated. Therefore, we sought to examine the consequences of genetically inactivating Pten on diet-induced atherosclerosis using the apoE-null mouse model, a well characterized animal model of atherosclerosis that recapitulates important features of the human disease (26,27). Since global Pten inactivation in the mouse causes lethality during embryogenesis, (29) we analyzed fat-fed apoE-/- and Pten+/−/apoE-/- mice. Inactivation of one allele of Pten led to a 50% reduction in PTEN protein expression in the aorta of Pten+/−/apoE-/- compared to apoE-/- mice. Importantly, PTEN inactivation had functional consequences, since phosphorylation of its target AKT/PKB in aortic tissue was increased by approximately 50% without changes in total AKT expression. However, we found no differences in aortic atherosclerotic lesion between apoE-/- and Pten+/−/apoE-/- mice. Likewise, analysis of the atheromatous lesions in these animals disclosed no differences in several histopathological parameters, including the area occupied by VSMCs, cellularity, proliferation and apoptosis.

It has been previously shown that Pten+/- mice spontaneously developed germ cell, gonadostromal, thyroid and colon tumours (29), demonstrating that Pten is haplo-insufficient for tumor suppression. Of note in this regard, adenovirus-mediated intraarterial delivery of Pten after balloon injury in the rat carotid artery inhibits neointimal lesion development (22), a pathological process characterized by abnormally high hyperplastic growth of VSMCs (7,33,34). Thus, alterations in PTEN expression have a major impact on the course of highly proliferative disorders, such as cancer and neointimal hyperplasia induced by mechanical injury of the vessel wall. In contrast, we show here that atheroma development in apoE-/- mice is not affected by partial inactivation of Pten. Although vascular cell hyperplasia is also a feature of atherosclerosis, this disease involves additional processes that might not be regulated by PTEN (e. g., arterial lipid accumulation, neointimal foam cell formation, abundant extracellular matrix formation by arterial cells, VSMC dedifferentiation) (1-6). On the other hand, Shen et al. have recently suggested that PTEN expression may contribute to cardiovascular diseases by causing p38 MAPK stress signal-induced inhibition of insulin-signaling and eNOS activation (35). Thus, alterations in PTEN expression may promote both pro- and anti-atherogenic effects. Finally, we cannot rule out the possibility that the 2-fold reduction in aortic PTEN expression in Pten+/−/apoE-/- mice (cf. Figure 2) might not be sufficient to aggravate atheroma development compared to apoE-/- mice. Since global Pten inactivation causes embryonic lethality (29), addressing whether total Pten gene inactivation in the artery wall might indeed exacerbate atheroma progression will require the generation of apoE-/- mice with Pten disruption targeted to cell types known to participate in atherosclerosis (e. g., EC, VSMC, macrophage).

5. DISCUSSION

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6. ACKNOWLEDGEMENTS

7. REFERENCES

PTEN and diet-induced atherosclerosis


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