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Deficiency of cyclin-dependent kinase inhibitors p21^{Cip1} and p27^{Kip1} accelerates atherogenesis in apolipoprotein E-deficient mice

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Abstract

Cyclin-dependent kinase inhibitors, p21^{Cip1} and p27^{Kip1}, are upregulated during vascular cell proliferation and negatively regulate growth of vascular cells. We hypothesized that absence of either p21^{Cip1} or p27^{Kip1} in apolipoprotein E (apoE)-deficiency may increase atherosclerotic plaque formation. Compared to apoE^{-/-} aortae, both apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} aortae exhibited significantly more atherosclerotic plaque following a high cholesterol regimen. This increase was particularly observed in the abdominal aortic regions. Deficiency of p27^{Kip1} accelerated plaque formation significantly more than p21^{-/-} in apoE^{-/-} mice. This increased plaque formation was in parallel with increased intima/media area ratios. Deficiency of p21^{Cip1} and p27^{Kip1} accelerates atherogenesis in apoE^{-/-} mice. These findings have significant implications for our understanding of the molecular basis of atherosclerosis associated with excessive proliferation of vascular cells.

Keywords

Proliferation; Atherosclerosis; Aorta; Neointima

1. Introduction

Atherosclerotic plaque development is associated with excessive vascular cell replication [1]. Many mitogenic factors induce the proliferation of vascular smooth muscle cells (vsmc) during atherogenesis. Diminished expression of cyclin-dependent kinase inhibitors (CKIs) has been proposed to regulate atherosclerosis development, but their direct role is not known. Two families of cyclin-dependent kinase inhibitors (CKIs) have been identified: the Kip/Cip proteins (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) and the INK proteins (p15^{INKB}, p16^{INKA}, p18^{INKC} and p19^{INKD}) [2]. The Kip/Cip proteins contained a conserved domain that is necessary and sufficient for cyclin/cyclin-dependent kinase (CDK) interaction and inhibition in replicating

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cells. p21^{Cip1} and p27^{Kip1} are upregulated during arterial remodeling and inversely regulate the growth of vsmcs, resulting in G₁ arrest of vsmcs [3,4]. Thus, p21^{Cip1} and p27^{Kip1} play an important role in the regulation of cell-cycle progression by inactivating the CDKs in the G₁ phase [2], leading to cell cycle arrest, thereby regulating wound repair and growth regulation in the vasculature. We and others have reported that deficiency of either p21^{Cip1} or p27^{Kip1} leads to increased proliferation of vsmcs after vascular injury, indicating that CKIs regulate vsmc proliferation as endogenous inhibitors [3,4,5]. Furthermore, we have demonstrated that overexpression of either p21^{Cip1} or p27^{Kip1} by adenoviral vectors resulted in decreased neointima formation after vascular injury [5]. Overexpression of mutated p21^{Cip1} transgene reduces smooth muscle cell proliferation, macrophage deposition, oxidation-sensitive mechanisms, and restenosis in hypercholesterolemic apolipoprotein E knockout (apoE^{-/-}) mice [6]. Interestingly, p27^{Kip1} downregulates cyclin-dependent kinase 2 activity and cyclin A promoter activity in vsmc within the neointima formation in the rat carotid artery [7]. Furthermore, p27-p16 fusion gene has been designed to inhibit angioplasty-induced neointimal hyperplasia and coronary artery occlusion [8,9].

In view of the role played by p21^{Cip1} and p27^{Kip1} in cell-cycle regulation, we hypothesized that the absence of either p21^{Cip1} or p27^{Kip1} would lead to accelerated atherosclerotic plaque formation. To test this hypothesis, we used apolipoprotein E (apoE)-deficient mice, a commonly used model of atherosclerosis, in combination with either p21^{Cip1} or p27^{Kip1} deficiency to determine whether these CKIs play a pivotal role during atherogenesis, to study which of these two proteins is more atherogenic, and to investigate whether there is a difference in atherosclerotic plaque formation between regions of the aortae. In this study, the impact of p16^{INKA} deficiency has not been tested since this protein, in contrast to p21^{Cip1} and p27^{Kip1}, has been reported as a weaker inhibitor of intimal vsmc proliferation after vascular injury [5]. This present study establishes a causal link between decreased expression of either p21^{Cip1} or p27^{Kip1} and atherogenesis in hypercholesterolemic mice.

2. Materials and methods

2.1. Animals

Animal groups were C57Bl/6 (wild-type) mice, mice deficient in apolipoprotein E (apoE^{-/-}), p21^{Cip1} (p21^{-/-}), p27^{Kip1} (p27^{-/-}), both p21^{Cip1} and apolipoprotein E (apoE^{-/-}/p21^{-/-}), and both p27^{Kip1} and apolipoprotein E (apoE^{-/-}/p27^{-/-}). Eight weeks after birth, both male and female animals were started on a diet consisting of either normal chow or high fat chow with 0.15% cholesterol (42% from fat). The animals were sacrificed after either 6 or 15 weeks of either diet. Each group consisted of between 4 and 16 mice, resulting in a total number of 224 mice analyzed. Compared to p21^{-/-}, intercrossing of p27^{-/-} mice with apoE^{-/-} was delayed due to their impaired fertility. All animal studies were approved by the Animal Care and Use Committee of the National Institutes of Health.

2.2. Tissue processing

In each group, at least four aortae were set aside for total *in situ* preparation for Oil-Red-O staining. For this purpose, they were fixed by 4% paraformaldehyde / 5% Sucrose in PBS overnight and then rinsed in 70% ethanol for at least 10 minutes. For cross sections, at least three aortae were set aside and stored in PBS-buffered formalin.

2.3. Oil-Red-O staining

Aortae were stained with Oil-Red-O staining solution [35 ml of 0.2% Oil-Red-O (Sigma; St. Louis, MO) in methanol, and 10 ml of 1N NaOH] for 50 minutes. The tissues were then destained by incubating in 70% ethanol for 30 minutes and washed with water. The tissues were moved onto a wax covered dissecting plate (Fisher; Pittsburgh, PA) and fixed by stainless

pin (0.1 mm diameter, Watkins & Doncaster; Cranbrook, UK). Since the aortic tissues are semitransparent, all adventitial fat tissues were removed prior to image capturing.

2.4. Morphometrical analysis

After being pinned to the dissecting plate, pictures of the inner surface of the aortae stained with Oil-Red-O were taken under the dissecting microscope. Then, the images were analyzed using computer software (Media Cybernetics; Image-Pro Plus 4.1; Silver Spring, MD) at the arch, thoracic, and abdominal regions. The aortic arch was defined as between the end of the aortic valves and the left subclavian artery, whereas the thoracic aorta was designated as between this artery and the end of the celiac artery. The remaining distal portion and iliofemoral bifurcation were defined as the abdominal aorta. Oil-Red-O-stained areas were measured and divided by total or segmental aortic surface area to determine the percentage of atherosclerotic plaque.

2.5. Histomorphological analysis

For cross sectional studies, formalin-fixed aortae were divided into 6 segments. From each paraffin-embedded segment, 30 sections (five μm thick) were taken and every tenth section stained with Hematoxylin & Eosin (H&E). The H&E-stained sections were then analyzed under a conventional light microscope by the same image analysis software as used for the Oil-Red-O stainings. Intimal and medial areas were measured, and then the intima /media ratios calculated. We found no significant changes between male and female genders in the percentage of plaque formation in all groups, and hence, data from male and female aortae were combined.

2.6. Statistical analysis

Mean $\pm$ SEM values of percentage of Oil-Red-O staining in the arch, thoracic, and abdominal segments of the aortae were presented. Each individual group was compared with its respective C57Bl/6 group. Statistical differences were analyzed using a database statistics program (StatView 5.0.1; SAS Institute; Cary, NC) by multiple variance analysis (ANOVA) and the Bonferroni/Dunn method. The significance level was set at $p < 0.05$.

3. Results

3.1. Deficiency of p27^{Kip1} accelerates atherogenesis more than p21^{Cip1} in apoE^{-/-} mice on a normal diet

We studied the impact of the absence of p21^{Cip1} and p27^{Kip1} on aortic atherogenesis induced by a high-fat diet. Since apoE-deficient mice develop atherosclerotic lesions similar to those in humans, we generated apoE^{-/-}/p21^{-/-} or apoE^{-/-}/p27^{-/-} mice by intercrossing apoE^{-/-} mice with mice deficient for p21^{-/-} and p27^{-/-}. This allowed us to study three sets of variables and their interactions: 1) p21 and p27 function; 2) apoE function; and 3) normal or high fat diet feeding for 6 and 15 weeks.

In mice with intact apoE fed a normal diet, we observed an increase in plaque formation in p27^{-/-} mice at 6 and 15 weeks of feeding, compared with p21^{-/-} or C57Bl/6 wild-type control mice (6 weeks: $2.38 \pm 1.00\%$ versus $0.00 \pm 0.00\%$ and $0.00 \pm 0.00\%$, respectively; 15 weeks $2.46 \pm 0.90\%$ versus $0.53 \pm 0.42\%$ and $1.65 \pm 1.52\%$, respectively) (Fig. 1A, upper panels), suggesting that p27 deficiency alone induces atherogenesis.

ApoE^{-/-} mice did not exhibit significant increases in aortic lesions at 6 or 15 weeks feeding on a normal chow diet compared to wild-type control mice (Fig. 1A, upper panel, right). However, the double knock-out mice, apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-}, demonstrated even greater degrees of plaque lesion formation at 6 and 15 weeks compared with apoE^{-/-} mice

alone; the apoE^{-/-}/p27^{-/-} plaques being more severe than the apoE^{-/-}/p21^{-/-} lesions (6 weeks: apoE^{-/-}/p27^{-/-} 7.17 ± 0.81% *versus* apoE^{-/-}/p21^{-/-} 1.71 ± 1.17% and apoE^{-/-} 0.40 ± 0.26%, *p* < 0.05; 15 weeks: apoE^{-/-}/p27^{-/-} 29.26 ± 11.61% *versus* apoE^{-/-}/p21^{-/-} 4.32 ± 1.01% and apoE^{-/-} 4.01 ± 0.77%, *p* < 0.001) (Fig. 1A, upper panel). These data suggest that CKI deficiencies are synergistic with pro-atherogenic lipids to enhance atherosclerosis development.

3.2. Deficiency of cyclin-dependent kinase inhibitors p27^{Kip1} and p21^{Cip1} accelerates atherogenesis in apoE^{-/-} mice on high fat diet

The addition of a high fat diet resulted in modest effects on lesion formation in p21^{Cip1} and p27^{Kip1} null mice at 6 and 15 weeks of feeding (p21^{-/-} 1.45 ± 0.88% at week 6 *versus* 0.78 ± 0.52% at week 15, and p27^{-/-} 3.36 ± 0.65% at week 6 *versus* 6.90 ± 1.66% at week 15) (Fig. 1A, lower panels and 1B). As expected, all apoE^{-/-} mice fed a high fat diet for 6 and 15 weeks developed larger aortic lesions than wild-type controls (0.24 ± 0.19% at week 6 and 0.80 ± 0.44% at week 15) (Fig. 1A, lower panels and 1B).

In contrast, compared to apoE^{-/-} aortae, formation of plaque was significantly increased in apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} mice after high fat cholesterol diet at both 6 (11.26 ± 3.19% *versus* 18.44 ± 0.97% and 30.43 ± 5.26%, respectively) and 15 (19.38 ± 1.56% *versus* 37.91 ± 3.90% and 48.19 ± 9.30%, respectively) weeks (Fig. 1A, lower panels and 1B). These data suggest that a high fat diet further accelerates plaque formation against a background of apoE and CKI loss of function.

3.3. Deficiency of p27^{Kip1} accelerates atherogenesis more than p21^{Cip1} in apoE^{-/-} mice on a high fat cholesterol diet

In the setting of apoE deficiency and high fat cholesterol feeding, apoE^{-/-}/p27^{-/-} aortae demonstrated significantly larger aortic atherosclerotic plaques compared with apoE^{-/-}/p21^{-/-} at 6 weeks (30.43 ± 5.27% *versus* 18.44 ± 1.00%, respectively, *p* < 0.01) (Fig. 1A, lower panels and 1B), but, this difference did not reach statistical significance at 15 weeks.

3.4. Deficiency of p21^{Cip1} and p27^{Kip1} in apoE^{-/-} accelerates atherosclerotic plaque formation in the thoracic and abdominal aortic segments

In apoE^{-/-} mice, most plaque formation was seen in the aortic arch (25.10 ± 4.95%) at 6 weeks of cholesterol-rich diet, but this was observed to a lesser extent in the thoracic (8.23 ± 3.92%, *p* < 0.05) or abdominal regions (8.60 ± 2.44%, *p* < 0.05) (Figs. 2A–C). Similarly, after 15 weeks of cholesterol-rich diet, most plaque formation in apoE^{-/-} mice was seen in the aortic arch (54.84 ± 2.77%) compared to the thoracic (11.50 ± 1.18%, *p* < 0.01) or abdominal regions (7.45 ± 1.87%, *p* < 0.01). In the aortic arch, there was no visible difference in the percentage of plaque formation between apoE^{-/-} (54.84 ± 2.77%), apoE^{-/-}/p21^{-/-} (62.49 ± 2.80%) and apoE^{-/-}/p27^{-/-} (59.07 ± 10.82%) mice following 15 weeks of cholesterol-rich diet (*p* = NS) (Fig. 2A). However, compared to apoE^{-/-} mice, a significant increase in plaque formation was seen in abdominal parts of apoE^{-/-}/p27^{-/-} (7.45 ± 1.87% *versus* 37.77 ± 7.25%, *p* < 0.01 and 51.58 ± 11.62%, *p* < 0.05, respectively) mice following 15 weeks of high fat cholesterol diet (Fig. 2C).

3.5. Histological characterization of atherosclerotic lesions in apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} mice

The above findings of percentage of plaque formation as detected by Oil-Red-O staining were confirmed by morphometrical analysis of cross-sections (Fig. 3A and B). At 15 weeks of high fat cholesterol diet, the intima/media ratios of apoE^{-/-} (0.47 ± 0.56, *p* < 0.05), apoE^{-/-}/p21^{-/-} (0.67 ± 0.62, *p* < 0.01) and apoE^{-/-}/p27^{-/-} (1.08 ± 0.30, *p* < 0.01) aortae was significantly

greater than C57Bl/6 wild-type controls (0.11 ± 0.03). In apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} aortae, the majority of neointimal cells consisted of infiltrating mononuclear cells and spindle-shaped vsmcs, and they were the source of intracellular lipid particles. Thus, apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} mice appeared to exhibit more advanced atherosclerotic lesions, with apoE^{-/-}/p27^{-/-} lesions being more severe.

4. Discussion

We have analyzed the inactivation of p21^{Cip1} and p27^{Kip1} kinase inhibitors on atherogenesis *in vivo*. Our results contribute to our understanding of the molecular basis of atherosclerosis associated with excessive vascular cell proliferation. The major findings in this study are that the Kip/Cip CKI proteins are differentially potent inhibitors of atherogenesis; this, in turn, leads to differences in atherosclerotic plaque development. It is noteworthy that inactivation of either p21^{Cip1} or p27^{Kip1} alone is not sufficient to promote significant atherogenesis in mice despite an atherogenic diet for 15 weeks. However, when generated in combination with an apoE^{-/-} genetic background, the CKI deficiency leads to significantly more pronounced lesions compared to apoE^{-/-} alone. This increase was observed especially in the abdominal aortic regions. In normal arteries, p27^{Kip1} is constitutively expressed in medial and intimal vsmcs, but it is rapidly decreased after vascular injury once mitogenic proliferation takes place. In contrast, p21^{Cip1} is not observed in vsmcs of normal arteries and it is upregulated along with p27^{Kip1} in the later phase of experimental arterial remodeling [4]. These patterns of protein expression have also been observed in human arterial tissues [10]. Furthermore, it has been reported that both p21^{Cip1} and p27^{Kip1} can block vsmc proliferation *in vitro* and decrease restenosis in experimental models [11,12].

Deficiency of p27^{Kip1} leads to greater atherosclerotic plaque development than p21^{Cip1} in apoE^{-/-} mice. The expression of both p21^{Cip1} and p27^{Kip1} in vsmcs inactivates cdk2 and cdk4 [5]. *In vivo*, overexpression of p27^{Kip1} inhibits intimal cell proliferation and thickening after vascular injury [5]. This effect is more potent than the inhibitory effect of p21^{Cip1}, whereas p16^{INKA} is a weak inhibitor in the arterial wall. These differences in molecular mechanisms have important implications for our understanding of cardiovascular disease. The most potent effect of p27^{Kip1} is likely due to downregulation of the cyclin A promoter [7]. Interestingly, p27^{Kip1} mice do not develop tumors, but exhibit benign hyperplasia in multiple organs [13]. Furthermore, vascular repair and regeneration is regulated by the proliferation of bone marrow-derived hematopoietic and nonhematopoietic cells through a p27^{Kip1}-dependent mechanism and that immune cells largely mediate these effects [11].

In most of the experimental models of atherosclerosis, plaque development is reported mainly in the aortic arch, whereas thoracic and abdominal parts of the aorta are either ignored or not separately demonstrated. In this study, most plaque formation in apoE^{-/-} mice was seen in the aortic arch, but rarely observed in the thoracic or abdominal regions. In apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} aortae, atherosclerotic plaque formation in the aortic arch was also seen as severe as in apoE^{-/-} mice; however, the apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} aortae exhibited more atherosclerotic lesions particularly in the abdominal regions. In these areas, excessive atherosclerotic lesions represent a life-threatening condition characterized by chronic inflammation, plaque rupture, destructive remodeling of the extracellular matrix, and increased local expression of matrix metalloproteinases (MMPs) [14]. Earlier studies demonstrated that inflammatory cell expression of both MMP-9 and -12 plays a critical role in an experimental model of aortic aneurysm disease [15,16]. Our experimental results may have clinical relevance since degradation of the abdominal aortic wall corresponds with human aneurysmal development.

Previous studies have suggested that high fat fed apoE^{-/-} mice develop comparable levels of hyperlipidemia regardless of p27^{Kip1} status [17]. However, these investigators evaluated, atherosclerotic plaques only in the aortic arch and one month after cholesterol-rich diet, suggesting that the time frame was likely insufficient to observe the full effects of genetic mutation on atherosclerosis development. Indeed, in the present study, we quantified plaque areas 6 and 15 weeks after cholesterol-rich diet and not only in the aortic arch, but also in the thoracic and abdominal segments. In addition, we simultaneously compared the rate of atherogenesis between p21^{Cip1} and p27^{Kip1} deficiencies in the same model. We observed that apoE^{-/-}/p27^{-/-} aortae exhibited significant atherosclerotic plaque compared to controls which was apparent at 6 weeks and quite progressive by 15 weeks, indicating the importance of observations over a sufficient period of time. Contrary to the previous data and our study suggesting a protective role for p21, Merched AJ et al. found that absence of p21, either globally or in bone marrow-derived cells, protects against atherosclerosis [18]. However, their study assessed the effect of p21 inactivation in apoE^{-/-} mice at 20 and 63 weeks. This covered more advanced stages of atherosclerosis development, but lacked assessment of initial and early atherosclerotic lesions.

Our findings have direct implications for the design of therapies to treat human vasculoproliferative disorders. Gene transfer of vectors that encode either p21^{Cip1} or p27^{Kip1} following mechanical vascular injury produces significant reduction in vsmc proliferation and neointimal thickening, demonstrating that cell cycle inhibition is an effective approach [3,5, 19]. Inactivation of these either proteins results in a loss of growth control and apoptosis of vascular cells and thus, failure to repair arterial wound healing. Thus, approaches to augment function of CKIs represent an effective tool to treat human vascular disease. Further insights into molecular control mechanisms of vascular wound repair are needed to dissect the pathogenesis of fibroproliferative vascular disease.

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Fig. 1. Percentage of plaque areas in aortae

(a) Mean $\pm$ SEM values of percentage Oil-Red-O staining in the entire aorta for all groups.

Each individual group was compared with its respective C57Bl/6 group. * $p < 0.05$ and

*** $p < 0.001$. (b) Representative Oil-Red-O-stained aortae from mice fed a cholesterol-rich diet for 15 weeks. Note excessive plaque formation in aortae of the apoE^{-/-}/p21^{-/-} and apoE^{-/-}/p27^{-/-} genotypes, as well as that deficiency of p27^{Kip1} accelerates atherogenesis more than p21^{Cip1} deficiency in apoE^{-/-} mice. The images of aortae fixed on a wax surface were captured under a surgical microscope and analyzed by image analysis software.

Fig. 2. Percentage of plaque areas in different aortic segments

Mean $\pm$ SEM values of percent Oil-Red-O stainings in the aortic arch (a), thoracic (b) and abdominal (c) segments in mice after high-cholesterol diet for 6 weeks or 15 weeks in all groups. Each individual group was compared with its respective apoE^{-/-} group. * $p < 0.05$ and ** $p < 0.01$.

Fig. 3. Histomorphological changes in the aortic wall

(a) Mean intima/media area ratios of abdominal segments of aortae in C57Bl/6, apoE^{-/-} and apoE^{-/-}/p21^{-/-}. *p < 0.05, **p < 0.01. (b) Representative H&E-stained histomorphological cross sections of the abdominal aortic segments after high cholesterol diet for 15 weeks. An average of three sections at 6 different levels of an aorta is quantified in each group. Different layers were manually circumscribed and their areas were quantified by image analysis software. Arrowheads denote internal elastic lamina bordering neointima formation and medial layer. Original magnification × 200.