Control of cell proliferation in atherosclerosis: insights from animal models and human studies

José J. Fuster¹, Patricia Fernández¹, Herminia González-Navarro¹, Carlos Silvestre¹,², Yafa Naim Abu Nabah¹, and Vicente Andrés¹,²*

¹Laboratory of Vascular Biology, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia-CSIC, C/Jaime Roig 11, 46010 Valencia, Spain; and ²Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Department of Atherothrombosis and Cardiovascular Imaging, Spanish National Cardiovascular Research Center (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, Spain

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1. Introduction

Atherosclerosis, restenosis (vessel renarrowing after successful angioplasty), and graft atherosclerosis after coronary artery bypass surgery are chronic inflammatory diseases that lead to the formation of obstructive vascular lesions. Excessive cell proliferation within the arterial wall is a key contributor to plaque growth; therefore, understanding the molecular mechanisms that control hyperplastic growth of vascular cells is of utmost importance for the development of efficient therapies against atherosclerosis and restenosis.

Mammalian cell proliferation is controlled by a large number of proteins that modulate the mitotic cell cycle (Figure 1). Progression through the cell cycle requires the activation of holoenzymes composed of a catalytic cyclin-dependent protein kinase (CDK) and the regulatory subunit cyclin. Specific CDKs are sequentially activated during different phases of the cell cycle by the oscillating synthesis and degradation of their cyclin partners. The activity of CDK/cyclins is also regulated by phosphorylation/dephosphorylation and by their interaction with CDK inhibitory proteins (CKIs) of the Cip/Kip (CDK interacting protein/kinase inhibitory protein: p21Cip1, p27Kip1, p57Kip2) and Ink4 (inhibitor of CDK4: p16INK4a, p15INK4b, p18INK4c, p19INK4d) families. All Cip/Kip proteins bind to and inhibit a wide spectrum of CDK/cyclin complexes, while the Ink4 proteins specifically inhibit cyclin D-associated CDKs. Mitogenic and antimitogenic stimuli affect the rates of CKI synthesis and degradation, as well as their redistribution among different CDK/cyclin heterodimers. In addition, other proteins, such as the transcriptional regulator p53, modulate the expression and function of CKIs to ensure that cells do not progress to the next phase of the cell cycle before appropriate conditions have been reached. CDK/cyclin activity modulates E2F/DP- and retinoblastoma protein (Rb)-dependent transcription of target genes involved in cell cycle control and DNA biosynthesis (Figure 2). In non-proliferating cells, lack of CDK/cyclin activity leads to the accumulation of hypophosphorylated Rb, which binds to and inactivates the dimeric transcription factor E2F/DP. In proliferating cells, CDK/cyclin activation causes the accumulation of hyperphosphorylated Rb during late G1-phase, thus causing the release of E2F/DP and...

* Corresponding author. Tel: +34 914531200, Fax: +34 914531265, Email: vandres@cnic.es

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the transactivation of various target genes necessary for cell cycle progression.

Cellular proliferation within atherosclerotic and restenotic plaques has been predominantly observed in vascular smooth muscle cells (VSMCs) and macrophages. Animal studies and tissue culture experiments have demonstrated that several cardiovascular risk factors promote the proliferation of these cell types, and there is evidence that these risk factors affect the expression or activity of CDK/cyclins and CKIs. For example, the mitogenic effects of oxLDL and homocysteine on VSMCs have been associated with higher expression of several cyclins and increased activity of CDKs. Similarly, angiotensin II treatment increases cyclin D1 and CDK4 expression and decreases p21 Cip1 and p27Kip1 levels in rat mesenteric arteries. This review discusses animal and human studies that examine the role of cell cycle regulators in native and graft atherosclerosis. Although these proteins also play an important role in restenosis post-angioplasty, this is beyond the objectives of this article and is reviewed elsewhere.

2. Cell cycle regulators in experimental atherosclerosis

This section reviews animal studies assessing the role in atherosclerosis of various proteins involved in cell cycle regulation, including the transcriptional regulators p53 and Rb, and the Cip/Kip family members p27Kip1 and p21Cip1 (summarized in Table 1). To date, no animal model studies have been reported on the role in atherosclerosis of p57Kip2, p15INK4b, p16INK4a, p18INK4c and p19INK4d. However, such studies appear warranted, given the recent genome-wide association studies suggesting a role for the INK4/ARF locus in the development of coronary artery disease (CAD) and myocardial infarction (MI) (see below).

2.1 p53

The tumour suppressor p53 mediates the cellular response to a variety of stresses mainly by regulating the transcription of over 150 genes. Active p53 can induce reversible growth arrest in the G1 or G2 phases of the cell cycle, as well as cellular senescence or apoptosis. The first evidence supporting a role for p53 in the development of atherosclerosis was reported by Guevara et al., who demonstrated that p53 deficiency accelerates atheroma development in the aorta of apolipoprotein E (apoE)-null mice, coinciding with increased cell proliferation within the atheroma. Later, Mercer et al. confirmed the enhanced aortic atherosclerosis of mice doubly deficient for p53 and apoE compared with apoE-null controls. However, in this latter study, p53 deficiency did not significantly increase atheroma development in the brachiocephalic artery, suggesting that the atheroprotective actions of p53 depend on the vascular bed being examined. Lesions in the brachiocephalic artery of p53-deficient animals contained an increased proportion of proliferating cells and reduced numbers of apoptotic cells.
cells. Apoptosis within the atheroma affected both macrophages and VSMC, whereas most proliferating cells were monocytes/macrophages.12

The role of p53 in atherosclerosis development has also been assessed by using bone marrow transplantation (BMT) strategies to selectively inactivate p53 in haematopoietic cells. Van Vlijmen et al.13 reported that lethally irradiated apoE*3-Leiden mice transplanted with p53-deficient bone marrow show a significant increase in aortic atherosclerosis compared with mice transplanted with p53-wild-type marrow. This finding coincided with a non-significant tendency towards decreased apoptosis in mice receiving p53-deficient marrow, and the authors suggested this to be the mechanism underlying their findings. In contrast to previous studies of the effect of global inactivation of p53,11,12 cell proliferation within the atheroma was not affected by p53-deficient BMT.13 Merched et al.14 used a similar approach to analyse the effect of p53 haematopoietic inactivation on atheroma development in LDLR-null mice, another widely used model of atherosclerosis. These authors found that mice receiving p53-deficient BMT develop significantly larger aortic atheromas than mice transplanted with p53-wild-type bone marrow. However, in contrast to the results of Van Vlijmen et al.,13 p53-deficient BMT was associated with exacerbated cell proliferation within the atheroma.14 Using a different approach, Mercer et al.15 found that transplantation of p53 wild-type bone marrow into p53-deficient apoE-null mice reduced aortic plaque formation and neointimal cell proliferation in brachiocephalic arteries, but also markedly reduced apoptosis. Very recently, Boesten et al.15 showed that atherosclerosis development in the aortic root, aortic arch, and thoracic aorta of fat-fed apoE-null mice is unaffected by Cre-loxP-mediated macrophage-selective inactivation of p53. Cell proliferation within the atheroma was also unaffected by macrophage-specific p53 deficiency, whereas plaque apoptosis was markedly reduced.

Therefore, while most studies suggest an atheroprotective role for p53, it is unclear whether this is achieved mainly through effects on cell proliferation or apoptosis. The conflicting data among studies might result from experimental differences; for example, type of diet, periods of fat-feeding, and use of animal models with different genetic modifications. Alternatively, they might reflect cell-type-specific effects of p53, since p53-deficient peritoneal macrophages exhibit reduced apoptosis under standard culture conditions, whereas p53-deficient VSMCs exhibit increased apoptosis.12 In this regard, it should be noted that adenovirus-mediated overexpression of p53 induces VSMC apoptosis in a collagen model of murine atherosclerosis, suggesting that the anti-apoptotic actions of p53 in VSMCs in culture might not occur in the atherosclerotic plaque.16 Another possible explanation is the different technologies used to achieve tissue-specific p53 inactivation, since BMT yields gene inactivation in both lymphoid and myeloid haematopoietic lineages, while the Cre-LoxP technology used by Boesten et al.15 inactivates the target gene only in myeloid cells. Moreover, BMT might also provoke gene inactivation in a subset of VSMCs and endothelial cells (ECs), since bone marrow progenitors can also differentiate into these cell types.17–19

Although the underlying mechanism of p53’s atheroprotective action remains undefined, strategies to increase its expression or function might be predicted to inhibit atherosclerosis. However, p53 gain-of-function studies performed in our laboratory appear to challenge this possibility, since atherosclerosis is unaffected in
apoE-null mice carrying an additional p53 allele that reproduces the normal expression and regulation of the endogenous p53 gene (apoE-null Super-p53 mice). In this study, increased p53 function did not provoke significant differences in the size of atherosclerotic lesions within the ascending aorta, aortic arch, or thoracic aorta, irrespective of feeding with a high-fat diet or standard chow. Cell proliferation and apoptosis within the atheroma were similarly unaffected. These findings demonstrate that moderate p53 gain-of-function does not limit atheroma development in mice, and thus shed doubt on the utility of p53 activation as a means of preventing atherosclerosis. However, it remains possible that more intense increases in p53 function might have therapeutic potential.

### 2.2 Retinoblastoma protein

Rb plays a major role in cell proliferation and apoptosis. It is well established that hypophosphorylated Rb induces cell cycle arrest in G1 by two mechanisms: first, it impedes E2F-mediated transcription of genes involved in cell cycle progression (Figure 2), and secondly it actively represses transcription through the formation of the Rb-E2F transcription repressor complex. The role of Rb in the regulation of apoptosis was identified in loss-of-function studies, showing that Rb deficiency in mice triggers p53-dependent apoptosis and results in embryonic lethality.

The effects of Rb disruption on atherogenesis have been studied in apoE-null mice with selective inactivation of Rb in macrophages (macrophage-Rb<sup>del</sup> apoE-null mice). These animals develop larger and more advanced aortic atheromas than apoE-null mice, with a decreased macrophage content and an increased percentage of VSMCs. Apoptosis within the atheroma is almost undistinguishable between the two groups, indicating that macrophage Rb plays a minor role in cell-death within the atheroma. In contrast, the authors found a significant increase (2.6-fold) in lesional macrophage proliferation in macrophage-Rb<sup>del</sup> apoE-null mice, suggesting that inhibition of macropage proliferation is the major mechanism underlying the atheroprotective properties of Rb. Since Rb also modulates VSMC proliferation, senescence, and apoptosis, future studies are warranted to investigate the effects on atherosclerosis development of selective Rb inactivation in VSMCs.

### 2.3 p27<sup>Kip1</sup>

The Cip/Kip family member p27<sup>Kip1</sup> is a CKI that plays a major role in the control of the mammalian cell cycle in various pathophysiological settings. p27<sup>Kip1</sup> is maximally translated and stable in quiescent cells, and contributes to growth arrest through the inhibition of cyclin-CDK complexes. Upon mitogenic stimulation, p27<sup>Kip1</sup> is rapidly downregulated, allowing activation of cyclin E–

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#### Table 1 Atherosclerosis development after genetic manipulation of cell-cycle regulators in the mouse

<table>
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<tr>
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<th>Genetic modification</th>
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<th>Effect on cell proliferation</th>
<th>Effect on apoptosis</th>
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<td>Increase (brachiocephalic)</td>
<td>Reduction</td>
<td>Mercer et al.</td>
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<td>p53&lt;sup&gt;+/+&lt;/sup&gt; BMT in p53&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Reduction (aorta); none</td>
<td>Reduction (brachiocephalic)</td>
<td>Reduction</td>
<td>Mercer et al.</td>
</tr>
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<td>None</td>
<td>Reduction</td>
<td>Boesten et al.</td>
</tr>
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<td>p53&lt;sup&gt;−/−&lt;/sup&gt; BMT in p53&lt;sup&gt;+/+&lt;/sup&gt; mice</td>
<td>Increase</td>
<td>Increase</td>
<td>None</td>
<td>Merced et al.</td>
</tr>
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<td>apoE-null</td>
<td>One extra p53 allele (normally regulated)</td>
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<td>None</td>
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<tr>
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<td>Macrophage-specific Rb deficiency</td>
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<td>Increase</td>
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</tr>
<tr>
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<td>p27&lt;sup&gt;Kip1&lt;/sup&gt; global inactivation</td>
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<td>Increase</td>
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<td>Diez-Juan and Andrés</td>
</tr>
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<td>apoE-null</td>
<td>p27&lt;sup&gt;Kip1&lt;/sup&gt;−/− BMT in p27&lt;sup&gt;Kip1&lt;/sup&gt;&lt;sup&gt;+/+&lt;/sup&gt; mice</td>
<td>Increase</td>
<td>Increase</td>
<td>Not studied</td>
<td>Diez-Juan et al.</td>
</tr>
<tr>
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<td>Increase</td>
<td>Merced and Chan</td>
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<td>Reduction</td>
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<td>Increase</td>
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<td>Increase</td>
<td>Not studied</td>
<td>Not studied</td>
<td>Khanna</td>
</tr>
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BMT, bone marrow transplant. Only studies reporting statistically significant effects are included.
CDK2 and cyclin A–CDK2 complexes and the subsequent transcriptional activation of genes required for the G1/S transition and the initiation of DNA replication. 25–26

p27 acts as a tumour suppressor. 27 Mouse studies performed in our laboratory revealed that it also has anapoptotic functions. We first showed that genetic inactivation of p27Kip1 greatly accelerates diet-induced atherogenesis in apoE-null mice ( ∼ 6-fold), coinciding with enhanced proliferation of macrophages and VSMCs within the atheroma ( ∼ 4-fold). 28 Moreover, analysis of apoE-null mice with one p27Kip1 allele inactivated revealed that a moderate decrease in p27Kip1 protein expression is sufficient to hasten atherosclerosis development in this model. In a subsequent study, we demonstrated that haematopoietic cell-selective disruption of p27Kip1 in apoE-null mice enhances arterial macrophage proliferation and accelerates aortic atherosclerosis. 29

Interestingly, this strategy also augmented aortic expression of the proinflammatory cytokines CCL2/MCP-1 and CCL5/RANTES, suggesting that p27Kip1 might have proliferation-independent functions that affect the inflammatory response within the atheroma. In this regard, in addition to modulating proliferation, p27Kip1 regulates other cellular processes that may contribute to the atherosclerosis development, such as migration, apoptosis, and autophagy. 30–37 Moreover, p27Kip1 interacts with a number of proteins that might influence atheroma development, such as the small GTPases RhoA 31 and Rac 36 and the signalling adaptor Grb2. 38,39 It remains to be demonstrated whether these proliferation-independent functions and/or interactions with signalling proteins contribute to the atheroprotective properties of p27Kip1.

The molecular mechanisms that regulate p27 expression and function in the arterial wall remain ill defined. It is well established that p27Kip1 protein levels are mainly regulated by post-translational modifications. One of the most consistently demonstrated modifications of p27Kip1 is its phosphorylation at threonine 187 (T187), which is required for the degradation of p27Kip1 at the G1/S transition and in the G2 phase prior to mitosis. 26 To investigate whether this post-translational modification of p27Kip1 plays a role in atherosclerosis, our laboratory generated apoE-null mice with both p27Kip1 alleles replaced by a version carrying a T A A mutation at position 187 to block phosphorylation at this residue (apoE-null p27T187A mice). 40 We found that aortic p27Kip1 expression was unaffected by the T187A mutation, and atheroma size, lesion cellularity, cell proliferation, and apoptosis were undistinguishable between fat-fed apoE-null p27T187A and apoE-null mice. Hence, it can be concluded that phosphorylation of p27Kip1 at T187 is not implicated in the control of aortic p27Kip1 expression and atherosclerosis in hypercholesterolemic mice. Future studies are therefore warranted to analyse the effect of additional post-translational modifications of p27Kip1 on atherosclerosis development.

2.4 p21Cip1

The Cip/Kip family member p21Cip1 is a major downstream target of p53 that modulates cell proliferation. Global inactivation of p21Cip1 reduces atherosclerosis burden in apoE-null mice fed either standard chow or a proatherogenic high-fat diet. 41,42 Moreover, transplantation of p21Cip1-deficient bone marrow into apoE-null mice also reduces atheroma size, thus demonstrating a protective effect of p21Cip1-deficiency in macrophages. This contrasts with studies on p53, p27Kip1, and Rb, whose total or partial inactivation in mice aggravates atherosclerosis (see above). In vivo incorporation of bromodeoxyuridine (BrDU) into 20-week-old apoE-null mice fed a normal chow diet revealed no differences in lesional cell proliferation between p21Cip1-deficient and p21Cip1-wild-type mice, suggesting that the atherogenic actions of p21 are unrelated to its role as a cell cycle regulator. However, cell proliferation in the atherosclerotic plaque probably occurs at specific stages of lesion formation; so additional studies, with mice at different ages and fed with high fat for different periods, are required to precisely establish the consequences of p21Cip1 inactivation on lesional cell proliferation. Merched and Chan 42 found significant upregulation of other growth suppressors upon inactivation of p21Cip1, such as p16Ink4a, Rb, and p53, and suggested that this might compensate the effect of p21Cip1 deficiency on cell proliferation. Specifically, the authors hypothesize that the increased levels of p16Ink4a, a strong inhibitor of CDK4, would displace other Cip/Kip family members (p27Kip1 and p57Kip2) from CDK4/cyclin D inhibitory complexes, making them available to inhibit CDK2 and thereby decelerating cell cycle progression and proliferation in the absence of p21Cip1.

Consistent with the previously reported antiapoptotic role of p21Cip1, 43 Merched and Chan 42 found increased lesion apoptosis after both global and macrophage-specific inactivation of p21Cip1 (1.8- and 3-fold, respectively). Moreover, gene expression profiling of thioglycolate-elicited peritoneal macrophages from p21Cip1-null mice revealed higher expression of putative atheroprotective factors (SR-BI, macrophage scavenger receptor A, and LDL-receptor related protein) and lower levels of proinflammatory molecules (macrophage inflammatory proteins 1 and 2 and interleukin-1α). p21Cip1-deficient macrophages also exhibited a 2-fold increased phagocytic activity towards fluorescent latex microspheres and apoptotic thymocytes. In summary, Merched and Chan’s 42 work demonstrates that p21Cip1 is a proatherogenic molecule, since its inactivation limits the production of proinflammatory cytokines, enhances immune cell apoptosis, and upregulates phagocytic macrophage activity, which facilitates apoptotic cell clearance and prevents necrosis, thereby promoting plaque stability.

Merched and Chan’s 42 conclusions contrast with a very recent study that reports that inactivation of p21Cip1 renders mice more susceptible to high fat diet-induced atherosclerosis. 44 However, in this second study, the dietary fat level was only moderate, and the mice used were on a mixed genetic background (FVB), had low plasma lipid levels (maximum 100 mg/dL), and did not readily develop atherosclerosis. Moreover, atheroma development was analysed only in the coronary artery. In contrast, Merched and Chan 42 validated their data through the use of two dietary regimens (regular chow and western diet), the study of global and macrophage-restricted ablation of p21Cip1 in a widely used mouse model of atherosclerosis (apoE-null mice), and analysis of atherosclerosis lesions in whole aortas and in cross-sections of the aortic sinus.
3. Cell cycle regulators in experimental graft atherosclerosis

Excessive cell proliferation and neointimal hyperplasia followed by atheromatous plaque development are common features of both vein graft and cardiac allograft atherosclerosis. In this section, we discuss different cell cycle-based strategies employed to inhibit experimental graft atherosclerosis (summarized in Table 2). We also discuss several animal studies that highlight the important role of the p53-p21Cip1 axis in this pathology.

3.1 Antisense oligodeoxynucleotides (ODNs) targeting cell cycle regulators

The use of complementary oligodeoxynucleotides (ODNs) to target CDK mRNAs and transcription factors involved in cell cycle regulation has been mainly investigated in experimental graft atherosclerosis. Mann et al.45,46 demonstrated the beneficial effects of selectively blocking the expression of the cell cycle regulators CDK1 (also called CDC2) and proliferating cell nuclear antigen (PCNA) in rabbit jugular veins grafted into carotid arteries. These engineered grafts underwent a phenotypic shift from hyperplasia towards hypertrophy, maintained a normal endothelial phenotype, and were resistant to diet-induced atherosclerosis. The benefits of this strategy have been confirmed by cotransfection of antisense ODNs to PCNA and CDK1 in a rat cardiac allograft model.47 Moreover, transfection of antisense ODNs to PCNA alone significantly inhibits intimal hyperplasia in experimental rabbit vein grafts,48 while transfection of antisense ODNs to CDK1 alone has protective effects in a mouse coronary allograft model.49 Increased expression of CDK1 has been detected in the coronary arteries of chronically rejected allografts of non-human primates,50 suggesting that this kinase is a key player in graft vasculopathies and a potential target for gene therapy. Another target of potential therapeutic interest is CDK2, since enhanced CDK2 mRNA expression has been found in the thickened intima of coronary arteries of mouse heterotopic cardiac allografts, and antisense CDK2 ODNs efficiently inhibit neointima formation and VCAM-1 expression in this disease model.51 Antisense strategies have also targeted several 'immediate-early' genes implicated in vascular cell proliferation. Suggs et al.52 reported increased expression of c-fos and c-jun after perfusion of rat vein grafts, and showed that treatment of grafts with antisense ODNs to these 'immediate-early' genes significantly reduced the thickness of the intimal layer in the perianastomotic and midgraft regions. Similarly, expression of the c-myc oncogene has been correlated with VSMC proliferation during the period of maximal intimal thickening in rat experimental vein grafts,53 and intraoperative application of antisense c-myc oligomers has been reported to inhibit neointima formation in a porcine vein graft model.54,55 Similar protective effects on intimal hyperplasia have been demonstrated for locally delivered antisense ODNs to the proto-oncogene c-myb in rabbit experimental vein grafts.56

3.2 Decoy ODNs targeting cell cycle regulators

Other genetic approaches have used double-stranded ODNs encoding the consensus-binding sequences for transcription factors involved in the activation of atherogenic genes ('decoy oligonucleotides'). Treatment of experimental grafts with decoy ODNs for E2F has been extensively explored, and has provided strong evidence of reduced neointimal hyperplasia in mouse and non-human primate cardiac allografts57–59 and in rabbit60,61 and canine62 vein grafts. In these studies, E2F blockade correlated with VSMC growth arrest and suppression of positive cell cycle regulatory genes in the graft wall, and also with downregulation of inflammatory mediators such as NFkappaB58 and E-selectin,59 without inhibiting endothelial repopulation after vein bypass.61 Moreover, in the setting of diet-induced hypercholesterolaemia, delivery of E2F decoy ODNs provides vein grafts with long-term resistance to atherosclerosis.60 Chimeric decoy ODNs, inhibiting both E2F and NFkappaB, reduced intimal hyperplasia and accelerated re-endothelialization in a rabbit model of vein graft, and these

<table>
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<td>c-fos/c-jun</td>
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effects correlated with reduced accumulation of VSMCs and macrophages and with decreased expression of PDGF, VCAM-1, and MCP-1. Although it is beyond the scope of this review, it is worth noting that the E2F decoy strategy (edifoligide) has been explored in a series of clinical trials known as the PRoject of Ex vivo Vein graft ENgineering via Transfection (PREVENT). While confirming the safety and viability of E2F decoy ODNs treatments, the four trials conducted to date have found that this treatment was clinically ineffective at preventing human graft failure (reviewed in 64).

3.3 The p53-p21\textsuperscript{Cip1} axis in experimental graft atherosclerosis

Several studies have highlighted the role of p53 and its target p21\textsuperscript{Cip1} in experimental graft atherosclerosis. Neointimal hyperplasia of vein grafts is significantly increased in p53-null mice, coinciding with significantly reduced apoptosis, and augmented and reduced lesional content of VSMCs and macrophages, respectively. Moreover, cultured VSMCs exhibit increased proliferation and migration and decreased apoptosis in response to sodium nitroprusside, thus suggesting that p53 deficiency accelerates neointima formation by promoting VSMC proliferation and abrogating cell apoptosis. In line with these findings, adenovirus-mediated p53 overexpression significantly upregulates apoptosis and reduces neointimal proliferation in porcine vein grafts, thereby limiting neointimal thickening.

In a rat model of vein arterialization, p21\textsuperscript{Cip1} is gradually down-regulated, reaching a minimum by day 7 that is sustained until day 90, suggesting that reduced p21\textsuperscript{Cip1} levels may contribute to vascular cell proliferation in this pathological model. Although the same study revealed no changes in p27\textsuperscript{Kip1} and p16\textsuperscript{Nk4a} expression, further studies are required in other graft models to conclusively address the role of CKIs in graft atherosclerosis, since regulation of these proteins appears to vary depending on the vascular bed. Interestingly, overexpression of p21\textsuperscript{Cip1} in rabbit vein grafts by intraoperative transfection not only significantly inhibits neointima formation but also induces maturation of VSMCs from the neonatal to the adult phenotype. Additionally, oral administration of the antithrombotic drug tranilast in murine cardiac transplantation models inhibits cardiac allograft vasculopathy in association with increased neointimal expression of p21\textsuperscript{Cip1} and decreased expression of PCNA.

4. Cell cycle regulators in human atherosclerosis

The importance of proliferation in the growth of atherosclerotic plaques has been extensively studied in human vascular obstructive lesions. In this section, we summarize several reports that analyse cell proliferation and the expression and localization of cell cycle regulators within human atherosclerotic plaques.

4.1 Cell proliferation in human atherosclerosis

The occurrence of cell proliferation in human vascular obstructive disease is demonstrated by the consistent finding of proliferation markers in human primary atheromatous plaques and restenotic lesions. However, it should be noted that some studies have reported very low proliferation rates, while others find high proliferative activity in human atherosclerotic and restenotic lesions. Several factors may contribute to these conflicting findings, including technical differences (differences in tissue fixatives, antigen accessibility, analysis of different proliferation markers, etc.), differences in the arteries being analysed (for example, peripheral, coronary, and carotid arteries), and variation in the state of development of the atheroma (for example, fatty streaks, fibrolipid plaques, or complicated atheromas). Supporting this last possibility, complicated atheromas in human carotid artery samples (type VI) have recently been reported to exhibit higher proliferation rates than non-complicated fibrous lesions (type V).

Proliferating cells within human atherosclerotic plaques include VSMCs, leukocytes, and ECs. Rekhter and Gordon demonstrated divergence in the proliferation rates of these different cell types in advanced atheromas from human carotid arteries. While monocyte/macrophages are the predominant proliferative cell type in the intima (46% monocyte/macrophages, vs. 9.7% α-actin immunoreactive VSMCs, 14.3% ECs, and 13.1% T-lymphocytes), proliferating VSMCs predominate in the media (44.4% VSMCs vs. 20% ECs, 13% monocyte/macrophages, and 14.3% T-lymphocytes). This study also revealed higher proliferation rates in the intimal lesion than in the underlying media (1.61 ± 0.35 vs. 0.05 ± 0.03%, respectively), suggesting that different distributions of growth regulatory proteins and stimuli exist in different regions of human atherosclerotic arteries.

4.2 Expression and localization of cell cycle regulators in human atherosclerosis

As mentioned earlier, studies in animal models have demonstrated both atheroprotective and atherogenic actions for different cell cycle regulators. Human studies have demonstrated differential expression of these proteins in atherosclerotic lesions and healthy vessels, thus highlighting the role of cell cycle regulators in atherosclerosis development.

Quantitative immunoblotting analyses have demonstrated low p27\textsuperscript{Kip1} levels in human atherosclerotic and restenotic coronary arteries compared with aorta, internal mammary artery, and carotid artery thrombendarterectomy specimens. In the same study, p21\textsuperscript{Cip1} was found to be upregulated in restenotic lesions compared with primary lesions and other vascular vessels. In another report, Tanner et al. analysed CKI expression in healthy and atherosclerotic specimens of human coronary artery. Expression of p27\textsuperscript{Kip1} was abundant in non-proliferating cells within normal and atherosclerotic arteries, whereas p21\textsuperscript{Cip1} was undetectable in normal arteries but markedly upregulated in atherosclerotic tissue. Moreover, p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} expression was most frequently found in non-proliferating regions within the atheroma, suggesting an inverse correlation between arterial cell proliferation and CKI expression. Interestingly, p27\textsuperscript{Kip1} and TGF-β receptors are co-expressed in human atherosclerotic coronary artery specimens, suggesting that the anti-mitogenic action of TGF-β in these lesions might be mediated by p27\textsuperscript{Kip1}. 
The transcriptional regulator p53 is overexpressed, but not mutated in human atherosclerotic tissue. Moreover, Ihling et al.89 reported that p53 and p21Cip1 are co-expressed in non-proliferating regions within advanced human carotid atherosclerotic plaques, thus indicating that p53-dependent transcriptional activation of p21Cip1 might protect against excessive vascular cell growth. The same group reported p53 expression in apoptotic cells within human atherosclerotic plaques.88 Moreover, p53 and MDM2, a nuclear protein which promotes p53 degradation, co-localize in a few Ki67-positive cells, suggesting that the p53-dependent apoptosis or re-entry into the cell cycle might depend on the relative abundance of these two proteins.

In addition to their roles as inhibitors of cell proliferation, p16Ink4a, p21Cip1, and p53 might also play an important role in cellular senescence, a process relevant to advanced atherosclerosis.90 Recent studies have revealed increased expression of p16Ink4a and p21Cip1 in human coronary plaques, co-localizing with the activity of the senescence marker β-galactosidase (SAβG).91 Interestingly, cells from atherosclerotic lesions contain shorter telomeres,91 another characteristic linked to cell senescence and cardiovascular disease.92 Marfella et al.93 also found that the expression of p16Ink4a and p53 is markedly upregulated in atherosomas from elderly patients compared with younger patients undergoing carotid endarterectomy.

4.3 Expression of cell cycle regulators in human graft atherosclerosis

As discussed earlier, animal studies highlight the role of the p53-p21Cip1 pathway in the pathophysiology of graft atherosclerosis. There is also evidence of p53 and p21Cip1 upregulation in human graft atherosclerosis. McLaren et al.94 found elevated expression of p53 in cardiac allograft recipients with acute rejection, and suggested this oncosuppressor as a potential myocyte damage marker for the diagnosis of acute cardiac allograft rejection. Similarly, high levels of p53 were observed in atherosclerotic areas of human aorto-coronary saphenous vein bypass grafts, with a strong correlation between p53 expression and apoptosis (as determined by TUNEL staining) in the intima, but not in the media of vein grafts.95 In contrast to these findings, Baas et al.96 found almost undetectable p53 expression in coronary arteries from transplanted hearts, although its transcriptional target p21Cip1 was highly expressed.

5. Genetic evidence for the role of cell cycle regulators in human atherosclerosis

It is now widely accepted that the classic environmental risk factors only partly explain the development of atherosclerosis, and that genetic risk factors are critically involved in this pathology and its clinical manifestations. Genetic polymorphisms associated with atherosclerosis usually affect endothelial function, inflammation, lipid metabolism, or the thrombosis and fibrinolysis cascades. However, recent genetic studies have found robust genetic polymorphisms in genes involved in cell cycle regulation (mostly CKIs), underlining the important role of cell proliferation in atherogenesis.

5.1 The 9p21 genetic polymorphisms and the INK4/ARF locus

The strongest and most replicated susceptibility locus for CAD and MI so far identified in humans is located on chromosome 9p21, and comprises several single nucleotide polymorphisms (SNPs) that have been consistently associated with these diseases in several independent Caucasian and Asian populations97–103 (and others, reviewed in104), but not in African American subjects.97,105 This susceptibility locus is also associated with other vascular diseases, such as abdominal aortic and intracranial aneurysms.106 The most replicated 9p21 SNPs associated with CAD and MI lie in a region 100 Kb centromeric to the INK4/ARF locus, thus suggesting a link between this locus and atherogenesis. The INK4/ARF locus plays essential roles in cell proliferation, apoptosis, and senescence, and encodes two CKIs (p15Ink4b and p16Ink4a), the p53-regulatory protein ARF, and a recently discovered non-coding RNA named ANRIL (for antisense noncoding RNA in the INK4a locus). Strikingly, expression of all INK4/ARF transcripts is reduced in the peripheral blood T-cells of individuals homozygotic for a common 9p21 SNP associated with increased risk of atherosclerosis (rs10757278).107 This provides a direct link between these atherosclerosis-associated SNPs and the INK4/ARF locus and suggests a key role for p15Ink4b, p16Ink4a, ARF, and ANRIL in protecting against atherosclerosis development. Studies with genetically modified mice are warranted to assess whether INK4/ARF gene products indeed affect atherogenesis.

5.2 Genetic polymorphisms in CKIs of the Cip/Kip family

Several genetic studies have identified MI-associated variants in human genes encoding CKIs of the Cip/Kip family. For example, CDKN1C, the gene encoding p57Kip2, contains two polymorphisms (a promoter GT-repeat and a variable number of repeats of the amino acid PAPA-motif) that were found to be associated with MI.108 Similarly, the ACGT > A SNP within the CDKN1B gene (encoding p27Kip1) was found to be associated with increased risk of MI and with reduced basal p27Kip1 promoter activity in proliferating human cells.109 However, this SNP has also recently been reported to confer a decreased risk of in-stent restenosis and a 20-fold increase in basal p27Kip1 promoter activity in quiescent human cells.110 This apparent discrepancy, between decreased risk of in-stent restenosis and increased risk of MI, might be explained by the divergent pathophysiological roles of VSMCs in restenotic and atherosclerotic lesions. In in-stent restenosis, decreased VSMC proliferation can reduce neointima formation, but in atherosclerotic plaques it can also diminish fibrous cap thickness, increasing plaque vulnerability and potentially accounting for the increased risk of MI. Regarding the divergent effects of this SNP on p27Kip1 promoter activity, it should be noted that this activity was assayed in different cell types (Jurkat vs. HEK293 cells) and in different culture conditions (proliferating vs. quiescent cells). Further studies are therefore required to conclusively define the consequences of the ACGT > A SNP on p27Kip1 expression.
Moreover, larger cohorts need to be analysed to validate these genotype-disease associations.

6. Concluding remarks

Mounting evidence demonstrates that excessive cell proliferation is an essential hallmark of atherosclerosis development, both in animal models and in human beings. Therefore, proteins involved in the regulation of cell proliferation in the vascular wall are receiving special attention as potential therapeutic targets for the treatment of vascular diseases. Studies in genetically engineered mice have conclusively demonstrated the atheroprotective properties of the growth suppressors p53, p27Kip1, and Rb and the surprising proatherogenic function of p21Cip1, despite its well-defined cyto-static activity. Interestingly, apart from their roles in regulating cell proliferation, these proteins also appear to modulate other cellular processes within the atheroma, such as apoptosis and cell senescence. Further animal studies are warranted to analyse the role of other cell cycle regulators in the development of atherosclerosis, especially in light of the genetic evidence suggesting important roles for CKIs and ARF in the development of CAD and MI in humans.

Although the evidence is conclusive that altering the cell cycle machinery affects atherosclerosis development in animal models, we are unaware of clinical trials targeting cell cycle regulators for the treatment of native atherosclerosis. However, given that administration of rapamycin (sirolimus) alleviates atherosclerosis in the mouse (reviewed in111), prospective long-term trials should be instigated to assess whether renal and heart transplant recipients treated with this immunosuppressive and antiproliferative drug exhibit a reduction in morbidity and mortality attributable to cardiovascular disease. In the case of graft atherosclerosis, results obtained from animal studies have already been translated into therapeutic strategies, although so far these have proved to be unsuccessful in humans. Therefore, as our knowledge of the role of cell cycle regulators in atherosclerosis grows, the challenge will be to translate all this information into valuable diagnostic and therapeutic tools for humans, as has been achieved in the setting of post-angioplasty restenosis with the use of stents engineered to deliver cytostatic drugs.

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