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Invited Review

Vascular smooth muscle cell proliferation in the pathogenesis of atherosclerotic cardiovascular diseases

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Summary. Atherosclerosis is the principal cause of myocardial infarction, stroke, and peripheral vascular disease, accounting for nearly half of all mortality in developed countries. For example, it has been estimated that atherosclerosis leads to approximately 500,000 deaths from coronary artery disease and 150,000 deaths from stroke every year in the United States (American Heart Association, 1996). Percutaneous transluminal angioplasty has become a well-established technique for revascularization of occluded arteries. However, the long-term efficacy of the procedure remains limited by progressive vessel renarrowing (restenosis) within the following few months after angioplasty. Abnormal vascular smooth muscle cell (VSMC) proliferation is thought to play an important role in the pathogenesis of both atherosclerosis and restenosis. Accordingly, considerable effort has been devoted to elucidate the mechanisms that regulate cell cycle progression in VSMCs. In the present article, we will review the different factors that are involved in the control of VSMC proliferation, especially in the context of cardiovascular disease. Ultimately, a thorough understanding of these regulatory networks may lead to the development of novel drug and gene therapies for the treatment of cardiovascular diseases. Therapeutic approaches that targeted specific cell-cycle control genes or growth regulatory molecules which effectively inhibited neointimal lesion formation will be also discussed.

Key words: Smooth muscle cell, Cell cycle, Atherosclerosis, Restenosis, Gene therapy

Importance of VSMC proliferation in the pathogenesis of atherosclerosis and restenosis

Atherosclerosis is a complex process characterized by the formation of a neointimal lesion that progressively occludes the arterial lumen. Neointimal thickening is due to the accumulation of cellular and extracellular substances in the space between the endothelial cell lining (intima) and the underlying medial VSMCs. According to the response-to-injury hypothesis, atherosclerosis is triggered by different risk factors (hypercholesterolemia, aging, hypertension, cigarette smoking and diabetes) that can somehow lead to endothelial dysfunction (Ross, 1993). The normal endothelium plays numerous physiological roles including: 1) provision of a nonthrombogenic, semi-permeable surface; 2) regulation of vascular tone by release of small molecules that modulate vasodilation (nitric oxide, PGI2) or vasoconstriction (endothelin); 3) secretion of different growth regulatory molecules and cytokines; 4) provision of a nonadherent surface for leukocytes and 5) ability to modify (oxidize) lipoproteins that are transported into the arterial wall. Once one or many of these endothelial properties have been altered, a succession of events can lead to the formation of fatty streaks, the earliest recognizable lesion of atherosclerosis, and ultimately to fibrous and fibrocalcified plaques. Studies in hypercholesterolemic animals and in human atherosclerotic arteries have identified three processes involved in the formation of the atherosclerotic lesion (Ross, 1993): 1) the proliferation of VSMCs, macrophages and possibly lymphocytes; 2) the formation by VSMCs of a connective tissue matrix comprising elastic fibre proteins, collagen and proteoglycans; and 3) the accumulation of lipid and mostly free and esterified cholesterol in the surrounding matrix and the associated cells. VSMCs play the principal role in the fibroproliferative component of the disease process, because it is the principal source of the connective tissue in the arterial wall (Ross, 1993). Numerous observations suggest that VSMCs in atherosclerotic lesions have changed from a ***contractile*** to a ***synthetic*** state (Campbell and Campbell, 1990). In the ***synthetic*** state, VSMCs can respond to different growth factors and synthetize extracellular matrix (Sjölund et al., 1990). "Activated" VSMCs can also migrate toward the arterial lumen and express abundant

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levels of novel matrix components and proteases that modify the surrounding matrix. This "growth and synthetic response" of VSMCs contributes to the development of the neointimal lesion that characterizes atherosclerosis.

Excessive VSMC proliferation is also believed to be involved in the pathophysiology of restenosis, the recurrence of arterial narrowing at the site of balloon angioplasty that occurs in 20-55% of coronary artery disease patients after successful angioplasty (Fuster et al., 1992; Libby et al., 1992; Ross, 1993). It is thought that the acute disruption of the protective endothelial lining at the site of angioplasty triggers this aggressive form of atherosclerosis, which is typically characterized by exuberant VSMC hyperplastic response (Bauters and Isner, 1997; Libby and Tanaka, 1997; Thyberg, 1998), extracellular matrix accumulation (Schwartz et al., 1992; Strauss et al., 1994) and local "remodeling" (elastic recoil) of the dilated vessel (Post et al., 1994; Wilensky et al., 1995). To date, among all the different pharmacological attempts to prevent restenosis (Franklin and Faxon, 1993), only antibody against platelet IIb/IIIa integrin (Topol et al., 1994) and probucol (Tardif et al., 1997; Yokoi et al., 1997; Rodés et al., 1998) have shown possible utility for the reduction of clinical restenosis. Angiographic restenosis rates have also been shown to be reduced following the implantation of intravascular prosthesis (stents) at the angioplasty site. However, the reduction in restenosis rates with these devices has only been modest, from 32% to 22% in one study (Serruys et al., 1994) or from 42% to 32% in another study (Fischman et al., 1994). This limited effect may be explained by the fact that although stents can provide a larger lumen following angioplasty and prevent the local elastic recoil, they are associated with a paradoxical increase in neointimal formation and "late loss" in the months following the procedure (Fischman et al., 1994; Serruys et al., 1994). Therefore, taking into account that a growing proportion of angioplasties are now being performed together with stent implantation, effective therapies to prevent restenosis may rely on reducing VSMC proliferation and migration after balloon injury.

Molecular control of cellular proliferation

In the adult organism, at homeostasis, VSMCs in the vessel wall express differentiation markers and their proliferation index is extremely low. However, mature VSMCs can undergo phenotypic modulation and reenter the cell cycle in response to several physiological and pathological stimuli (Thyberg, 1998). Using different animal models of angioplasty *in vivo*, several investigators have demonstrated a rapid proliferative response of VSMCs in the media, followed by a second peak of proliferation in the neointima which then declines to basal levels within 2 to 6 weeks after vascular injury (Stemerman et al., 1982; Clowes et al., 1983; Clowes and Schwartz, 1985; Majesky et al., 1987; Hanke et al., 1990; Ohno et al., 1994; Stadius et al., 1992; Geary et al., 1996). Moreover, VSMC proliferation (Indolfi et al., 1995b) and neointimal hyperplasia (Asada et al., 1996) have been shown to be influenced by the degree of balloon injury in different animal models. Recently, increased VSMC proliferation after angioplasty has been associated with up-regulation of different components of the cell cycle machinery (see below).

Progression through the cell cycle in mammalian cells is driven by several cyclin-dependent protein kinases (CDKs) that function at different phases of the cell cycle (Motokura and Arnold, 1993; Heichman and Roberts, 1994; Hunter and Pines, 1994; King et al., 1994; Nurse, 1994; Peeper et al., 1994; Sherr, 1994; Morgan, 1995). Activation of CDKs requires their association with members of a family of structurally related proteins called cyclins. The levels of individual cyclins, which fluctuate during the different phases of the cell cycle, are controlled transcriptionally and by the ubiquitin-dependent proteolytic machinery. Different

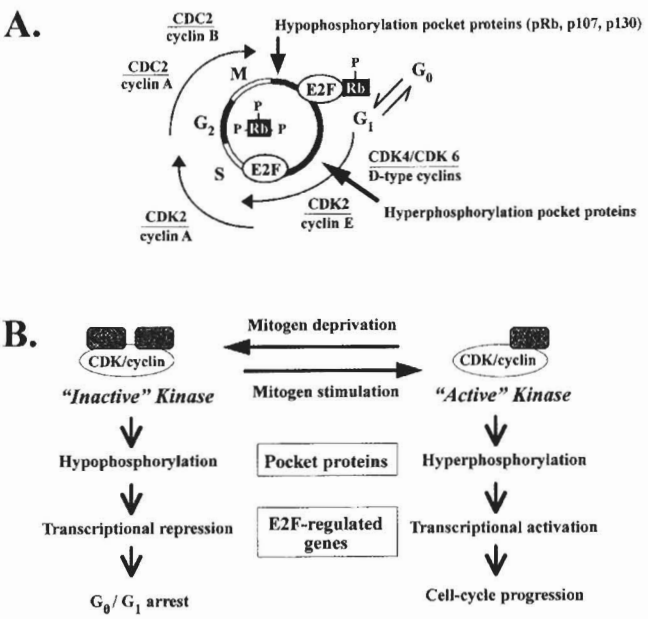

Fig. 1. Cell cycle control in mammalian cells. **A** Progression through the mammalian cell cycle requires the sequential activation of specific CDK/cyclin complexes. Active CDK/cyclin holoenzymes phosphorylate cellular substrates, including the pocket proteins pRb, p107 and p130. It is accepted that hyperphorylation of pocket proteins during G1 blocks their interaction with the transcription factor E2F, thus causing transactivation of genes with functional E2F sites that are required for DNA synthesis (S phase). Subsequently, hypophosphorylated pocket proteins interact again with E2F and repress transcription of E2F-regulated genes. **B.** CDK inhibitory (CKI) proteins associate with CDK/cyclin complexes. Although active CDK/cyclin holoenzymes containing a single CKI molecule have been demonstrated in cultures of proliferating cells, binding of multiple CKI molecules inhibit CDK activity. For example, mitogen deprivation causes upregulation of CKIs, hypophosphorylation of pocket proteins and ultimately G0/G1 arrest. In contrast, mitogen restimulation of starvation-synchronized cells is associated with downregulation of CKIs, thus allowing activation of CDK/cyclin holoenzymes and progression through the cell cycle.

CDK/cyclin complexes are orderly activated at specific phases of the cell cycle (Fig. 1A). Progression through the first gap-phase (G1) requires cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2 holoenzymes. Functional cyclin A/CDK2 complexes are required for DNA synthesis (S phase) and, subsequently, cyclin A/CDC2 and cyclin B/CDC2 pairs are assembled and activated during the second gap-phase (G2) and mitosis (M phase), respectively. Recent evidence has been provided suggesting the requirement of CDK2 for entry into mitosis as a positive regulator of cyclin B/CDC2 kinase activity (Guadagno and Newport, 1996).

Active CDK/cyclin holoenzymes are presumed to hyperphosphorylate the retinoblastoma susceptibility gene product (pRb) and the related pocket proteins p107 and p130 (Fig. 1A,B). The interaction among members of the E2F family of transcription factors and individual pocket proteins is complex and determines whether E2F proteins function as transcriptional activators or repressors (Helin and Harlow, 1993; Weinberg, 1995; Mayol and Graña, 1998). Simplified, it is accepted that phosphorylation of pocket proteins from mid G1 to mitosis is involved in the transactivation of genes with functional E2F sites. The genes activated by E2F include several growth and cell-cycle regulators (i.e., c-myc, N-myc, CDC2, cyclin E, and cyclin A), as well as genes encoding proteins that are required for DNA synthesis (Farnham et al., 1993; Helin and Harlow, 1993; DeGregori et al., 1995; Mayol and Graña, 1998).

CDK activity is negatively regulated by members of a new class of cell cycle regulators, termed CDK inhibitors (CKIs), which associate with and inhibit the activity of CDKs (Elledge and Harper, 1994; Peter and Herskowitz, 1994; Graña and Reddy, 1995; Morgan, 1995) (Fig. 1B). To date, the list of cloned mammalian CKIs includes p15, p16, p18, p19, p21, p27 and p57. In addition to its inhibitory effect on CDK2, p21 can also inhibit DNA replication through direct interaction with proliferating cell nuclear antigen (PCNA) (Flores-Rozas et al., 1994; Waga et al., 1994), and separate domains of p21 are involved in these two activities (Chen et al., 1995; Luo et al., 1995).

Positive regulators of VSMC proliferation

Mechanical stress

In hypertension, VSMCs are exposed to a chronic increased mechanical stress, which is associated with enhanced VSMC proliferation. Likewise, a marked increase in tension occurs transiently at the site of balloon angioplasty. *In vitro* systems have been developed to test whether mechanical stress alone may be transduced into growth stimulatory signals similar to those produced by growth factors. Chronic cyclic strain promoted DNA synthesis in VSMCs isolated from diverse vascular beds and species, including human VSMCs (Sumpio and Banes, 1988; Predel et al., 1992; Wilson et al., 1993; Hishikawa et al., 1994; Calara et al., 1996; Cheng et al., 1996). This stimulatory effect appears to involve activation of phospholipase C and protein kinases A and C in a process mediated by secreted platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Wilson et al., 1993; Hishikawa et al., 1994; Calara et al., 1996; Cheng et al., 1996; Mills et al., 1997). Whereas release of basic FGF (bFGF, also known as FGF-2) from VSMCs was negligible in response to the small strains that may occur in the normal artery, increased mechanical strain induced bFGF release depending on both the frequency and the amplitude of deformation (Cheng et al., 1997). Mechanical stress also increased *c-fos* expression and phosphoinositide turnover in cultured VSMCs (Lyall et al., 1994). Of note is that a single transient mechanical strain can induce VSMC proliferation, in part by autocrine or paracrine release of bFGF (Cheng et al., 1996). Thus, sustained and transient mechanical strain may elicit a proliferative response of VSMCs during hypertension and after angioplasty, respectively. Collectively, these findings suggest that VSMC hyperplasia induced by mechanical stretch and growth factors are mediated, at least in part, by common signal transduction pathways.

Growth factors

Many growth factors and cytokines have been shown to stimulate VSMC proliferation *in vitro* and *in vivo* (Majack, 1987; Majesky et al., 1988; Banskota et al., 1989; Raines et al., 1989; Majack et al., 1990; Sjölund et al., 1990; Thyberg et al., 1990; Hultgårh-Nilsson et al., 1991; Salhany et al., 1992; Ross, 1993). Growth factors that induce VSMC proliferation and are generally upregulated in atherosclerotic lesions include PDGF, bFGF, tumor necrosis factor-α (TNF-α), insulin-like growth factor-1 (IGF-1), heparin-binding epidermal growth factor-like growth factor, interleukin-1 and transforming growth factor-ß (TGFß). Further evidence that bFGF and PDGF might be physiological regulators of VSMC growth has been provided using neutralizing antibodies directed against these growth factors, which inhibited neointimal VSMC accumulation after angioplasty (Ferns et al., 1991; Lindner and Reidy, 1991). Similarly, inhibition of PDGF-ß receptor subunit expression suppressed neointimal thickening (Sirois et al., 1997; Banai et al., 1998; Hart et al., 1999). Conversely, overexpression of bFGF and PDGF promoted neointimal hyperplasia (Nabel et al., 1993a,b).

Signal transduction molecules

As described above, protein kinases A and C participate in VSMC proliferation after being stimulated by growth factors like bFGF and PDGF. However, recent studies indicate that signal transduction molecules can also be directly upregulated by stretch injury that follows balloon angioplasty. Lai et al. (1996) documented an induction of MAPKs p42 (ERK-1) and p44 (ERK-2)

from day 2 to day 8 after injury of rat carotid artery with a slight decrease at day 14. Hu et al. (1997) showed a sustained induction of ERK-2 from 5 minutes to 14 days after rat carotid balloon injury. This was followed by an increase in the expression of the protooncogenes c-fos and c-jun and enhanced AP-1 DNA-binding activity. MAPK activity increased markedly from 15 to 30 minutes after rat carotid artery injury with a return toward basal levels by 11 hours (Lille et al., 1997). However, later time points (>24 hours) were not analyzed in that study. Similarly, a recent study documented a very rapid (within 5 minutes) induction of ERK activity after angioplasty of porcine carotid and coronary arteries (Pyles et al., 1997). These studies suggest that activation of MAPK in response to mechanical manipulation of arteries may be biphasic. In the first phase (<2 hours), there is a rapid activation of the kinase in response to stretch. In the second phase, there is a more slowly developing increase that occurs over the time scale of days. This protracted increase in activity may reflect the transformation of VSMCs from a contractile to a secretory phenotype, or may be mediated by the growth factors induced locally by the injury.

Transcription factors and components of the cell cycle machinery

Numerous studies have identified transcription factors that positively regulate VSMC growth. Expression of a constitutive NF-κB-like activity appears to be essential for proliferation of cultured bovine VSMCs (Bellas et al., 1995). Several protooncogenes (i.e., *c-fos*, *c-jun*, *c-myc*, *c-myb*, *egr-1*) are activated in serum-stimulated VSMCs, and in some cases their overexpression is sufficient to induce VSMC proliferation in vitro (Castellot et al., 1985; Kindy and Sonenshein, 1986; Reilly et al., 1989; Brown et al., 1992; Campan et al., 1992; Bennett et al., 1994; Rothman et al., 1994; Gorski and Walsh, 1995). Higher levels of *c-myc* mRNA are present in VSMCs cultured from atheromatous plaques than in VSMCs from normal arteries (Parkes et al., 1991), and arterial injury induced protooncogene expression (Miano et al., 1990, 1993; Sylvester et al., 1998). Moreover, *c-myc* and *c-myb* antisense oligonucleotides inhibited VSMC proliferation *in vitro* (Pukac et al., 1990; Brown et al., 1992; Simons and Rosenberg, 1992; Bennett et al., 1994; Shi et al., 1993, 1994), and their application prior to balloon angioplasty reduced neointima formation (Simons et al., 1992; Bennett et al., 1994; Shi et al., 1994). Collectively, the above studies have identified peptide growth factors and protooncogenes that are likely to stimulate VSMC growth *in vitro* and *in vivo*.

VSMC proliferation in the balloon-injured rat carotid artery is associated with a temporally and spatially coordinated expression of CDK2 and its regulatory subunits, cyclin E and cyclin A (Wei et al., 1997). Induction of these factors correlated with increased CDK2-, cyclin E- and cyclin A-dependent kinase activity, indicating the assembly of functional CDK2/cyclin E and CDK2/cyclin A holoenzymes in the injured arterial wall. Expression of CDK2 and cyclin E was also detected in human VSMCs within restenotic lesions (Kearney et al., 1997; Wei et al., 1997), suggesting that induction of positive cell-cycle control genes is a hallmark of injury-induced VSMC hyperplasia.

Recent studies have provided significant insight into the control of cell-cycle gene expression in VSMCs. Overexpression of protein kinase C δ (PKC δ) inhibited VSMC proliferation, and this effect was associated with suppression of cyclin D1 and cyclin E expression (Fukumoto et al., 1997). Consistent with the stimulatory effect of Ras-dependent mitogenic signaling on cellular proliferation, evidence has been presented implicating Ras in the activation of the G1 CDK/cyclin/E2F pathway (Winston et al., 1996; Aktas et al., 1997; Kerkhoff and Rapp, 1997; Leone et al., 1997; Lloyd et al., 1997; Peeper et al., 1997; Zou et al., 1997). Moreover, inactivation of Ras inhibited neointimal lesion formation after angioplasty (Indolfi et al., 1995a; Ueno et al., 1997b), suggesting an important role of Ras on VSMC proliferation *in vivo*. Since cyclin A is essential for cell cycle progression and its expression is induced after angioplasty (Wei et al., 1997), we explored a potential link between Ras and cyclin A gene expression in VSMCs (Sylvester et al., 1998). Our results show that Ras is critical for the normal induction of cyclin A promoter activity and DNA synthesis in mitogen-stimulated VSMCs, and overexpression of the AP-1 transcription factor c-fos efficiently circumvented this requirement via interaction with the cAMP-responsive element (CRE, also known as ATF) at position –79 to –72 in the cyclin A promoter (Fig. 2). Binding of endogenous c-fos and CRE binding (CREB) factors to the cyclin A CRE correlated with VSMC proliferation induced by serum *in vitro* and by angioplasty *in vivo* (Sylvester et al., 1998), and angioplasty induced the localized expression of c-fos in VSMCs (Miano et al., 1990, 1993; Sylvester et al., 1998). Thus, c-fos expression and binding to the cyclin A CRE is spatially and temporally consistent with a role for this factor in the stimulation of cyclin A expression and VSMC proliferation after balloon angioplasty. Notably, the E2F site at position –37 to –32 in the cyclin A promoter was essential for both serum- and c-fos dependent induction of cyclin A expression in VSMCs (Sylvester et al., 1998). Taken together, these findings suggest that c-fos and E2F are important components of the signaling cascade that link Ras activity to cyclin A transcription and VSMC proliferation (Fig. 2).

Negative regulators of VSMC proliferation

Using several animal models of arterial injury, it has been shown that "activated" VSMCs resume a quiescent phenotype within 2-6 weeks after angioplasty (Stemerman et al., 1982; Clowes et al., 1983; Clowes

and Schwartz, 1985; Hanke et al., 1990; Geary et al., 1996). Recent studies have identified some of the molecules and regulatory networks responsible for VSMC growth arrest *in vivo*. Balloon angioplasty resulted in the induction of the CKIs p21 and p27 in VSMCs at time points that correlated with reduced CDK2 activity and the decline in VSMC proliferation (Chen et al., 1997; Tanner et al., 1998). Moreover, overexpression of p27 efficiently blocked mitogen- and c-fos-dependent induction of cyclin A promoter activity in cultured VSMCs (Chen et al., 1997; Sylvester et al., 1998). Thus, upregulation of p21 and p27 may contribute to VSMC growth arrest at late time points after angioplasty. In agreement with this hypothesis, adenovirus-mediated overexpression of p21 (Chang et al., 1995; Yang et al., 1996; Ueno et al., 1997a) and p27 (Chen et al., 1997) attenuated neointimal thickening in balloon-injured arteries. It has also been shown that induction of p27, but not p21, is associated with inhibition of VSMC proliferation in cells stably transfected with PKC δ (Fukumoto et al., 1997). Whether PKC δ is involved in the upregulation of p27 after angioplasty *in vivo* remains to be explored. The regulation of CDK inhibitors by integrins and extracellular matrix components in VSMCs is discussed below.

Endothelium-derived nitric oxide (NO), synthesized by a constitutive NO synthase, is thought to play an important role as a physiological vasodilator and inhibitor of VSMC growth (Moncada et al., 1991; Nava et al., 1995). Teleologically, the lack of endothelium-derived NO production due to disruption of the protective endothelial lining after balloon angioplasty might be expected to contribute to VSMC hyperplasia. Consistent with this notion, eNOS-null mice have a much greater degree of intimal growth after arterial injury when compared to wild-type mice (Moroi et al., 1998; Rudic et al., 1998). Moreover, arterial delivery of EC mitogens that accelerated reendothelization also attenuated neointimal hyperplasia after vascular injury (Bjornsson et al., 1991; Asahara et al., 1995; Van Belle et al., 1997). High production of NO by neointimal VSMCs via an inducible pathway (iNO synthase) may also contribute to the restoration of the quiescent phenotype after balloon angioplasty (Yan and Hansson, 1998). Administration of the NO precursor L-arginine (McNamara et al., 1993; Hamon et al., 1994; Le Schwarzacher et al., 1997; Tourneau et al., 1999), or in vivo transfer of NO synthase gene (von der Leyen et al., 1995; Shears et al., 1997, 1998; Chen et al., 1998; Janssens et al., 1998; Varenne et al., 1998) inhibited neointimal lesion development in several animal models, including balloon angioplasty, cholesterol-induced atherosclerosis and allograft atherosclerosis. Conversely, chronic inhibition of NO production accelerated neointima formation in hypercholesterolemic rabbits (Cayatte et al., 1994). Collectively, these results implicate NO as a negative regulator of neointimal hyperplasia. Recent studies have provided significant insight into the mechanism underlying NO-induced VSMC growth arrest (Ishida et al., 1997; Guo et al., 1998). Addition of NO donors to starvation-synchronized VSMCs induced p21 expression and inhibited the activation of CDK2 and phosphorylation of pRb normally seen upon serum restimulation. NO donors also blocked mitogen-dependent upregulation of cyclin A promoter activity and mRNA levels. These studies suggest that repression of cyclin A transcription and p21-dependent inhibition of CDK2 activity contribute to NO-induced VSMC growth arrest. The molecular mechanisms underlying NO-dependent induction of p21 expression and repression of cyclin A transcription need to be explored further.

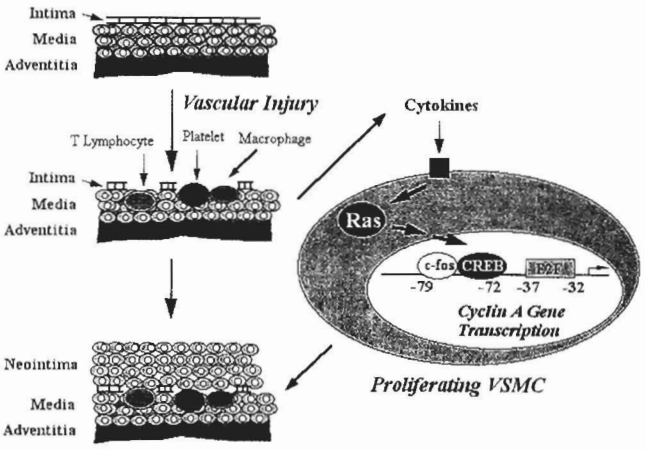

Fig. 2. Ras-dependent regulation of cyclin A gene expression and VSMC proliferation. Ras activity is critical for VSMC proliferation *in vivo* and *in vitro*. Forced overexpression of c-fos can induce cyclin A gene transcription in the absence of Ras, and this effect requires the CRE site at position –79 to –72 in the cyclin A promoter (Sylvester et al., 1998). Moreover, VSMC proliferation induced by serum *in vitro* and by angioplasty *in vivo* correlated with binding of c-fos and CREB factors to the cyclin A CRE, and angioplasty induced c-fos expression in VSMCs. Both the CRE and the E2F site (position –37 to –32) in the cyclin A promoter are essential for mitogen-dependent induction of cyclin A expression in VSMCs. These findings suggest that c-fos, CREB and E2F factors are important components of the signaling cascade that link Ras activity to cyclin A transcription and VSMC proliferation (see text for details).

Little is known about transcription factors that repress VSMC proliferation whose downregulation and/or inactivation might play an important role in atherosclerosis and restenosis. Inactivation of p53 in apoE-null mice has recently been shown to increase cellular proliferation and accelerate atherosclerosis (Guevara et al., 1999). Conversely, over-expression of p53 has been shown to inhibit VSMC proliferation *in vitro* and *in vivo* (Yonemitsu et al., 1998). The homeobox gene *Gax* is highly expressed in cultures of quiescent VSMCs, and its mRNA is rapidly downregulated upon growth factor stimulation of VSMCs *in vitro* and following angioplasty *in vivo*

(Gorski et al., 1993; Weir et al., 1995). Moreover, overexpression of *Gax* inhibited VSMC proliferation *in vitro* and following balloon injury of the rat carotid artery in a p21-dependent manner (Smith et al., 1997a,b).

Regulation of VSMC proliferation by extracellular matrix components

Accumulating evidence indicates that specific components of the extracellular matrix (ECM) and integrins are physiological cell-cycle control elements in atherosclerosis and restenosis (Assoian and Marcantonio, 1996). Neointimal VSMCs within atherosclerotic lesions synthesize novel ECM components and induce the expression of matrix-degrading proteases that remodel the surrounding ECM. For example, matrix-degrading metalloproteinase (MMP) expression is induced within atherosclerotic plaques and after balloon angioplasty (Bendeck et al., 1994; Galis et al., 1994; Zempo et al., 1994; Southgate et al., 1996). Moreover, MMP inhibitors repressed VSMC proliferation in vitro and after angioplasty *in vivo* (Southgate et al., 1992; Zempo et al., 1996; Cheng et al., 1998). Accordingly, these ECM enzymes have been implicated in the induction of neointimal VSMC hyperplasia during atherosclerosis and restenosis. The serine proteases tPA and uPA have also been found to be upregulated following arterial injury in the rat model (Clowes et al., 1990; Jackson et al., 1993; More et al., 1995; Reidy et al., 1996). In addition to their role in migratory activity of VSMCs, it has recently been discovered that human VSMCs express high-affinity tPA receptors (Ellis and Whawell, 1997). Indeed, tPA has been shown to be a potent mitogen for human aortic VSMCs (Herbert et al., 1994). Interestingly, uPA and its receptor (uPAR) seem to be essential for the migration of VSMCs (Noda-Heiny and Sobel, 1995; Noda-Heiny et al., 1995; Okada et al., 1996). In addition, both uPA and uPAR have been detected in different stages of human atherosclerotic lesions (Lupu et al., 1995; Noda-Heiny et al., 1995; Raghunath et al., 1995). The development of mice lacking different components of the plasminogen system has helped to better define the role of these factors in VSMC proliferation *in vivo*. In plasminogen-deficient mice, VSMCs fail to migrate toward the intima, but their replication is unaffected (Carmeliet et al., 1997a,b). Neointimal formation is reduced in the uPA-deficient mouse and in mice deficient in both uPA and tPA, but not in tPA-null mice, suggesting that uPA is the major player in this process (Carmeliet et al., 1997a,b).

Integrins are transmembrane heterodimers that bind to a number of ligands, primarily ECM molecules, and stimulate a variety of transduction pathways (Hynes, 1992). One integrin in particular, αvß3, is thought to interact with osteopontin and play a critical role in regulating cellular functions deemed essential for restenosis including migration, ECM invasion and proliferation of VSMCs (Panda et al., 1997). αvß3 has been found to be expressed by VSMCs in the intima of diseased human coronary arteries (Hoshiga et al., 1995) and is upregulated following balloon injury of baboon brachial arteries (Stouffer et al., 1998). Further evidence of the importance of this integrin in the pathogenesis of restenosis has been provided by showing that selective αvß3 blockade could potently limit neointimal hyperplasia in animal models of arterial injury (Choi et al., 1994; Srivatsa et al., 1997). Interestingly, it has been suggested that inhibition of αvß3 could constitute a potential mechanism for the beneficial effects on clinical restenosis of abciximab (an inhibitor of platelet glycoprotein IIb/IIIa) in patients undergoing high-risk percutaneous coronary interventions (Topol et al., 1994).

Changes in collagen content have been well documented in different animal models of atherosclerosis and angioplasty (Strauss et al., 1994; Karim et al., 1995; Coats et al., 1997). To investigate whether changes in collagen may regulate VSMC proliferation, Koyama et al. (1996) studied the growth properties of VSMCs cultured on monomer collagen fibers and on polymerized collagen. The rationale for these studies is that polymerized collagen may resemble the scenario of a normal artery composed of quiescent VSMCs, and monomer collagen might mimic the ECM surrounding proliferating VSMCs within atherosclerotic plaques. Consistent with this interpretation, mitogen-stimulated VSMCs proliferated in culture dishes coated with monomer collagen, but were arrested in G1 when grown on polymerized collagen. The inhibitory effect of polymerized collagen on VSMC growth appeared to be mediated by α2 integrins, and was associated with suppression of p70 S6 kinase and upregulation of the CKIs p21 and p27. The ability of polymerized collagen to inhibit VSMC proliferation is consistent with a low proliferative index of VSMCs in the normal arterial wall (Koyama et al., 1996). This interpretation would predict that p21 and p27 might be involved in the maintenance of the quiescent state in the VSMCs residing in an intact artery. However, although p21 and p27 are expressed at high levels in balloon-injured arteries at time points that coincide with the decline in VSMC proliferation, expression of these growth suppressors is low or undetectable in normal arteries (Yang et al., 1996; Chen et al., 1997; Tanner et al., 1998). Despite this apparent discrepancy, however, the findings by Koyama et al. (1996) provide convincing evidence that the ability of VSMCs to respond to growth signals is highly dependent on changes in specific ECM components through regulation of CKIs *in vitro*. Further studies are required to determine whether integrins and ECM components are involved in the control of CKI expression in VSMCs *in vivo*.

The glycoprotein thrombospondin 1 (TSP1) is a component of the ECM synthesized and secreted by activated platelets (Lawler et al., 1978) and a variety of cell types including ECs (McPherson et al., 1981; Reed et al., 1995), macrophages (Jaffe et al., 1985), fibroblasts (Jaffe et al., 1983) and VSMCs (Mumby et al., 1984).

TSP1 is a 450 kD homotrimeric that interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of functions (Asch et al., 1991; Frazier, 1991). TSP1 can induce EC growth arrest in vitro (Bagavandoss and Wilks, 1990; Taraboletti et al., 1990), and inhibits the spontaneous development of angiogenic tube-like structures both *in vitro* and *in vivo* (O'Shea and Dixit, 1988; Good et al., 1990; Iruela-Arispe et al., 1991). In marked contrast, TSP1 promotes VSMC proliferation and migration (Majack et al., 1986; Yabkowitz et al., 1993), and plays a stimulatory role in platelet activation and aggregation (Dixit et al., 1985; Tuszynski et al., 1988). Of note is that TSP1 expression has been associated with atherosclerotic lesions, acute vascular injury, hyper-cholesterolemia and hypertension (Wight et al., 1985; Raugi et al., 1990; Botney et al., 1992; Liau et al., 1993; Van Zanten et al., 1994; Reed et al., 1995; Roth et al., 1998). Taken together, these findings suggest that TSP1 may play an important role in the pathogenesis of atherosclerosis and restenosis. We have recently shown that VSMC growth arrest upon blockade of TSP1 requires the CKI p21 (Chen et al., 1999b). Moreover, antibody blockade of TSP1 accelerated reendothelialization and reduced neointima formation in balloon-injured rat carotid artery (Chen et al., 1999a).

Effect of aging on VSMC proliferation

Aging leads to changes in the cardiovascular system that are associated with an increased risk of atherosclerosis (Kannel and Gordon, 1980; Folkow and Svanborg, 1993; Marín, 1995; Bilato and Crow, 1996). However, very little is known about age-related mechanisms causing cardiovascular dysfunction and enhanced atherosclerosis. Stemerman et al. (1982) compared the *in vivo* kinetics of VSMCs from young adult rats (3-4 months) to those of old rats (21-24 months). They performed aortic endothelial denudation and found that [^{3}H]thymidine incorporation into VSMCs and intimal growth was increased with aging. These authors concluded that the more pronounced atherosclerotic plaque growth seen with aging may be the result of an age-related response to injury rather than merely the accumulation of time-related intimal change. Using a different approach, Spagnoli et al. (1992) showed that aging results in increased aortic atherosclerosis in hypercholesterolemic rabbits. Hariri et al. (1986) used transplantation of aortic segments into young or old recipients and studied the myointimal hyperplasia seen after aortic endothelial injury with a nondistending coiled wire catheter. They demonstrated that the vascular response to endothelial injury appears to be a function of the age of the arterial segment rather than the host environment. *In vitro* studies have confirmed that VSMCs isolated from old rats have a significantly higher mitogen-mediated proliferative response than young cells. Cultures of old VSMCs disclosed a greater percentage of their population in the S phase and a decrease in the percentage of cells in the G_0/G_1 phase as compared with young VSMCs (Hariri et al., 1988). Old VSMCs also showed an increased response to stimulatory growth factors (PDGF) and a decreased response to inhibitory growth factors (TGF-ß) (McCaffrey et al., 1988; McCaffrey and Falcone, 1993). Taken together, these findings suggest that age-dependent increase in VSMC proliferation may contribute to the increased prevalence and severity of atherosclerosis and restenosis in the elderly.

In recent experiments, we used a rabbit model to elucidate potential mechanisms involved in the age-dependent increase in VSMC proliferation (Rivard et al., 2000). We found that enhanced proliferation in VSMCs isolated from old animals is associated with augmented levels of cyclin A and CDK2 protein expression. In marked contrast, expression of cyclin E in VSMCs did not appear to change during aging. We also showed that aging results in increased transcription from the cyclin A promoter and expression of c-fos, a member of the AP1 family of transcription factors that interacts with the cyclin A promoter and mediates induction of cyclin A transcription and VSMC proliferation (Sylvester et al., 1998). Consistent with this notion, electrophoretic mobility shift assays demonstrated age-dependent increase in AP1 DNA-binding activity in VSMCs. These findings suggest that augmented cyclin A expression via the action of AP1 transcription factors contributes to increased VSMC proliferation with advanced age. They also establish, for the first time, a direct link between the transcriptional and cell cycle machinery that may contribute to the increased prevalence and severity of atherosclerosis in the elderly.

Antiproliferative therapies to inhibit vascular smooth muscle cell hyperplasia

As discussed in further detail above, excessive proliferation of VSMCs contributes to neointimal thickening during atherosclerosis and restenosis. Therefore, inhibiting this pathological response might be a suitable approach to the treatment of vascular proliferative disease. A variety of therapeutic strategies that targeted specific components of the cell-cycle machinery have been shown to successfully reduce neointimal lesion formation in response to arterial injury. These studies include inhibition of CDK2 (Abe et al., 1994; Morishita et al., 1994a), CDC2 (Morishita et al., 1993, 1994b; Abe et al., 1994), cyclin B1 (Morishita et al., 1994b), cyclin G1 (Zhu et al., 1997), E2F (Morishita et al., 1995), and PCNA (Morishita et al., 1993; Frimerman et al., 1999), as well as overexpression of the growth suppressor molecules p21 (Chang et al., 1995; Yang et al., 1996; Ueno et al., 1997a), p27 (Chen et al., 1997), p53 (Yonemitsu et al., 1998) and pRb (Chang et al., 1995; Smith et al., 1997). Likewise, inactivation of CDC2/PCNA (Mann et al., 1995) and CDK2 (Suzuki et al., 1997) attenuated graft atherosclerosis. Several investigators have also demonstrated a significant

reduction of neointimal cell proliferation after gene transfer of herpesvirus thymidine kinase and administration of ganciclovir following angioplasty in normal and atheromatous arteries (Ohno et al., 1994; Simari et al., 1996; Steg et al., 1997). Gene therapy strategies that targeted signal transduction molecules and transcription factors implicated in the regulation of cell-cycle control gene expression and VSMC proliferation also attenuated neointimal thickening *in vivo*. These include overexpression of the homeobox gene *Gax* (Maillard et al., 1997; Smith et al., 1997), antisense oligonucleotides against the protooncogenes *c-myc* (Bennett et al., 1994; Shi et al., 1994) and *c-myb* (Simons et al., 1992), and inhibition of cellular Ras (Indolfi et al., 1995a; Ueno et al., 1997b). Currently, clinical trials have been initiated to examine the safety and efficacy of some of these gene therapy approaches, including *c-myc* antisense oligonucleotides to reduce restenosis after stenting, and the E2F decoy strategy to treat atherosclerosis after coronary bypass graft.

An alternative approach to treat vascular disease associated with VSMC growth is the use of anti-proliferative drugs. It is important to emphasize, however, that several drugs that efficiently inhibited VSMC hyperplasia in animal models of vascular injury failed to reduce the incidence of restenosis in patients (Califf et al., 1991; Popma et al., 1991; Franklin and Faxon, 1993). The lack of correlation between animal studies and human clinical trials is likely to be due to differences in the response of arteries of diverse species to mechanical injury. Nevertheless, other therapeutic strategies have shown promising results in preclinical and clinical trials. For example, animal models of arterial injury have shown that restenosis may be prevented by local radiation therapy, and this effect is associated with reduced proliferation in the media and the adventitia of irradiated vessels (Waksman, 1997). Similarly, intracoronary radiotherapy has shown positive results in reducing the rate of restenosis in patients (Condado et al., 1997; Teirstein et al., 1997, 1999). Another example is probucol, which reduced luminal narrowing after arterial injury in animal models (Ferns et al., 1992; Schneider et al., 1993) and after balloon coronary angioplasty in patients (Tardif et al., 1997; Yokoi et al., 1997; Rodés et al., 1998). It should be noted that in addition to its antioxidant and antiproliferative properties, probucol also influences the lipoprotein profile. Moreover, it was recently proposed that probucol exerts its antirestenotic effects by improving vascular remodeling after angioplasty rather than by inhibiting neointimal formation per se (Cote et al., 1999). Therefore, future studies are required to elucidate the precise mechanisms underlying the beneficial effect of probucol.

Concluding remarks

Abnormal VSMC proliferation plays an important role in the pathogenesis of cardiovascular diseases, including atherosclerosis and restenosis. Because of the public health importance and economic impact of these pathological processes, elucidating the regulatory factors and molecular mechanisms that control VSMC growth is currently the subject of active research. In this review, we have discussed mechanisms underlying cell-cycle control in VSMCs and their implication in vascular occlusive diseases. Gene therapy strategies that targeted specific cell-cycle control genes or growth regulatory molecules have been effective at inhibiting VSMC proliferation and preventing arterial narrowing in several animal models of vascular injury. The safety and efficacy of some of these approaches are currently being tested in clinical trials. These include inactivation of *c-myc* to treat restenosis after coronary stenting, and inhibition of E2F function to prevent neointimal hyperplasia in bypass grafts. Local radiation therapy and probucol have already shown positive results in recent clinical trials for the treatment of restenosis. Despite these encouraging results, it is important to emphasize that several antiproliferative drugs that inhibited vessel narrowing in animal models of angioplasty have failed to reduce the incidence of restenosis in patients. It is therefore essential to continue our efforts to elucidate the molecular mechanisms governing the control of VSMC *in vitro* and *in vivo*. Ultimately, a thorough understanding of these regulatory networks may lead to the development of novel drug and gene therapies for the treatment of atherosclerotic cardiovascular diseases.

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