Cell Cycle–Dependent Regulation of Smooth Muscle Cell Activation

Ruediger C. Braun-Dullaeus, Michael J. Mann, Daniel G. Sedding, Steven W. Sherwood, Heiko E. von der Leyen, Victor J. Dzau

Objective—Although numerous diseases involving cellular proliferation are also associated with phenotypic changes, there has been little direct evidence that cell phenotype and the cell’s response to external stimuli are modified during passage through different phases of the cell cycle. In this study, we demonstrate that an association exists between cell cycle progression and the expression of genes involved in cellular activation.

Methods and Results—Early cell cycle arrest of aortic smooth muscle cells was found to inhibit the tumor necrosis factor α (TNFα)-induced upregulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, important markers of vascular cell activation in diseases such as atherosclerosis. A combination of immunocytochemistry and flow cytometry were used to document that TNFα-induced adhesion molecule upregulation was inhibited during G1-phase and S-phase, but not in G0-phase or G2/M-phase cells. The inhibition of adhesion molecule expression occurred at the level of transcription, as demonstrated by changes in the patterns of mRNA and protein accumulation in cycling and arrested cells.

Conclusions—Early cell cycle phases may represent states in which the responses to a variety of stimuli that influence cell fate can be modulated, and these observations may have novel implications for the prevention and/or therapy of vascular proliferative, neoplastic, and inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2004;24:845-850.)

Key Words: smooth muscle ■ cell cycle ■ adhesion molecule ■ VCAM-1 ■ proliferation ■ atherosclerosis ■ restenosis

Vascular smooth muscle cells (VSMCs) are known to play a critical role in the pathogenesis of cardiovascular proliferative disorders such as atherosclerosis, postangioplasty restenosis, bypass vein graft failure, and cardiac allograft vasculopathy. VSMCs not only enter cell cycle and proliferate, thereby contributing to neointima formation, but also become “activated,” a term referring to the expression of cytokines, adhesion molecules, chemotactants, proteolytic enzymes, and other molecules not normally expressed in the quiescent, contractile VSMCs of the medial layer of the vessel wall. For example, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) have been documented on neointimal VSMCs of animal models of atherosclerosis, restenosis, and transplant vasculopathy, as well as in human plaques. These molecules, in turn, likely mediate and facilitate a robust inflammatory response and the further proliferation and migration of VSMCs.

Because both activated VSMCs and proliferating VSMCs were detected within the same neointimal lesion, it was generally assumed that proliferating cells are activated cells. However, this supposition has never been proven. Furthermore, it is not known whether activation and proliferation of cells during atherogenesis are parallel-occurring but independent phenomena, or whether the activation state of a cell is influenced by its entry into and its progression through the different phases of the cell cycle. In a series of recent studies, our laboratory and others have found that vascular cell cycle arrest not only inhibits cellular proliferation and, as a result, neointima formation, but also ameliorates changes in vascular cell phenotype and actually reduces the heightened susceptibility of certain vessels to atherosclerosis or vasculopathy. The current study, therefore, tested the hypothesis that an association exists between cell cycle progression and the susceptibility of vascular cells to cytokine induced adhesion molecule expression on the cell surface. We were able to demonstrate that VCAM-1 and ICAM-1 expression were inhibited during the G1-phase and S-phase of cell cycle.

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From Cardiothoracic Surgery (M.J.M.), University of California, San Francisco Medical School, San Francisco, Calif; Cardiovascular Research (V.J.D.), Brigham and Women’s Hospital/Harvard Medical School, Boston, Mass; the Department of Internal Medicine I/Cardiology (R.C.B.-D.), Dresden University of Technology, Dresden, Germany; the Department of Internal Medicine I/Cardiology (D.G.S.), Giessen University, Giessen, Germany; Molecular Probes Inc (S.W.S.), University of Oregon, Eugene, Ore; and the Department of Internal Medicine/Cardiology (H.E.v.d.L.), Hannover Medical School, Hannover, Germany.
Correspondence to Dr Victor J. Dzau, Tower 1, Office of the Chairman, Department of Medicine, Brigham and Women’s Hospital/Harvard Medical School, 75 Francis Street, Boston, MA 02115. E-mail vdzau@partners.org

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These observations support the notion that cell cycle manipulation may provide a means of therapeutic intervention in vascular pathobiology involving cellular proliferation and inflammation.

Methods

Cell Culture
Primary cultures of VSMCs were initiated by enzymatic dissociation from the aortae of 7- to 8-week-old male Sprague-Dawley rats (Charles River Breeding Laboratories, Sulzfeld, Germany) by the method of Owens et al.1 VSMCs were maintained in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12 (DMEM/F12; Gibco) and 10% heat-inactivated fetal bovine serum (Gibco). Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂. Using this technique, VSMCs exhibit typical, spindle-shaped morphology and a multilayered hill-and-valley growth pattern. Expression of α-actin was demonstrated by immunohistochemical staining with a smooth muscle-specific anti-α-actin antibody (Sigma). Studies were conducted on VSMCs (passages 7 to 12) after achieving confluence for 2 days in 20% fetal bovine serum (FBS)/DMEM/F12, followed by serum-withdrawal for 2 days to induce quiescence.

Immunocytochemistry
For immunocytochemical staining, VSMCs, grown on chamber slides, were rinsed with phosphate-buffered saline (PBS) and fixed in −20°C acetone for 10 minutes. The slides were blocked with goat serum for 10 minutes and the primary antibody was applied (mouse anti-VCAM-1, 15 μg/mL; a generous gift from Dr Lobb, Biogen, Cambridge, Mass) for 1 hour, diluted in PBS with 3% BSA. After washing times with PBS, a biotinylated goat anti-mouse antibody (Zymed) was applied, followed by avidin-biotin peroxidase complex. Antibody binding was visualized with 3-amino-9-ethyl carbazole (AEC; Zymed). Cells were counterstained with hematoxylin. Omission of primary antibodies and the staining with mouse nonimmune IgG served as a negative control.

Flow Cytometry
Cells were harvested by trypsinization, fixed overnight with 75% methanol, washed, and incubated for 1 hour at 37°C in PBS containing 100 μg/mL RNase, 10 μg/mL propidium iodide (PI), 3% FBS, and 15 μg/mL mouse anti-VCAM-1 antibody (Seikagaku) or 5 μg/mL mouse anti-ICAM-1 (Santa Cruz Biotechnology), (mouse nonimmune IgG was used as control). For double staining, rabbit anti-cyclin D1 antibody (1:200, Santa Cruz Biotechnology) was added simultaneously. After 2 washes in PI-PBS, a secondary, fluorescein isothiocyanate-(FITC)-labeled goat anti-mouse (Cy5-labeled donkey anti-rabbit) antibody was applied (1:1000, Caltac) for 1 hour at 37°C. After another 2 washes, the cells were resuspended in 500 μL PI-PBS and analyzed for DNA content (PI) and FITC (VCAM-1) positivity. Samples were analyzed using standard methods on a Coulter Epics XL-MCL flow cytometer. Data were computer-analyzed with Multiple Option Cell Cycle Fitting Version 2.50 (Phoenix Flow Systems).

RT-PCR and Competitive RT-PCR
RNA was extracted from VSMCs using Ultraspec (Biotex). Reverse transcription (RT) was performed by incubation of 0.25 μg of total RNA in a reaction buffer containing 20 mmol/L Tris/HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 μmol/L of each oligonucleotide, 10 U/mL RNase inhibitor (Perkin Elmer), 2.5 μmol/L random hexamers, and 26 U of avian myeloblastosis virus reverse transcriptase (Gibco) for 1 hour at 42°C. Specific primers were directly added to the reverse-transcriptase-reaction product and polymerase chain reaction (PCR) was performed with 2.5 U Taq polymerase (Perkin Elmer) in a total volume of 50 μL. Thirty-two cycles were used with cycle times of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute. Twenty μL of the PCR product were electrophoresed in a 1.6% agarose gel and visualized with ethidium bromide. The following oligonucleotide primers were used: VCAM-1, 5'-CCA-CAG-GGC-TAC-ATG-AGG-GT-3' (sense), 5'-TGC-CAA-TTT-CCT-CCC-TTA-AA-3' (antisense). The resultant PCR product was 674 bp.

Competitive RT-PCR with RNA mimics was performed as previously described.12 Briefly, a RNA mimic for rat VCAM-1 was generated to yield a RT-PCR amplification product of 447 bp using aforementioned primers, which yielded a cDNA of 674 bp from native VCAM-1 mRNA. Varying known amounts of mimic RNA were mixed with 0.25 μg of total sample RNA for quantitation. RT was performed as described. Electrophoresis allowed comparison of mimic and sample cDNA products in each reaction tube; the reaction mixture that yielded bands of equivalent visual density represented the point at which sample mRNA approximately equaled the known concentration of mimic RNA (when no tubes had equivalent bands, the equivalence point was taken to be the midpoint of the “crossover” concentrations).

Quantitative Analysis of Apoptosis by Fluorescence Microscopy
Fluorescent DNA-binding dyes were used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells to be analyzed were stained with Hoechst33342 (5 μg/mL), added to the culture medium for 20 minutes at 37°C. The media and the PBS rinses were collected and the cells were trypsinized. Media, PBS, and trypsinized cells were pooled and collected by centrifugation at 1200 rpm 5 minutes at 4°C. Cell pellets were resuspended in a small volume (50 μL) of serum-containing medium with 1 μg/mL Hoechst33342 and 5 μg/mL PI. An aliquot (25 μL) was placed on a glass slide, covered with a glass coverslip, and viewed under fluorescence microscopy. Individual nuclei were visualized at ×400 to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells.

Statistical Analysis
Data are given as mean ± SEM. Statistical analysis was performed by ANOVA. Posttest multiple comparison was performed by the method of Bonferroni. All experiments were independently repeated at least 3 times.

Results

TNFα Stimulates VCAM-1 Expression in Quiescent and Cycling VSMC
In vitro VSMC activation and proliferation can occur as distinct processes.13 In the current study, quiescent rat aortic VSMC were found to double in cell number within 48 hours of mitogenic stimulation with FBS (Figure IB, available online at http://atvb.ahajournals.org). Serum stimulation was chosen rather than treatment with any single factor to more closely mimic the more complex type of mitogenic stimulation experienced by cells in vivo during the development of vascular proliferative disease. This rapid proliferative response, however, was not associated with any stimulation of the expression of VCAM-1, a marker of cellular activation that is known to play a role in macrophage invasion during atherogenesis (Figure IA, available online at http://atvb.ahajournals.org). In contrast, treatment of quiescent VSMCs with TNFα, a potent VSMC activator, resulted in a robust upregulation of VCAM-1. Because these in vitro studies established the potential independence of VSMC proliferation and activation, we then exposed the cells simultaneously to mitogens and cytokines to simulate a condition elaborated in the vessel wall during the pathogenesis of vascular proliferative diseases such as atherosclerosis, postangioplasty re-
TNF-α has been found to inhibit proliferation in certain cell types but not in others. Under our conditions of co-stimulation with TNF-α and serum, VSMC proliferation was not inhibited. Furthermore, the cells expressed VCAM-1 at 48 hours as when treated with TNFα alone (Figure IA and IB).

Although VCAM-1 expression at 48 hours was similar under both sets of conditions, the kinetics of VCAM-1 upregulation were markedly different in quiescent cells and in cells that were stimulated to re-enter cell cycle in a synchronized fashion. Whereas TNFα triggered a rapid increase in VCAM-1 mRNA expression that peaked at 8 hours in nonproliferating cells, quantitative RT-PCR documented that VCAM-1 mRNA levels did not increase significantly until 24 hours after co-stimulation with TNFα and FBS (Figure 1).

The time course of VCAM-1 protein upregulation was similarly shifted in TNFα-treated cells co-stimulated with FBS, and this shift correlated to the time course of cell cycle entry and progression documented by fluorescence-activated cell sorting (FACS) analysis (Figure 2). Synchronized VSMCs required ~18 hours for passage of a significant number of cells (40%) through G1-phase and into S-phase. By 24 hours, cells began to accumulate in G2/M phase (as indicated by tetraploid DNA content), and the subsequent decrease in the proportion of identifiable S-phase cells reflected both desynchronization and a reduction in proliferative rate caused by overgrowth.

**G1-Phase and S-Phase Cells Are Resistant to TNFα-Induced VCAM-1 Upregulation**

The temporal correlation of early cell cycle progression and resistance to TNFα-induced activation was corroborated by FACS analysis of cells stained immunocytochemically for both VCAM-1 expression and DNA content (Figure 3A). VCAM-1 expression was observed both in diploid (G0/G1) and tetraploid (G2/M) cells at 48 hours, but was markedly lower in S-phase cells (4.3%±0.5% in S-phase versus 54.1%±0.6% in G0/G1 and 39.8%±4.1% in G2/M). However, of those cells being positive for VCAM-1 in S-phase, fluorescence intensity on single-cell level was comparable to cells in G0/G1 phase (64.9±8.9 versus 59.3±4.5 relative units, respectively), whereas G2/M phase cells expressed significantly more VCAM-1 on the cellular surface (145.4±1.3 relative units). Figure 3B demonstrates a representative graph of a FACS analysis 18 hours after TNFα and FBS stimulation.

Differential activation of VSMC during cell cycle entry and progression was further verified by using a second adhesion molecule. ICAM-1 expression was similarly delayed during TNFα and FBS co-stimulation in comparison to TNFα stimulation alone (data not shown). Although to a lesser extent, ICAM-1 expression was also determined lower in S-phase cells (12.5%±5%, P<0.001) than in cells in G0/G1 phase (43.6%±5%) and G2/M phase (37.2%±5%) at 48 hours of co-stimulation.

Because adhesion molecule expression seemed inhibited in the general population of VSMC throughout the early phases of synchronized cell cycle progression, we hypothesized that resistance to TNFα activation may be a characteristic of G1-phase as well as S-phase cells. To distinguish between G0-phase and G1-phase VSMCs on FACS analysis, cells at 8 hours after co-stimulation with TNFα and FBS (at a time point shown in Figure 2B to precede entry into S-phase) were double-stained for VCAM-1 and cyclin D1, an early G1 marker used to differentiate quiescent G0 cells from those that have re-entered the cell cycle. Figure 4 demonstrates that cyclin D1-positive (G1) cells were VCAM-1-negative,
whereas VCAM-1-positive cells were found only among the cyclin D1-negative (G0) cells that had not yet mounted a proliferative response to FBS. Similar data were obtained for ICAM-1 as well (data not shown). Taken together with the data from S-phase cells, these results indicate that VSMC cell cycle entry and progression through both G1-phase and S-phase are associated with a resistance to TNFα/H9251-induced upregulation of these markers of activation.

This conclusion was further tested via pharmacologic arrest of VSMCs and accumulation of cells at different points in the cell cycle. TNFα/FBS co-stimulation was performed in the presence of either rapamycin (10 nM), which arrests cells in G1 phase, aphidicolin (5 μg/mL), which inhibits the G1-S transition, or nocodazole (40 ng/mL), a blocker of mitosis and cell division.16-18 A prolonged resistance to TNFα induction of VCAM-1 and ICAM-1 expression was observed both with rapamycin (4.5±1.5% VCAM-1 and 6.4±2.1% ICAM-1-positive cells) and aphidicolin (13.6±2.9% VCAM-1 and 9.8±3.2% ICAM-1-positive cells), respectively, (versus 40.5±3.3% VCAM-1 and 35.4±4.7 ICAM-1-positive cells in nonarrested, co-stimulated cells at 48 hours) as cells accumulated in the early phases of the cell cycle (Figure 5).

In contrast, cells that were allowed to proceed to the late G2/M phases before arrest of cell division with nocodazole expressed VCAM-1 and ICAM-1 to an extent similar to that seen in nonarrested cells (51.5%±6.4% versus 30.4%±8.6%, respectively). Neither rapamycin nor aphidicolin inhibited TNFα-induced VCAM-1 expression in quiescent cells that were not stimulated with FBS; the effects of these drugs on activation in cycling cells therefore suggests a link between cell cycle phase and inhibition of cell activation, rather than a non-cell cycle-related drug effect.

Both TNFα and aphidicolin have been known to trigger programmed cell death.14,19 However, apoptosis, as indicated by nuclear condensation with Hoechst 33342-staining, was reduced in VSMCs co-stimulated with TNFα and FBS compared with TNFα alone, suggesting that cell death was not likely to account for the reduction of VCAM-1 expression in those experimental groups (Figure 6).
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were treated with serum (FBS, 20%), TNFα, and aphidicolin (APC, 5 μg/mL) for 48 hours as indicated. Cells were harvested and quantitative analysis of apoptosis was performed after Hoechst-33342 staining by fluorescence microscopy (n=6, P<0.001 compared with cells treated with neither TNFα nor APC).

Figure 6. Serum prevents TNFα-induced or aphidicolin-induced programmed cell death (apoptosis). Confluent, quiescent VSMC were treated with serum (FBS, 20%), TNFα (200 U/mL), and aphidicolin (APC, 5 μg/mL) for 48 hours as indicated. Cells were harvested and quantitative analysis of apoptosis was performed after Hoechst-33342 staining by fluorescence microscopy (n=6, P<0.001 compared with cells treated with neither TNFα nor APC).

Discussion

It has long been recognized that the cell cycle phases coordinate proliferation through the rapid activation of a number of cell cycle–specific genes. Furthermore, an understanding is evolving of cell cycle–dependent regulation of factors that belong to the signaling systems of growth factor–induced proliferation. For example, the activation of the mitogen-activated kinase cascade and its downstream transcription factors have been found linked to specific phases of the cell cycle.22,23 Beyond proliferation, however, intricate connections between cell cycle regulation and control of other cellular processes are coming to light as the cell cycle machinery become better-understood.24,25 Proliferation and activation of VSMCs, for example, are distinct phenomena with separate signaling mechanisms, as evidenced by the long-standing observation that VSMC proliferation in vitro is not associated with activation, and that activation can be induced in nonproliferating cells via treatment with cytokines. These 2 processes, however, are found together during the pathogenesis of vascular proliferative diseases in vivo, and our group was intrigued by the observations that therapeutic cell cycle arrest was able to preserve a nonactivated cellular phenotype in animal models of either chronic vein graft stenosis or transplant vasculopathy.26 This study, therefore, examined whether VCAM-1 and ICAM-1 expression, both markers of activated atherogenic VSMCs, are modulated through entry into and progression through the cell cycle. We co-stimulated VSMC with TNFα and serum and were able to demonstrate that TNFα, a strong inducer of VCAM-1 and ICAM-1 in quiescent (G0) cells, had only a minor effect on cells passing through G1-phase or S-phase after serum stimulation. VCAM-1 and ICAM-1 expression, however, were inducible in later cell cycle phases (G2/M).

In this study, we used TNFα for activation, because it has been found to be a strong inducer of VCAM-1 and ICAM-1 expression in VSMC in vitro and has been detected in human intimal VSMC in vivo and in animal models of neointimal hyperplasia.27 Although TNFα has been demonstrated to have antiproliferative effects on certain cell types,14 this finding has not been observed in other cells, for example, in fibroblasts and in our in vitro system of primary VSMCs. VCAM-1 and ICAM-1 were thought to be suitable markers of activation, because they represent cytokine-inducible cell surface molecules, which are directly involved in the development of vascular proliferative diseases and which have been documented in neointimal VSMCs both in human plaques and in animal models of atherosclerosis, restenosis, or transplant vasculopathy.3,5–7 Nonendothelial adhesion molecule expression, including VCAM-1 and ICAM-1, have also been associated with intimal leukocyte accumulation and the progression and stability of atherosclerotic plaque. The regulation of classic phenotypic markers, such as smooth muscle α-actin or smooth muscle myosin heavy chain during serum stimulation of quiescent VSMCs, was not evaluated. These makers are not directly involved in atherogenesis, and they were found to be slow and inconsistently regulated. Accordingly, there is controversy about the relation between cell growth and cytodifferentiation.11,20

Although protein synthesis rate is highly activated during G1-phase and S-phase, VCAM-1 and ICAM-1 expression levels were reduced in TNFα-treated cells, indicating that the inflammatory response of a cell is a highly ordered process that does not allow simultaneous cell cycle activity and vice versa. Selective induction of cyclooxygenase-2 expression has also been found only in G0-phase fibroblasts, supporting the idea that quiescent G0 cells, which constitute a large cell population in the human body, play an important role in inflammation.30 Interestingly, the low number of cells expressing VCAM-1 even in S-phase did so with the same fluorescence intensity as TNFα-stimulated quiescent VSMCs. This finding implies a molecular on/off switch with an escape phenomenon of single cells. However, it could also represent a distinct population of VSMCs more susceptible for activation.

Because a significant number of activated cells were in the diploid (G0/G1) population at 48 hours after TNFα/FBS co-stimulation, postmitotic G1 cells appear to be able to continue to express adhesion molecules on their cell surface, in contrast to the apparent resistance to VCAM-1 and ICAM-1 upregulation and/or re-establishment of contact inhibition immediately after G0–G1 transition. Interestingly, cells pharmacologically arrested within G1-phase remained resistant to adhesion molecule upregulation, indicating that passage through G2/M phase is needed for VCAM-1 and ICAM-1 expression in G1 phase. S-phase cells, however, remained negative for adhesion molecule expression at postmitotic time points, although the absolute number of S-phase cells was low after 48 hours, reflecting desynchronization and/or re-establishment of contact inhibition.

The VCAM-1 and ICAM-1 genes are transactivated primarily by different subunits of the transcription factors nuclear factor-kappa B (NF-κB).31 Cyclin-dependent kinases, phase-specific key enzymes of orderly cell cycle progression, have also been found to regulate transcriptional gene activation by NF-κB.32 This finding could provide a mechanism for coordination of adhesion molecule expression with cell cycle progression. Induction of VCAM-1 has, however, also been shown to be dependent on a functional cooperation of the cell cycle regulatory proteins cyclin C and c-Myc.33 Further
studies are needed to reveal the mechanism the findings observed in our study.

Our observations add to the understanding that the cell cycle machinery is being used for other tasks apart from organization of proliferation24 and also might have novel implications for the future prevention and therapy of vascular proliferative, neoplastic, and inflammatory diseases. Preventive and therapeutic cell cycle arrest, whether pharmacologic or using gene therapy, may go beyond inflammatory diseases. Preventive and therapeutic cell cycle arrest, which could be prevented by enhanced proliferation has been associated with enhanced extracellular matrix production, which could be prevented by antisense oligonucleotides.10 Also, VSMC migration has been shown to be inhibited when cell cycle progression is blocked by camptothecin or aphidicolin can occur in all phases of the cell cycle. Biochem Soc Trans. 1992;20:84S.

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Figure 1. VCAM-1 expression in quiescent vs. proliferating VSMC. Confluent, quiescent VSMC were stimulated with serum (FBS, 20%) and/or TNFα (200 U/ml) for 48 hours. (A) VCAM-1 was detected immunocytochemically and (B) cell number was determined with a Coulter Counter ZN (Coulter Electronics) (n= 6, *p<0.001).