Regulation of cdk2 Activity in Endothelial Cells That Are Inhibited From Growth by Cell Contact

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Abstract—Endothelial cells (ECs) are quiescent in normal blood vessels but undergo rapid bursts of proliferation after vascular injury and during angiogenesis. Here we show that the activity of cyclin-dependent kinase-2 (cdk2), a key regulator of the G1 and S phases of the cell cycle, is expressed at high levels in proliferating ECs but at low levels in ECs that are contact-inhibited for growth. Despite these differences in kinase activity, the protein levels of cdk2 and 1 of its activating subunits, cyclin E, are not modulated by these different growth conditions. The cdk inhibitor p27 is highly expressed in contact-inhibited but not proliferating ECs, whereas the level of cyclin A protein is preferentially expressed in proliferating ECs. p27 protein was detected in immunoprecipitable complexes with cdk2 or cyclin E in cultures that were contact-inhibited for growth. The functional significance of the p27 induction was indicated by the detection of a heat-stable cdk2 inhibitory activity that was induced by endothelial cell-cell contact and could be immunodepleted with anti-p27 antibodies. In a confluent EC monolayer, cdk2 kinase activity was activated by a scraping injury that led to cell migration and proliferation. The injury-induced activation of cdk2 coincided with the downregulation of p27 and the induction of cyclin A. These data demonstrate that p27 is induced in confluent cultures of ECs. They also indicate that both p27 induction and cyclin A downregulation contribute to the inhibition of cdk2 and cell proliferation by cell-cell contact in ECs. (Arterioscler Thromb Vasc Biol. 2000;20:629-635.)

Key Words: cell cycle ■ contact inhibition ■ endothelium

Under normal conditions during adult life, the endothelium of many organ systems remain in a quiescent, nonproliferative phenotype.1,2 The inhibition of endothelial cell (EC) growth by cell-cell contact is in marked contrast to the rapid burst of proliferation that occurs when the endothelial monolayer is disrupted, such as during abrasion of the vessel wall by balloon angioplasty. High levels of EC proliferation are also associated with microvascular morphogenesis, which accompanies normal vascular development, and with the abnormal ingrowth of new blood vessels that occurs during tumor-induced angiogenesis.3,4 Despite the importance of EC proliferation during pathogenesis and normal development, little is known about the mechanisms that tightly couple changes in cell cycle activity with changes in the EC microenvironment.

Cell cycle progression is controlled by the periodic activation of cyclin-dependent kinases (cdks). cdk2 becomes activated by their association with activating subunits, referred to as cyclins.5,6 The cdk4/cyclin D complexes function in the early G1 phase of the cell cycle, whereas cdk2/cyclin E complex is activated later in the G1 phase when the retinoblastoma gene product becomes phosphorylated. Previous studies have demonstrated that cyclin E plays a key role in regulating the transition from G1 to S. Overexpression of cyclin E induces retinoblastoma protein hyperphosphorylation in an osteosarcoma cell line7 and shortens the G1 phase in human fibroblasts.8,9 Microinjection of anti-cyclin E antibody10 or anti-cdk2 antibody11 into human fibroblasts prevents these cells from entering the S phase. Cyclin A, another cdk2 partner, is upregulated when cyclin E levels are downregulated. Expression of the cdk2/cyclin A holoenzyme peaks in the S phase and is required for the elongation of initiated replication and continuation of the S phase.12–14 cdk activity is also modulated through association with negative regulatory subunits.15,16 A number of these cdk inhibitors have been reported, including p21, p27, p16, p15, p19, and p57.17–19 Two classes of cdk inhibitors have been identified: those that are specific for cdk4 and/or cdk6 (p16, p18, and p19) and those that have a broader specificity and are referred to as general inhibitors (p21, p27, and p57). These cdk inhibitors play a key role in cell growth inhibition during cellular differentiation or in response to environmental conditions that modulate cell growth, including exposure to γ-irradiation, growth factors, and cytokines. Induction of the cdk inhibitor p21 is associated with cell cycle withdrawal and cell survival during differentiation of skeletal muscle.20,21 hematopoietic,22 neuronal,23 and hepatic24 cells. Induction of the p27 cdk inhibitor has been observed in mink lung epithelial cells that are exposed to transforming growth factor-β or subjected to density-dependent growth inhibi-
EC proliferation is regulated by soluble growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), as well as insoluble components of the extracellular matrix. It has been shown that in vitro stimulation of EC proliferation by both VEGF and bFGF requires activation of protein kinase C. However, little is known about the regulation of cdk complexes and activity in vascular ECs. Previously it has been reported that the level of cyclin A mRNA is downregulated in ECs that are contact-inhibited for growth, but modulations in cyclin A protein level or their corresponding effects on cdk kinase activity were not evaluated. Moreover, the role of p27 in controlling cdk activity in ECs has not been elucidated. Here we show that cdk2 and cyclin A–and cyclin E–associated kinase activities are markedly reduced in contact-inhibited ECs, although levels of cdk2 protein are not affected by these different growth conditions. We found that cyclin A protein levels decline under conditions of cell-cell contact, but levels of cyclin E do not change. In contrast, p27 protein and cdk inhibitory activity are expressed in ECs that are contact-inhibited for growth, and p27 is found in association with the cdk/cyclin E complex in lysates prepared from these cells. We also found that p27 is downregulated and cyclin A upregulated in EC monolayers after a scraping injury that induces cdk2 activity. These data suggest that alterations in p27 and cyclin A expression regulate EC proliferation during the transition from contact inhibition to the proliferative state. This study represents the first systematic analysis of cdk activity and complex formation in cell-cell contact–inhibited ECs.

Methods

Cell Culture

Bovine aorta ECs (BAECs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein ECs (HUVECs) were isolated as described and grown in medium 199 (Life Technology) with 20% FBS, EC growth supplement (100 μg/mL), and heparin (50 U/mL). HUVECs between passages 1 and 5 were used in all experiments. Human microvascular ECs (HMECs) of dermal origin were purchased from Clonetics Corp (San Diego, Calif) and were grown in EBM medium containing human epidermal growth factor (10 ng/mL), hydrocortisone (10 μg/mL), 5% FBS, and 0.4% bovine brain extract (Clonetics Corp). HMECs were used between passages.

Injury of the EC Monolayer

Injury to EC monolayers was performed by a protocol described previously. In brief, contact-inhibited BAEC monolayers grown on 100-mm plates were scraped with a stainless steel rake, which uniformly swept out ~50% of the cells. Wounded BAEC monolayers were washed to remove floating cells and cellular debris and incubated in fresh medium to induce cell migration and proliferation. Cells were collected at different time points after injury and subjected to immunoblotting analysis.

Results

Regulation of cdk2 Kinase Activity and Cell Cycle Protein Levels in ECs

We first measured the DNA synthesis rates in subconfluent versus confluent BAECs under the culture conditions that were employed for the subsequent cell cycle analyses. Rates of [3H]thymidine incorporation were 40-fold higher in subconfluent cultures of BAECs than in confluent cultures on a per-cell basis despite the presence of 10% FBS in the medium (Figure 1A). The cell cycle distribution of BAECs in subconfluent and confluent cultures was also determined by flow
In cultures of subconfluent BAECs, 58% of the cells were in the G0/G1 phases of the cell cycle, whereas 24% were in the S and 17% in the G2/M phases. In contrast, in confluent cultures of BAECs, only 13% of the cells were in the G2/M phases of the cell cycle. These data, consistent with a previous report, demonstrate that under conditions of confluence, BAECs display cell-cell contact growth inhibition that arrests cells in the G0/G1 phases of the cell cycle.

To investigate the molecular basis of cell-cell contact growth inhibition in ECs, the levels of the cdk2-associated kinase activities were measured in extracts prepared from subconfluent and confluent cultures of BAECs. Immunoprecipitates of cdk2, cyclin E, or cyclin A prepared from proliferating cultures of BAECs all exhibited high levels of histone H1 kinase activity, whereas immunoprecipitates prepared from confluent cultures uniformly displayed low levels of kinase activity. Thus, the levels of cdk2 activity were correlated with the proliferative activities displayed by BAEC cultures under these different growth conditions.

The expression levels of cdk2, cyclin A, and cyclin E proteins were analyzed in BAECs. Immunoblot analyses revealed that the levels of cdk2 and cyclin E were similar between cultures of proliferating BAECs and that cultures of BAECs had been contact-inhibited for growth. In contrast, cyclin A was expressed at much lower levels in the contact-inhibited cells. This expression pattern of cyclin A protein parallels that of its transcript under these different growth conditions. A low level of cyclin A protein is likely to contribute to the reduced cdk2/cyclin A kinase activity that is...
Figure 2. Downregulation of cdk2-associated kinase activity in BAECs that were contact-inhibited for growth. Cell lysates from proliferating (P) or confluent (C) cultures of BAECs were immunoprecipitated with anti-cdk2, anti–cyclin (cyc) A, or anti–cyclin E antibodies. The immunoprecipitates were incubated with [γ-32P]ATP and histone H1. The reaction mix was separated on SDS–polyacrylamide gel electrophoresis gels, and the relative kinase activity of the immunoprecipitates is indicated by histone H1 incorporation of [γ-32P]ATP. The relative kinase activities detected in lysates of confluent cultures are presented in the histogram as percentages of the kinase activity in lysates of proliferating BAECs. Mean values and SDs were calculated from 3 independent experiments.

Figure 3. Immunoblotting analysis of cyclins (Cyc), cdk2, and p27 in proliferating versus confluent ECs. A, Cell lysates (40 μg) from proliferating BAECs (P) or BAECs that were inhibited for growth by cell-cell contact (C) were subjected to immunoblotting analysis with the indicated cyclin, cdk2, and p27 antibodies. The experiment was repeated 3 times, and a similar result was obtained. On average, levels of cdk2 and cyclin E were similar between proliferating and contact-inhibited BAECs. The levels of cyclin A in contact-inhibited BAECs decreased by a factor of 3.2, whereas the levels of p27 increased 4.3-fold. B, Protein levels of cdk2 and p27 in proliferating (P) or confluent (C) HUVECs were determined by immunoblot. The experiment was repeated 3 times, and a similar result was obtained. On average, levels of cdk2 were similar between proliferating and contact-inhibited BAECs. The levels of p27 increased 2.4-fold.

Figure 4. Immunoprecipitation coupled with immunoblotting revealed an association of p27 with cdk2 and cyclin E in contact-inhibited BAECs. A, Cell lysates from proliferating (P) or contact-inhibited (C) BAECs were immunoprecipitated with anti–cyclin E antibody in the absence (−) or presence (+) of immunogenic peptide for cyclin E. The immunoprecipitates were loaded on an SDS–polyacrylamide gel electrophoresis gel and immunoblotted with anti-cdk2, anti-p27, and anti–cyclin E antibodies sequentially. Both cdk2 and p27 were present in immunoprecipitable cyclin E complex from contact-inhibited BAEC lysates. In contrast, only cdk2 was present in the cyclin E complex from proliferating BAEC lysates. B, cdk2 immunoprecipitates from proliferating or contact-inhibited BAEC lysates were subjected to immunoblotting with anti-p27 and anti–cdk2 antibodies sequentially. cdk2 was associated with p27 in contact-inhibited but not proliferating BAECs. These experiments were repeated 3 times with similar results.

Potential negative regulators of cdk2 activity also include p21 and p57. Using an antibody that can detect p21 from a broad range of species, we were not able to detect significant levels of p21 in BAECs or HUVECs by Western blot analysis. This result is consistent with a previous report that p21 was not detected in ECs and smooth muscle cells from normal porcine iliofemoral arteries. Using an antibody specific for human p57, we were not able to detect significant levels of p57 in HUVECs. Thus, in contrast to findings in muscle and other cell types, Western blot analyses failed to detect significant levels of p21 or p57 expression (data not shown) in ECs.

p27 Is Bound to cdk2/Cyclin E Complexes in Contact-Inhibited ECs

Immunoprecipitation-coupled immunoblotting analyses were performed to determine the proteins associated with p27 in BAEC extracts (Figure 4A). The cyclin E–associated proteins were immunoprecipitated with anti–cyclin E antibody and subsequently subjected to immunoblotting with anti-cdk2 and...
anti-p27 antibodies. cdk2, but not p27, was detected in cyclin E immunoprecipitates from lysates of proliferating BAECs. In contrast, both cdk2 and p27 were detected in cyclin E immunoprecipitates from lysates of contact-inhibited BAECs. Similarly, p27 could be detected in cdk2 immunoprecipitates from lysates of contact-inhibited BAECs but not from lysates of proliferating BAECs (Figure 4B).

cdk2 Inhibitory Activity in Contact-Inhibited ECs Is Immunodepleted With Anti-p27 Antibodies

A number of cdk inhibitors are small, heat-stable molecules. Thus, heat-treated lysates can be used to analyze cdk inhibitor activities,35 in the absence of interfering cdks that are heat-labile. Heat-treated cdk2 inhibitory activity was detected in contact-inhibited but not proliferating BAEC lysates (lanes 2 and 4). Preclearance by immunoprecipitation with anti-p27 antibody largely depleted this cdk2 inhibitory activity (lane 5). The presence of p27 immunogenic peptide prevented immunodepletion of this cdk2 inhibitory activity (lane 6). The mean values from 3 independent experiments are expressed in the histogram with standard error bars. Film exposure from 1 representative experiment is shown.

p27 Downregulation Is Correlated With EC Migration and Proliferation in Response to a Scraping Injury

To measure the cell density–dependent expression level of cdk2, cyclin A, cyclin E, and p27, we implemented an endothelial wound-healing model and examined the steady-state levels of these proteins during the cellular response to injury, which occurs as cell-cell contacts are disrupted and the induction of cell migration ensues. With this model system, a uniform population response to injury is achieved by using a specially designed rake, which sweeps out 400- to 600-μm-wide zones of cells in concentric arrays, leaving an endothelial population that is migrating and proliferating (in unison) during the response to injury. Cell lysates were prepared and assayed for cdk2 activity and for the expression of p27, cyclin A, cyclin E, and p27 protein by immunoblotting with appropriate antibodies. The relative levels of p27, cyclin A, cyclin E, and cdk2 were also measured with a densitometer and expressed on the histogram (upper panel). The experiment was performed multiple times with similar results.
oxyuridine incorporation revealed active DNA synthesis in the forefront of migrating cells between 8 and 14 hours after injury but not at later time points (data not shown). These data demonstrate a good correlation between the induction of DNA synthesis by a scraping injury and changes in p27 and cyclin A levels. Furthermore, these data suggest that the coordinate regulation of p27 and cyclin A may have a role in controlling EC proliferation during wound healing.

**Discussion**

cdk2 performs key regulatory roles in the G1 and S phases of the cell cycle. Here we have demonstrated a marked reduction of cdk2 kinase activity in ECs that are contact-inhibited for growth compared with cells that are proliferating. To determine the regulatory mechanisms that contribute to this reduction in cdk2 activity, we analyzed the expression and protein interactions of cdk2 and its regulatory subunits in EC cultures under these different growth conditions. Initial measurements revealed similar levels of cdk2 protein in proliferating and growth-arrested ECs, suggesting that differential interactions with cdk2-associated protein might play a role in cdk2 regulation under these conditions. The experiments described herein demonstrate that at least 2 mechanisms contribute to the reduction in cdk2 activity in ECs that are contact-inhibited for growth.

The cdk2/cyclin E holoenzyme functions in the G1 phase of the cell cycle. In view of the important role of cyclin E in early cell cycle control, it is interesting that this peptide is not downregulated in ECs when they are contact-inhibited for growth and that immunoprecipitable cdk2/cyclin E complexes can be detected in both proliferating and growth-arrested ECs. The observation that lysates prepared from contact-inhibited ECs do not exhibit appreciable cyclin E–associated kinase activity suggested that inhibitory subunits might be involved in the regulation of cdk2 under these conditions. Our data show that p27 expression was markedly enhanced in confluent EC cultures that were contact-inhibited for growth. These data are consistent with a previous report that an elevated p27 protein level is associated with cell-cell contact or transforming growth factor-β treatment–induced growth arrest.

Immunoprecipitation-coupled immunoblot analyses were performed on EC extracts to test whether the binding of p27 to cdk2 might be responsible for the reduction in cdk2 kinase activity in ECs that were contact-inhibited for growth. Lysates prepared from confluent cultures of growth-arrested ECs demonstrated interactions between p27 and cdk2, p27 and cyclin E, and cyclin E and cdk2. Furthermore, lysates prepared from growth-arrested but not proliferating ECs displayed a heat-stable cdk2 inhibitory activity that could be immunodepleted by pretreatment with anti-p27 antibodies. Collectively, these data indicate that the cdk2/cyclin E holoenzyme, whose expression is not repressed by cell-cell contact in ECs, is inactivated by the association of p27 inhibitory subunits under conditions of cell-cell contact–mediated growth inhibition. Furthermore, the p27 level is rapidly and transiently downregulated after a scraping injury to EC monolayers, which induces transient EC proliferation and migration. These data suggest that p27 plays a key role in maintaining the growth-inhibitory status of ECs under condition of cell-cell contact and that its downregulation is involved for these cells to reenter the cell cycle.

We have also show here that cyclin A protein levels were substantially reduced in the confluent cultures of the growth-arrested ECs. This finding is consistent with a previous report showing that cyclin A mRNA level and promoter activity were lower in confluent cultures of ECs than in subconfluent cultures of ECs. We demonstrated that EC lysates contained reduced levels of cyclin A–associated kinase activity under conditions that lead to cell contact–induced growth arrest. Since the levels of the cdk2/cyclin A holoenzyme peak during the S phase and it is required for both the initiation and elongation of DNA replication, reductions in the level of this cyclin are likely to contribute to the growth arrest of ECs by inhibiting the cell cycle machinery that functions during the S phase of the EC cycle. In support of this hypothesis, we found that cyclin A was induced by a scraping injury to EC monolayers and that its induction was maintained for up to 14 hours.

Recently, it was reported that p27 can block cyclin A transcription in fibroblasts and vascular smooth muscle cells. This inhibitory effect of p27 requires the E2F binding site located at position −37 to −32 in the cyclin A promoter. Increased levels of p27 can inhibit cdk2/cyclin E activity and release p107 and the retinoblastoma tumor suppressor protein that, in turn, can inhibit E2F activity. Thus, it is plausible that an elevated level of p27 is instrumental in the repression of cyclin A expression in ECs under cell-cell contact conditions. However, this may not be the only pathway, since the cAMP response element–binding site at position −79 to −72 in the cyclin A promoter has also been shown to be required for the repression of cyclin A transcription in confluent ECs.

Interestingly, it has been reported that expression of the p21 cdk inhibitor can be regulated by the extracellular matrix through a p53-dependent pathway in ECs. Agonists of EC integrin αVβ3 have been shown to suppress p53 transcription activity, inhibit p21 expression, and increase the bcl-2/bax level, which promotes EC survival. Conversely, αVβ3 antagonists activate p53 and increase p21 expression, leading to EC apoptosis. Therefore, it appears that p27 is involved in cell-cell contact–mediated EC growth inhibition, whereas p21 is involved in EC survival mediated by the extracellular matrix.

Protein kinase Cs are involved in both VEGF- and bFGF-mediated EC proliferation. Recent reports indicate that protein kinase Cs exert their effects on EC proliferation via regulation of the cell cycle machinery, at least in part. It has also been shown that phorbol ester and diacylglycerol inhibit cdk2 activity and the G2/M transition in ECs through the suppression of cdc25B expression. Harrington et al reported that forced expression of protein kinase Cδ in ECs resulted in delayed passage through the S phase of the cell cycle. Collectively, these reports suggest that a diverse array of cell cycle proteins is involved in regulating EC proliferation in response to different environmental conditions.

In summary, we have shown here that cdk2 activity is low in ECs that are inhibited for growth by cell-cell contact. Two mechanisms can account for this reduction in cdk2 activity: (1) a reduction in cyclin A and (2) the upregulation of p27, which binds and inactivates the cdk2/cyclin E holoenzyme. The integrity of the endothelial monolayer is a key component in the development of atherosclerotic lesions, and the acceleration of endothelial healing after arterial injury can inhibit the formation of restenotic lesions. Therefore, further studies on EC cycle...
regulation may have important implications for understanding the basic mechanisms of atherogenesis and postinterventional restenosis.

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