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Angiostatin-Induced Inhibition of Endothelial Cell Proliferation/Apoptosis Is Associated With the Down-Regulation of Cell Cycle Regulatory Protein cdk5

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Abstract Endothelial cells (ECs) are quiescent in normal blood vessels, but undergo rapid bursts of proliferation after vascular injury, hypoxia or induced by powerful angiogenic cytokines like fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Deregulated proliferation of ECs facilitates angiogenic processes and promotes tumor growth. In dividing cells, cell cycle-associated protein kinases, which are referred as cyclin-dependent kinases (cdks), regulate proliferation, differentiation, senescence, and apoptosis. Cyclin-dependent kinase-5 (cdk5) is expressed in neuronal cells and plays an important role in neurite outgrowth, of neuronal migration and neurogenesis, its functions in non-neuronal cells are unclear. Here, we show for the first time that the cdk5 is expressed at high levels in proliferating bovine aortic endothelial (BAE) cells, by contrast insignificant low levels of cdk5 expression in quiescent BAE cells. In addition, bFGF up-regulates cdk5 expression in a dose-dependent fashion. Interestingly, temporal expression data suggests that cdk5 expression is very low between 24–48 h, but high level of cdk5 expression was detected during 60–72 h. This later time corresponds to the time of completion of one cell cycle (doubling of cell population) of BAE cell culture. Angiostatin (AS), a powerful inhibitor of angiogenesis inhibits ECs proliferation in dose-dependent manner with concomitant down-regulation of cdk5 expression. The role of cdk5 in ECs, proliferation and apoptosis was confirmed by selective inhibition of cdk5 expression by the purine derivative roscovitine, which inhibits bFGF-stimulated BAE cells proliferation and induces apoptosis in dose-specific manner. By contrast, the roscovitine analog olomoucine, which is a specific inhibitor of cdk4, but not of cdk5 failed to affect ECs proliferation and apoptosis. These data suggest for the first time that neuron specific protein cdk5 may have significant role in the regulation of ECs proliferation, apoptosis, and angiogenesis and extends beyond its role in neurogenesis. J. Cell. Biochem. 91: 398–409, 2004.

Key words: angiostatin; cdk5; cycline-dependent kinase; roscovitine; apoptosis

Angiogenesis, the process of new blood vessel growth, is essential for the growth of solid tumors and their metastases. Blood capillaries are composed of endothelial cells (ECs), which are usually quiescent under physiological conditions [Hanahan and Folkman, 1996]. These quiescent ECs can be induced to proliferate by direct exposure to the proangiogenic cytokines, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [Schulze-Osthoff et al., 1990; Folkman, 1992; Weindel et al., 1994; Hatva et al., 1995; Bussolino et al., 1997; Harris, 1998]. Normally in the G_0 phase of the cell cycle, growth and cell division are arrested, but several growth factors are able to initiate a complex series of events causing cells to traverse G_1, replicate DNA during the S-phase, and ultimately divide. On entering the cell cycle, certain gene products are activated, such as MAP kinase and cell cycle kinase. However, only limited information is

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available regarding the regulation of cyclin-dependent kinases (cdks) and their control of cell cycle progression in ECs. Growth and development of tumors is dependent on angiogenesis [Folkman, 1993]. Based in large part on the critical role of angiogenesis in tumor growth, much effort has focused on the development of diverse anti-angiogenic strategies, many of which target the regulation of ECs homeostasis [Auerbach and Auerbach, 1994]. ECs of the adult vasculature divide less than once per month on average [Engerman et al., 1967]. This endothelial quiescence provides an opportunity to target the proliferating ECs as a mean to inhibit pathological angiogenesis. When exposed to appropriate stimuli, quiescent ECs engage the cell cycle at early G_1, and transit through the cell cycle as a consequence of activation of specific cyclin-dependent kinases by cyclins or their regulatory partners [Sherr, 1994, 1996]. Proliferation of ECs is a prerequisite for neovascularization along with migration, differentiation, tube formation, and sprouting of new capillary branches [O'Reilly et al., 1994b; Cao et al., 1996; Bussolino et al., 1997; Folkman, 1997; Risau, 1997]. Angiostatin (AS), an internal fragment of plasminogen (PLG), was discovered based on its potent and selective endothelial cytostatic activity in vitro [O'Reilly et al., 1994b]. AS was shown to posses potent anti-angiogenic and anti-tumor activity without any visible toxicity in animal studies [O'Reilly et al., 1996; Wu et al., 1997]. Promising preclinical studies showed that AS inhibits the growth of solid tumors and prevents their metastasis, this anti-angiogenic compound was one of the earliest to enter anti-tumor clinical trials.

Despite many pharmacological studies, the current knowledge of AS's molecular mode of action is limited. A previous study has shown that *AS* gene therapy selectively inhibits ECs proliferation and disrupts the cell cycle progression (G_2/M transition) induced by M-phase-promoting factors. *AS* gene transfected ECs showed a marked mitosis arrest that correlated with the down-regulation of the M-phase phosphoproteins [Griscelli et al., 1998].

Other studies have demonstrated that AS in vitro inhibits ECs proliferation, migration, and tube formation [O'Reilly et al., 1994a,b; Ji et al., 1998] and induces apoptosis [Claesson-Welsh et al., 1998; Griscelli et al., 1998] without affecting the S-phase progression [Lucas et al., 1998]. It is quite clear from these reports that AS induces mitotic arrest of ECs at G_2/M-phase and does not affect the G_1/S-phase of cell cycle progression.

Various mechanism(s) of AS's mode of action have been suggested [Moser et al., 1999; Tarui et al., 2001; Troyanovsky et al., 2001; Tuszynski et al., 2002]. Our laboratory proposed that AS binds to an in vivo tyrosine kinase substrate annexin II on the EC surface [Tuszynski et al., 2002] and may be blocking plasmin generation. Plasmin, a strong serine protease facilitates angiogenesis via degradation of extra cellular matrix (ECM) [Bajou et al., 2001; Pepper, 2001; Tuszynski et al., 2002]. Despite the identification of multiple receptors for AS, the question still remains to be answered, how AS inhibits ECs proliferation and induces apoptosis? Is there any down-stream cellular signaling mechanism initiated by AS or is AS simply cytotoxic to ECs? Our knowledge of the role of AS in regulation of cell cycle and cellular signaling is limited and unexplored.

To explore the role of AS in the control of cell cycle regulatory proteins, we investigated whether AS targets one of the cdk through a mechanism unique to ECs. In this report, we provide evidence that AS targets cdk5, which in turn specifically regulates ECs proliferation and apoptosis.

MATERIALS AND METHODS

Human lysine-PLG, purified bovine elastase, and lysine sepharose were obtained from (Sigma Chemical Company, St. Louis, MO). The chromatography and electrophoretic reagents were obtained from (BioRad, Richmond, CA). Recombinant bFGF, anti-cdk5, anti-cdk4, monoclonal antibodies were purchased from (R&D System, Minneapolis, MN). Anti-k1-3 (AS) monoclonal antibody was purchased from (Enzyme Research, Inc., Chicago, IL). The purine analogues roscovitine and olomoucine were purchased from (Biomol Research Lab, Plymouth meeting, PA). The cell proliferation assay kit was purchased from (Promega, Madison, WI). Annexin V and DNA fragmentation detection kits were purchased from Oncogene (San Diego, CA). All other chemicals used in this study were of analytical grade.

AS Generation and Purification

AS was generated by limited proteolytic digestion of human PLG and purified by affinity

chromatography in our laboratory as described earlier [Tuszynski et al., 2002].

Cell Lines Maintenance and Treatments

Bovine aortic endothelial (BAE) cells were grown in Ham's F12 K media as we described previously [Tuszynski et al., 2002] containing either 10% fetal calf serum (FCS) or 0.1% BSA supplemented with L-glutamine and antibiotics. BAE cells cultured in the presence of 0.1% BSA showed no growth and were considered as quiescent cells as described [Martinez et al., 1999]. BAE cells were induced by bFGF and then treated with AS, roscovitine, and olomoucine in the growth medium for time periods and at doses as indicated in the figure legends.

Endothelial Cell Viability/Proliferation Assay

About 10,000 BAE cells were seeded in a 96-well tissue culture plate in triplicate either in the presence of 0.1% BSA or 10% FCS and induced with bFGF. After 72 h, the numbers of viable cells were determined using Promega's colorimetric cell proliferation assay kit [Tuszynski et al., 2002]. This assay actually determines the metabolic activity of live cells by a dehydrogenase enzyme, which converts MTS (Owen's reagent) into soluble formazan.

Western Blot Analysis

BAE cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Ne_3VO, 1 mM PMSF, 1% Triton X-100 after the treatment with the test compounds and proteins. The samples with 10 μg of protein were fractionated on 12% SDS–polyacrylamide gel before being transferred onto a nitrocellulose membrane. After a 2 h incubation in blocking buffer (TBS-5% milk-0.05% Tween 20), the membranes were incubated for 1 h with anti-cdk5 monoclonal antibody (dil 1:1,000) and 1 h with a horseradish peroxidase-conjugated goat anti-mouse IgG. After washing, the membranes were developed with enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation

Anti-cdk5 monoclonal antibodies were immobilized on protein A sepharose at 4°C for 3–4 h. BAE cells lysate (100 μg) was incubated with antibody-immobilized matrix for 2–3 h at 4°C with gentle shaking. The protein antibody immune complex was separated by centrifugation and washed extensively with PBS to remove any unbound proteins and re-suspended in 50 μl of SDS loading buffer, separated on a 12% SDS–PAGE, and electroblotted on nitrocellulose membrane. Membranes incubated with anti-cdk5 antibody (dil 1:1,000) for 1 h at room temperature and analyzed by Western blotting as described above.

Apoptosis Assays

Degradation of nuclear DNA and exposure of phosphatidyl serine (PS) on the cell surface are the key features of apoptosis. We have measured roscovitine-induced ECs apoptosis by two independent techniques. BAE cells were cultured in 6-well tissue culture plates and treated for 36 h with either roscovitine or olomoucine (5 μg/ml). Cells were washed and processed according to manufacturer's instructions. BAE cells were treated with equal amount of vehicle were considered as control. A positive control (actinomycin treated HL60 cells) was used in experiments supplied with the assay kit. Nuclear DNA extracted from control and treated BAE cells according to manufacturer's instruction was fractionated on 1.5% agarose gels following ethidium bromide staining. For annexin V staining, adherent cells were washed and detached with 0.5× trypsin. The cell suspension was labeled with annexin V-FITC and propidium iodide (PI). The cell suspension (25–50 μl) was transferred to microscopic slide and viewed under fluorescent microscopy equipped with FITC filter.

RESULTS

Identification of cdk5 in BAE Cells and Its Up-Regulation by the Angiogenic Cytokine bFGF

To investigate the mechanism by which AS interferes with cell cycle progression, we evaluated the expression of cdk5 protein in BAE cells. BAE cells were synchronized by serum starvation [Martinez et al., 1999; Yeh et al., 2000]. Serum-starved BAE cells did not show any proliferation (Fig. 1A) whereas, cells grown in 10% FCS and induced by bFGF showed exponential growth (Fig. 1A). We subsequently determined cdk5 expression by Western blot analysis in serum-starved and cells grown in serum and induced by bFGF. We found high levels of cdk5 protein expression in cells grown with FCS and

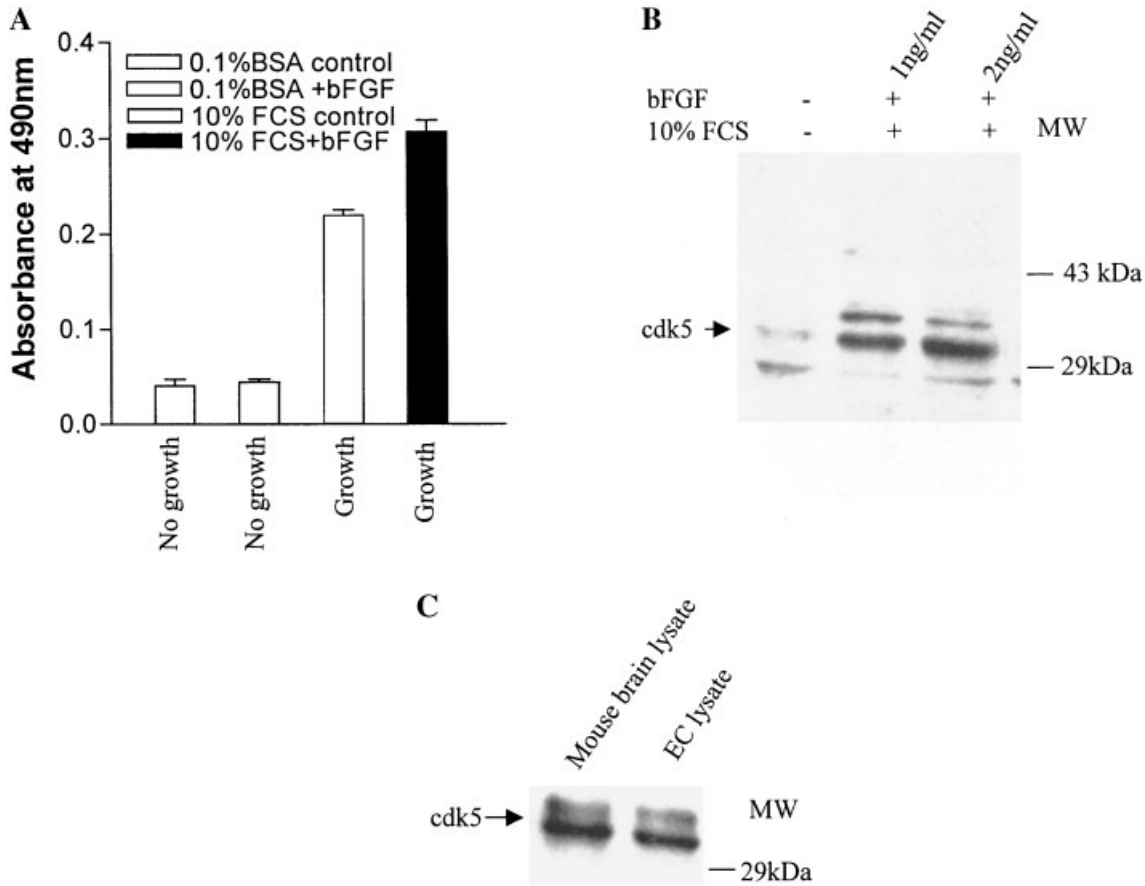

Fig. 1. Identification of cyclin-dependent kinase-5 (cdk5) expression in bovine aortic endothelial (BAE) cells and its up regulation by basic fibroblast growth factor (bFGF). Serum-starved BAE cells cultured in presence of 0.1% BSA showed no growth. In contrast cells grown with 10% fetal calf serum (FCS) in presence of bFGF (1 ng/ml) showed exponential growth (**A**). BAE cells treated with bFGF and analyzed for cdk5 expression using Western blot (**Panel B**). Identity of cdk5 in BAE cells was confirmed by using brain cell lysate as positive control. Brain and ECs lysates were immunoprecipitated with anti-cdk5 monoclonal antibody. These immunoprecipitates were separated on SDS–PAGE, transferred on to nitrocellulose membrane, incubated with anti-cdk5 monoclonal antibody (dil 1:1,000), and analyzed by Western blotting (**Panel C**). In this experiment anti-cdk5 antibody recognized a 33 kDa protein band in positive control (**left lane**) as well as in BAE cells (**right lane**). Anti-cdk5 cross-reactive bands in brain and BAE cells has exactly the same molecular weight confirming the expression of cdk5 in BAE cells.

bFGF as compared to cells grown in serum-starved medium. Furthermore, bFGF induced expression seemed dose-specific (Fig. 1B). Since cdk5 protein expression has been demonstrated exclusively in brain [Nikolic et al., 1996; Sharma et al., 1999], we examined whether endothelial cdk5 is the same as neuronal cdk5 or a cross-reactive isoform. To test this, we immunoprecipitated brain and BAE cell lysates in parallel experiments using anti-cdk5 monoclonal antibodies. Immunoprecipitates were fractionated on SDS–PAGE, transferred on to membrane and analyzed by Western blotting by probing with anti-cdk5. Our data (Fig. 1C) clearly suggests that brain and BAE cell cdk5 have exact the same molecular weight and they are immunochemically identical.

cdk5 Expression Correlates With BAE Cell Proliferation

To investigate the role of cdk5 in BAE cells proliferation, we studied the time course of cdk5 protein expression by Western blotting. BAE cells were grown in 10% FCS and induced by bFGF (1 ng/ml) and cell lysates were prepared after 0, 24, 48, 60, and 70 h intervals. These lysates were separated on SDS–PAGE and analyzed by Western blotting as before. Band densities of cdk5 protein were quantified using flurochem 8000 image analyzer (Alpha Innotech, San Leandro, CA) and areas of cdk5 bands are represented in graphical form in Figure 2. cdk5 expression was barely detected between 24–48 h after cell culture, but started to appear

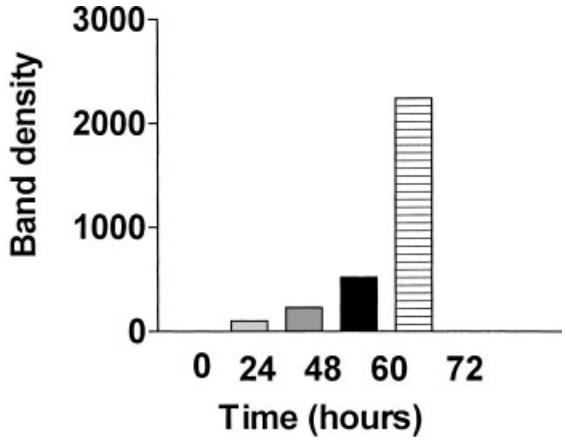

Fig. 2. Growth dependent expression of cdk5 in BAE cells. BAE cells seeded in 96-well plates and cultured in 10% FCS in presence of bFGF (1 ng/ml). Cells were harvested at time points indicated, 10 μg protein electrophoresed on SDS–PAGE and cdk5 expression was determined by Western blotting using anti-cdk5 antibody (dil 1:1,000). Densities of cdk5 bands were quantified by image analyzer. Data were analyzed using GraphPad Prism software.

from 60 h and peaked at 72 h. It is interesting to note that ECs needed approximately 68 h to complete one cell cycle (doubling cell population) [Martinez et al., 1999]. In our experiments the highest level of cdk5 expression was detected during the time frame when BAE cells prepare for division. These data may suggest that cdk5 expression regulates BAE cell proliferation.

AS Induced Inhibition of BAE Cells Proliferation Correlates With the Down-Regulation of cdk5 Expression

AS is a well known inhibitor of ECs proliferation, AS also induces apoptosis [Cao et al., 1996; Claesson-Welsh et al., 1998; Lucas et al., 1998] arrests cell cycle progression in G_2/M-phase of cell cycle [Griscelli et al., 1998]. We therefore investigated whether AS induced inhibition of BAE cells proliferation correlates with the level of cdk5 expression. To test this mechanism, two sets of BAE cells were cultured in the presence of bFGF and incubated with various concentrations of AS for 72 h. After 72 h one set of cells was analyzed for proliferation and the other set of cells was analyzed for cdk5 protein expression as seen in Figure 3. AS treatment down-regulates bFGF induced cdk5 protein level in a dose-specific manner (Fig. 3A) and directly correlates with the inhibition of cell proliferation (Fig. 3B,C).

cdk5 Expression in BAE Cells Correlates With Selective Sensitivity of AS

Sensitivity to AS differs among cells of different origin. For example, the growth of fibroblasts cells (NIH3T3) is not affected by AS [O'Reilly et al., 1994b]. We compared cdk5 protein levels and its correlation with AS sensitivity in NIH3T3 and ECs. Our Western blot analysis revealed lack of cdk5 expression in NIH 3T3 cells, while both BAE and HUVEC cells showed consistently expression of cdk5 (Fig. 4A). Next, we tested sensitivity of these cells to AS treatment. We found that AS failed to affect NIH3T3 cells growth, as also reported previously [O'Reilly et al., 1994b] while it inhibits the growth of BAE cells (Fig. 4B). These data raise the possibility that AS's anti-proliferative activity and induction of cell death could be in part due to down-regulation of the cell cycle regulatory protein cdk5, specifically in ECs.

Targeted Disruption of cdk5 Inhibits BAE Cells Proliferation and Induces Apoptosis In Vitro

Roscovitine is a selective inhibitor of cdk5 at very low concentration and known to induce cell apoptosis and inhibit proliferation [Havlicek et al., 1997; Meijer et al., 1997; Mgbonyebi et al., 1999; Edamatsu et al., 2000; Somerville and Cory, 2000; Sharma et al., 2002b]. To validate our findings that AS targets cdk5 in BAE cells and to determine whether specific blocking of cdk5 expression inhibits ECs proliferation and induces apoptosis, BAE cells were grown in two sets in the presence of bFGF as described before and incubated with various concentrations of roscovitine for 72 h. The cells were then analyzed respectively for cdk5 expression and proliferation. Immunoblot analysis revealed up-regulation of cdk5 expression by bFGF treatment. This bFGF-induced up-regulation of cdk5 was blocked by roscovitine treatment in a dose-specific manner (Fig. 5B). Cell proliferation assay data showed a dose-dependent inhibition by roscovitine and correlated with cdk5 protein expression level (Fig. 5A). Olomoucine a roscovitine analog is a specific inhibitor of cdk4, but does not inhibit cdk5 [Meijer et al., 1997]. Olomoucine failed to inhibit BAE cell proliferation as well as cdk5 expression (Fig. 5A,B). BAE cells were insensitive to olomoucine even at higher dose (50 μg/ml). Interestingly, cdk4 expression was not detected in BAE cells in

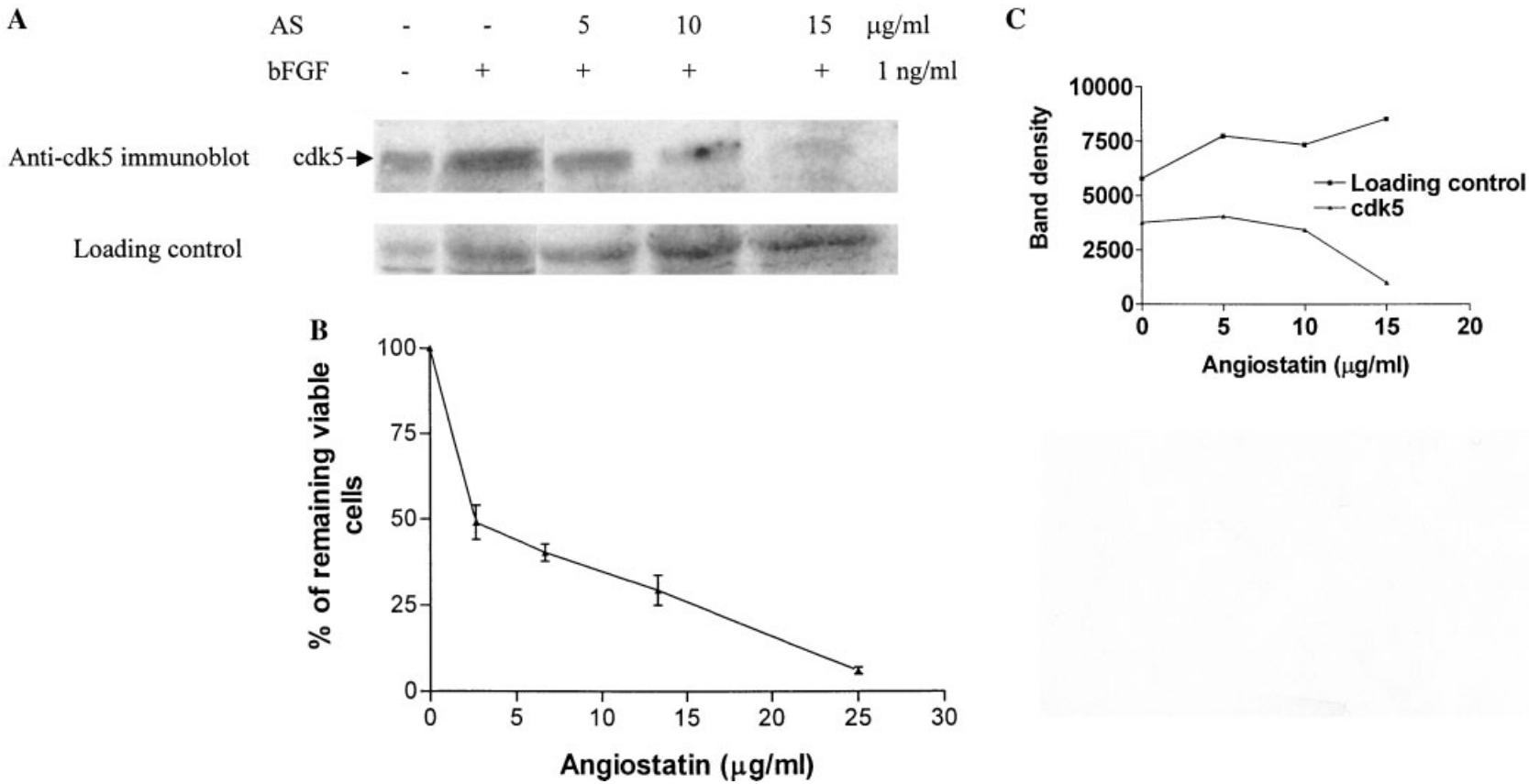

Fig. 3. Angiostatin (AS) treatment inhibits BAE cell proliferation and correlates with down-regulation of cdk5. Effect of AS on the expression of cdk5 was determined by Western blot analysis. About 10,000 BAE cells were plated on 96-well plates in 10% FCS and stimulated with bFGF (1 ng/ml). Cells were treated with AS for 72 h. After 72 h, 10 µg protein was analyzed for cdk5 expression (**Panel A**). The blot was striped subsequently and reprobed with anti-tubulin antibody (**bottom**) as control for protein loading. Densities of cdk5 and loading control band were quantified by image analyzer. Data were analyzed using GraphPad Prism software (**Panel C**). Viable cells were detected by Promega's cell proliferation kit (**Panel B**).

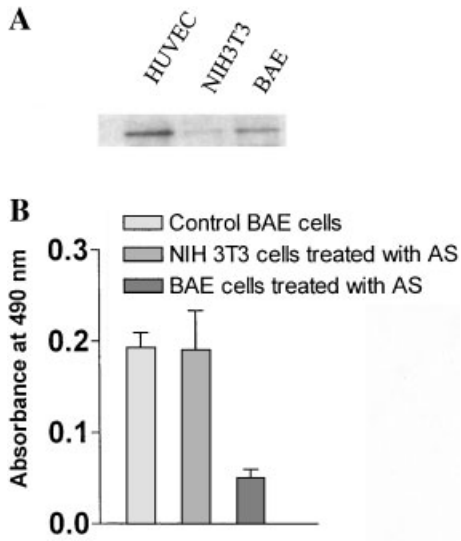

Fig. 4. AS selectively inhibits the proliferation of BAE, but not the fibroblast cells and correlates with cdk5 expression. NIH 3T3, HUVEC, and BAE cells were cultured in 96-well plates in two sets and treated with AS for 72 h. After 72 h treatment, 10 μg protein was immunoblotted with anti-cdk5 antibody (dil 1:1,000) and immunoreactive protein bands were identified by ECL (**Panel A**). Cell proliferation assay (**Panel B**).

presence or absence of bFGF (Fig. 5C). Roscovitine-treated cells showed DNA fragmentation, annexin V staining and extensive detachment from the surface, characteristics of cell apoptosis (Fig. 6). In sharp contrast, roscovitine analog olomoucine failed to induce apoptosis (Fig. 6). Taken together these data suggest that targeted disruption of cdk5 in BAE cells induces cell cycle arrest, apoptosis, and inhibits proliferation.

DISCUSSION

Since the discovery of AS as anti-angiogenic molecule, much of the research has focused on exploiting it for therapeutic use. Our laboratory is interested in understanding AS's molecular mode of action in ECs. In this context, we previously reported that AS binds to the ECs surface receptor annexin II [Tuszynski et al., 2002] and may regulate plasmin generation—one of the key proteolytic activities mediating the angiogenic process [Hajjar and Menell, 1997; Hajjar and Krishnan, 1999; Kang et al., 1999; Pepper, 2001; Tarui et al., 2002].

To gain further insight in the intracellular events triggered by AS, we have developed several lines of evidence suggesting that the serine threonine kinase cdk5 may regulate proliferation and cell apoptosis in BAE cells. The first major finding of this study is the identification of cdk5 expression in BAE cells and its up-regulation by one of the most potent stimulators of ECs proliferation and angiogenesis, bFGF [Biro et al., 1994]. Secondly AS, a powerful inhibitor of angiogenesis which down-regulated cdk5 expression in a dose-dependent fashion and inhibited BAE cells viability (Fig. 3A,B). Thirdly, roscovitine, a selective inhibitor of cdk5 [Havlicek et al., 1997; Meijer et al., 1997; Sharma et al., 2002b] completely inhibited BAE cells proliferation and induced cell death with concomitant down-regulation of cdk5 expression in a dose-dependent manner (Fig. 5A,B). To our knowledge this is the first report describing that in addition to its interaction with cell surface receptors [Sharma et al., 2002a] AS targets the cell cycle regulatory protein cdk5 and may be eliciting down-stream intracellular signaling pathways mediated by cdk5 [Sharma et al., 2002b].

Recent reports demonstrated that *AS* gene transfected ECs displayed a disrupted cell cycle progression (G_2/M-phase) and down-regulated M-phase phosphoproteins [Griscelli et al., 1998] without disruption of the G_1/S-phase of cell cycle [Lucas et al., 1998; Luo et al., 1998]. These reports indicate that AS targets proliferating ECs in the G_2/M-phase without any noticeable effect on DNA synthesis. Other anti-angiogenic drugs, e.g., TNP-470 and endostatin, have also been reported to interfere with cell cycle progression by targeting cdks and arrest ECs growth [Yeh et al., 2000; Hanai et al., 2002]. These reports prompted us to investigate the regulation of cdk by AS.

cdks have recently gained considerable interest in view of their essential role in the regulation of the cell division cycle, proliferation, and programmed cell death [Nigg, 1995; Lew and Kornbluth, 1996; Sherr, 1996; Havlicek et al., 1997]. cdk5 is a serine/threonine kinase and member of the cyclin-dependent protein kinase family (cdc2, cdc28, and other structural-related cdks) [Lew and Wang, 1995]. Although cdk5 is predominantly expressed in neurons and reportedly plays important role in neurogenesis involving neuronal migration, differentiation, and neurite outgrowth [Tsai et al., 1994; Nikolic et al., 1996; Ohshima et al., 1996; Chae et al., 1997; Ohshima et al., 1999; Sharma et al., 1999], its expression in ECs and role in angiogenesis has not been described before. Here, we show that the cell cycle protein cdk5 is

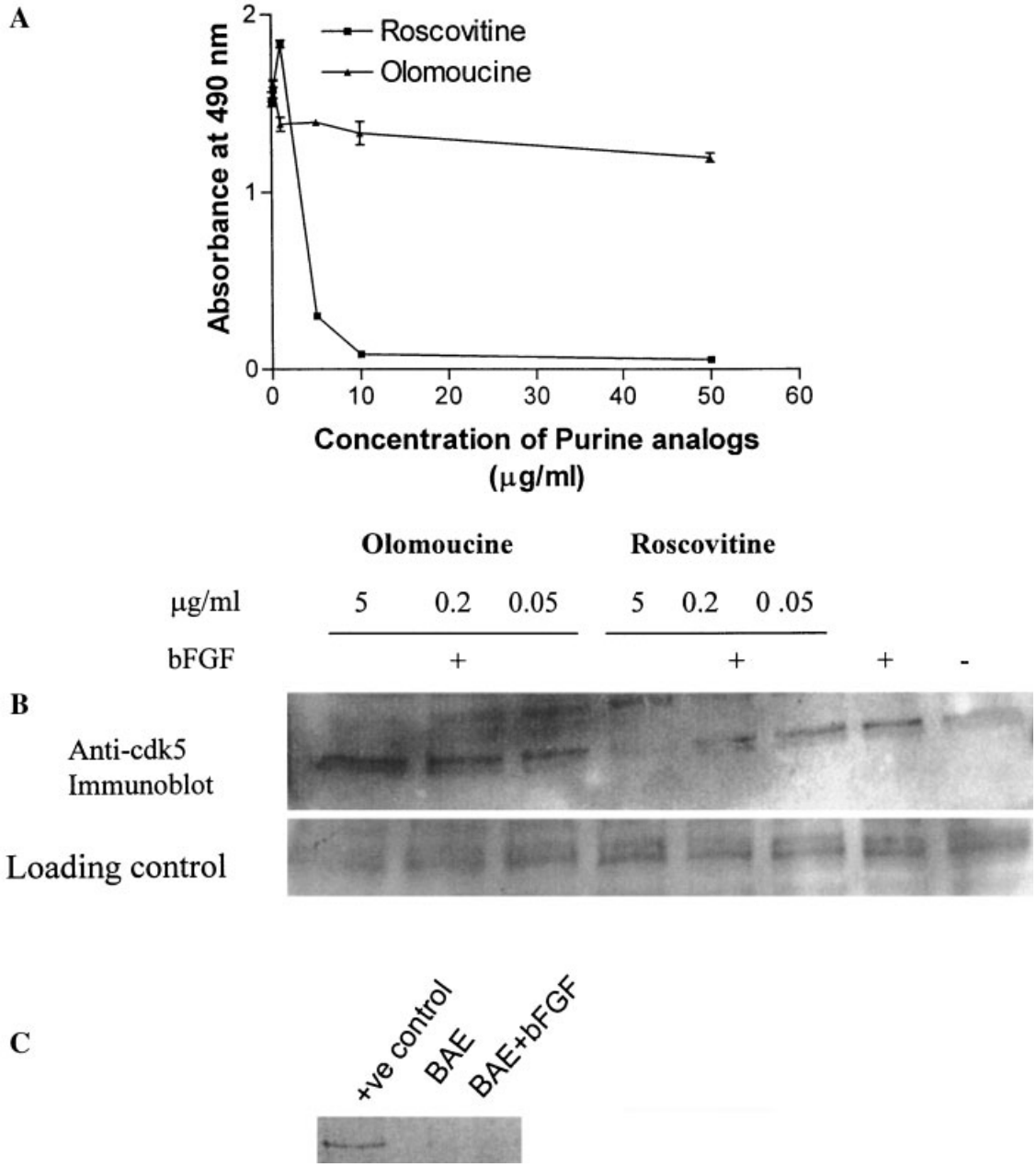

Fig. 5. Selective inhibition of cdk5 protein by roscovitine inhibits proliferation. Two sets of BAE cells were seeded in triplicate in Ham's medium containing 10% FCS and bFGF (1 ng/ml). After 24 h, BAE cells were exposed to either vehicle-containing medium (DMSO) or various concentrations of roscovitine and olomoucine for 72 h. After 72 h, viable cells were assayed by Promega's cell proliferation kit as describe previously (**Panel A**). Cells were lysed and 10 μg of protein was used to determined cdk5 protein expression using anti-cdk5 antibody (dil 1: 1,000). After immunoblot analysis, blot was striped and reprobed with anti-tubulin antibody to ensure equal amount of protein loading (**Panel B**, **bottom**). Western blot (**Panel C**) shows lack of cdk4 protein expression in BAE cells, A431 cell lysate supplied with monoclonal antibody used as positive control.

specifically linked to ECs proliferation and the apoptotic activity of AS.

In this report, we show that the angiogenic cytokine bFGF induces proliferation of BAE cells significantly as compared to quiescent cells (Fig. 1A). This increase in proliferation was directly correlated to the time-specific expression of cdk5. bFGF induced the expression of cdk5 only after 60 h of cell culture peaks at 72 h (Fig. 2). It has been reported earlier that BAE cells in vitro need about 68–70 h to complete one cell cycle (doubling of cell population). Cultured

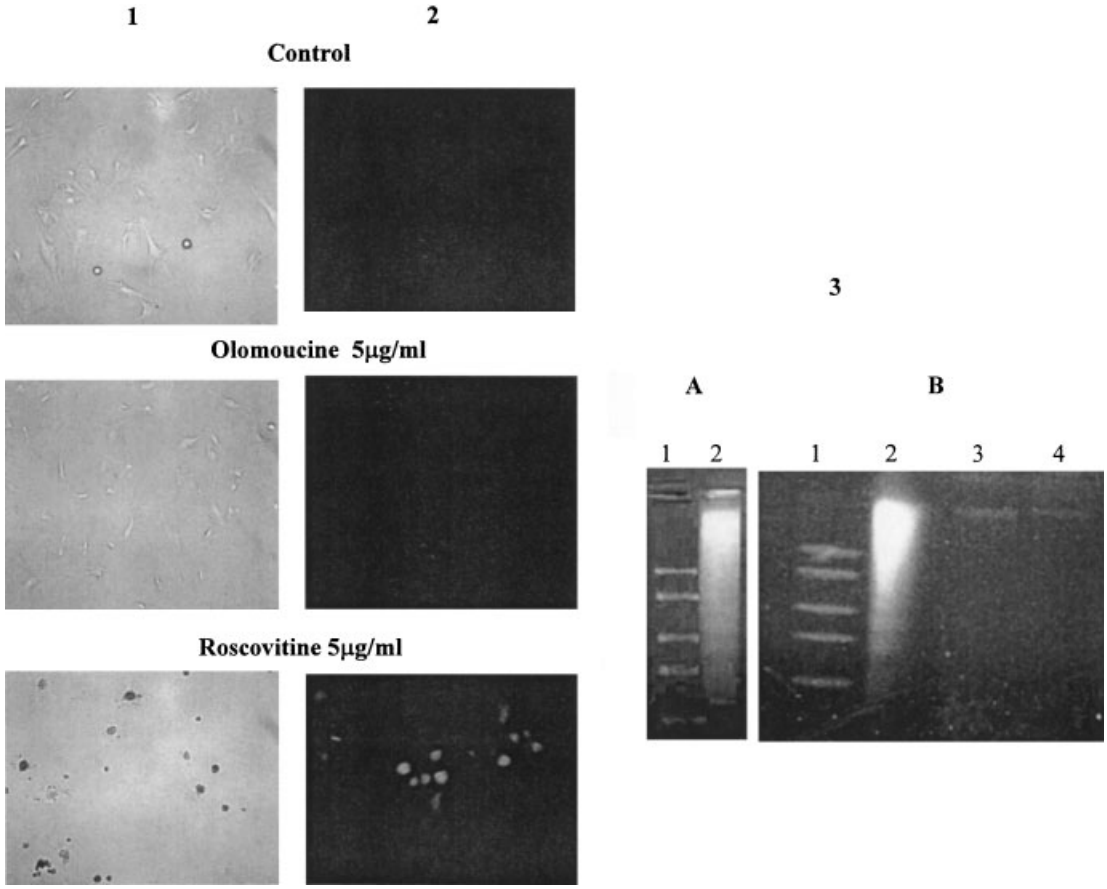

Fig. 6. Targeted disruption of cdk5 induces BAE cell apoptosis: BAE cells were treated with roscovitine, olomoucine (5 μg/ml) and vehicle. **Panel 1** is photomicrograph of control (DMSO), olomoucin and roscovitine treated BAE cells under light microscopy. Morphology of the cells treated with roscovitine showing characteristics of apoptosis (**bottom**). To detect apoptosis, BAE cells were stained with annexin V after treatments and visualized by fluorescent microscopy. Only roscovitine treatment showed positive staining with annexin V (**Panel 2**, **bottom**). Roscovitine induced BAE cells apoptosis was further confirmed by nuclear DNA fragmentation technique (**Panel 3B**, **lane 2**). Olomoucine treatment failed to induce DNA fragmentation (Panel 3B, **lane 3**) and annexin V staining (Panel 2, **middle**). Panel 3B, **lane 4**: DNA from control BAE cells. **Panels 3A** and B, **lane 1**: DNA ladder. Panel 3A, lane 2: Actinomycin treated HL60 cells (positive control).

BAE cells spend 36 h in G_1phase, 8 h in S-phase, and 24 h in G_2/M-phase (68 h) [Martinez et al., 1999]. Our time specific expression data suggests that cdk5 expression in 72 h is typically during the G_2/M-phase of cell cycle. It is likely that AS mediated disruption of G_2/M-phase [Griscelli et al., 1998] may be due to inhibition of cdk5 protein expression and activity. It is quite obvious from our results shown in Figure 3 that AS treatment of BAE cells inhibits cdk5 expression (Panel A) with concomitant inhibition of proliferation in a dose-specific manner (Panel B).

AS's activity is shown to be specific to ECs not to the fibroblasts cells (NIH 3T3) [O'Reilly et al., 1994b]. In this context, we tested the specificity of cdk5 expression in NIH 3T3 and compared to ECs. Surprisingly, cdk5 expression was not detected in NIH3T3 cells, AS treatment also failed to inhibit proliferation of NIH3T3 cells (Fig. 4A,B), consistent with previous observations [O'Reilly et al., 1994b]. These findings indicate that cdk5 may be an important molecule of endothelial cell cycle and critical for proliferation, apoptosis, and angiogenesis.

Selective Inhibition of cdk5 Protein Expression Induces EC Death and Inhibits Proliferation

To provide further support for the role of cdk5 in ECs survival and proliferation, we specifically blocked cdk5 expression by its selective inhibitor, roscovitine [Meijer et al., 1997; Sharma et al., 2002b]. Roscovitine, an efficient inducer of apoptosis and inhibitor of cell proliferation [Havlicek et al., 1997; Schutte et al., 1997; Edamatsu et al., 2000; Somerville and

Cory, 2000], being considered as potential anticancer agent. Our results show that roscovitine treatment inhibits bFGF induced cdk5 expression in a dose-dependent manner (Fig. 5A) and directly correlates with the inhibition of BAE cells proliferation (Fig. 5B). Roscovitine treatment also showed progressive loss of BAE cells attachment. Interestingly, the same dose (5 μg/ml) of roscovitine treatment which detached almost 95% cells from substratum with complete disappearance of cdk5 expression also caused cell death (Fig. 6 Panel 1, Bottom) [Mgbonyebi et al., 1999]. In sharp contrast, olomoucine an analog of roscovitine and specific inhibitor of cdk4 failed to inhibit cdk5 expression (Fig. 5B). Olomoucine also failed to induce BAE cells apoptosis and inhibition of proliferation. These results further suggest that at least cdk4, a specific serine threonine kinase expressed during G_1/S-phase, is not involved in EC proliferation and apoptosis. These findings might also explain why AS does not affect G_1/S-phase of cell cycle [Lucas et al., 1998; Luo et al., 1998].

ECs attachment to ECM components is prerequisite for survival, migration, proliferation, and eventually angiogenesis [Bussolino et al., 1997; Risau, 1997]. Recent evidence suggests that cell-matrix adhesion is mediated by over expression of cdk5 [Negash et al., 2002] and activation of focal adhesion kinase (FAK) [Kornberg et al., 1992]. Cell ECM adhesion accompanied by phosphorylation and recruitment of number of related signaling molecules thereby transducing anchorage and survival messages to nucleus [Leventhal et al., 1997; Marushige and Marushige, 1998]. It is likely that extensive detachment of cells from cell culture substratum and apoptotic BAE cell death observed in our experiment after roscovitine and AS treatment may be due to disruption of cdk5 mediated adhesion or intracellular signaling. In support of our argument recently it has been reported that cdk5 mediated phosphorylation results in inhibition of MAP kinase kinase 1 (MEK1) catalytic activity and hence the phosphorylation of ERK1/2 [Sharma et al., 2002b]. The role of MAP kinase in cellular proliferation, survival and differentiation is well established [Pearson et al., 2001].

Analysis of cell cycle related gene expression in post mitotic neurons during apoptosis demonstrated a striking down-regulation of *cdk5* and cyclin *D3* genes, in line with our observations [Freeman et al., 1994]. The association of cdk5 with apoptosis has also been reported previously and support our observations [Ahuja et al., 1997]. It is possible that the inhibition of cdk5 may deny the cells anchorage and traction needed for proliferation, migration, and angiogenesis.

In summary, the results of this study clearly demonstrate that bFGF up-regulates cdk5 expression in a time and dose-specific manner and correlates with ECs growth. On the other hand an anti-angiogenic molecule AS, known to specifically induce ECs apoptosis and inhibit their proliferation, inhibited cdk5 expression in dose-dependent manner. AS induced cdk5 down-regulation correlated with inhibition of BAE cells proliferation and of cell death. These findings may be highly relevant to elucidate the anti-angiogenic function of AS.

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