Retinoblastoma Protein Modulates the Inverse Relationship between Cellular Proliferation and Elastogenesis*

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The mechanism that leads to the inverse relationship between heightened cellular proliferation and the cessation of elastic fibers production, observed during formation of the arterial occlusions and dermal scars, is not fully understood. Because the retinoblastoma protein (Rb), responsible for cell cycle initiation, has also been implicated in insulin-like growth factor-I-mediated signaling stimulating elastin gene activation, we explored whether differential phosphorylation of Rb by various cyclin-cyclin-dependent kinase complexes would be responsible for promoting either elasticogenic or pro-proliferative signals. We first tested cultures of dermal fibroblasts derived from Costello syndrome patients, in which heightened proliferation driven by mutated oncogenic H-Ras coincides with inhibition of elastogenesis. We found that Costello syndrome fibroblasts display elevated level of Rb phosphorylation on serine 780 (Ser(P)-780-Rb) and that pharmacological inhibition of Ras with radicicol, Mek/Erk with PD98059, or cyclin-dependent kinase 4 with PD0332991 not only leads to down-regulation of Ser(P)-780-Rb levels but also enhances Rb phosphorylation on threonine-821 (Thr(P)-821-Rb), which coincides with the recovery of elastin production. Then we demonstrated that treatment of normal skin fibroblasts with the pro-proliferative PDGF BB also up-regulates Ser(P)-780-Rb levels but treatment with the pro-elasticogenic insulin-like growth factor I activates cyclinE-cdk2 complex to phosphorylate Rb on Thr-821. Importantly, we have established that elevation of Thr(P)-821-Rb promotes Rb binding to the Sp1 transcription factor and that successive binding of the Rb-Sp1 complex to the retinoblastoma control element within the elastin gene promoter stimulates tropoelastin transcription. In summary, we provide novel insight into the role of Rb in mediating the inverse relationship between elastogenesis and cellular proliferation.

Elastic fibers are composed of a microfibrillar scaffold made up of several glycoproteins and a polymeric elastin core. They are particularly numerous in the extracellular matrix of blood vessels lungs, heart, skin, ligaments, periosteum, and auricular cartilage (1–3). Mature elastic fibers, deposited almost exclusively during the late gestation and the early childhood, constitute the most durable element of the extracellular matrix that in undisturbed tissues may last over the entire human lifespan (4, 5). However, after local inflammation or mechanical injuries, the elastic fibers can be degraded (6–9). Interestingly, the healing of wounded connective tissue frameworks and the remodeling of injured blood vessels or heart mostly engages the intense proliferation of local fibroblasts or smooth muscle cells and seldom concludes with the recovery of normal elastic fibers that are otherwise replaced with the abundant collagen fibers (6–15). On the other hand, patients with genetic diseases caused by primary haploinsufficiency of the elastin gene (supravalvular aortic stenosis or Williams-Beuren syndrome) develop the hyperproliferative intimal thickenings of major conduit arteries in early childhood (16–18). Previous studies from our laboratory showed that cultured fibroblasts or arterial smooth muscle cells derived from Williams-Beuren syndrome and supravalvular aortic stenosis patients (17) as well as from patients with other genetic deficiencies characterized with impaired elastic fiber assembly (19–22) also develop arterial and cardiac lesions associated with heightened cellular proliferation. We also studied cells derived from Costello syndrome (CS)3 (MIM 218040) a developmental disorder caused by germ line mutations of the H-Ras gene that induces a persistent hyper-activation of the downstream mitogenic signaling pathway involving Ras-Raf-Mek-Erk. This in turn leads to the development of peculiar phenotype that also includes formation of diverse malignant tumors (19–21, 23, 24). Interestingly, these patients are also characterized with the excessively wrinkled skin, respiratory problems, skeletal deformations, hypertrophic cardiomyopathy, cardiac valve malformations, and arterial occlusions, which can all be linked to impaired elastogenesis (19–21, 25, 26). Although results of our previous studies demonstrated an inverse relationship between cellular proliferation and elastic fiber production (17, 20–22), the molecular mechanism responsible for this phenomenon has not been elucidated.

It has been previously demonstrated that IGF-I facilitates delivery of the Sp-family of transcription factors (Sp1/Sp3) to a specific DNA domain located within elastin gene promoter (27, 28). This sequence is homologous to the DNA sequences capable of interaction with the tumor suppressor retinoblastoma protein (Rb) located in promoters of the multiple genes

*This work was supported by Canadian Institute of Health Research Grant PG 13920 and by Heart and Stroke Foundation of Ontario Grant NA 4381 (to A.H.).
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1 The abbreviations used are: CS, Costello syndrome; Rb, retinoblastoma protein; IGF-I, insulin-like growth factor; Sp1, specificity protein 1; RCE, retinoblastoma control element; cdk, cyclin-dependent kinase.

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involved in both cellular proliferation (29–34) and differentiation (35–38). This observation was followed by the suggestion that such retinoblastoma control elements (RCE) would also modulate transcription of tropoelastin gene. However, the particular mechanism facilitating binding of Sp transcription factors to Rb and their successive interaction with RCE in the elastin gene promoter has not been described yet.

It has been previously documented that in its hypophosphorylated state Rb represses the G1-S transition by sequestration of E2F-family of transcription factors (41, 42). The pro-proliferative signals that activate cyclin-D-cdk4/6 complexes to induce Rb phosphorylation on its serine residues 780 and 795 (Ser-780/795) cause the release of E2F and allow for transcription of E2F-responsive genes, required for the cell cycle initiation (29–34, 40, 43). Although the activations of the cyclin A and cyclin E complexes with cdks have been linked to the propagation through S-phase the cell cycle (44, 45), these active complexes have also been identified in the non-proliferating cells, suggesting their role(s) in other cellular processes (46–49). Meaningfully, the binding of Rb to the transcription factors belonging to the Sp-family of zinc-finger proteins has been linked to the activation of numerous genes contributing to cellular differentiation (50–54) as well as to the activation of elastin transcription (27, 28).

Thus, in the present study we investigated whether the phosphorylation status of Rb that can be differentially modulated by different cyclin-cdk complexes would constitute a crucial step in the mechanism responsible for the inverse relationship between cellular proliferation and deposition of elastic fibers. To test this hypothesis we used primary cultures of highly proliferative dermal fibroblasts derived from CS patients that also exhibit low elastic fiber deposition as well as cultures of normal fibroblasts exposed to the pro-proliferative factor PDGF BB or to the elastogenic factor IGF-I in the presence and absence of specific cdk4 and cdk2 inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemical grade reagents, inhibitor of Ras/Raf interactions, radicicol (#R2146), Mek1/2 inhibitor, PD98059 (#P2125), growth factors human insulin-like growth factor (IGF-I) and human recombinant platelet-derived growth factor (PDGF-BB), polyclonal anti-Ki67 antibody, secondary antibodies fluorescein-conjugated goat anti-rabbit, goat antimouse, and anti-phospho-Thr-821-Rb antibody were all obtained from Sigma. The inhibitor of cdk4, PD0332991, was a generous gift from Pfizer Canada Inc. (Pointe-Claire/Dorval, QC). The inhibitors of cdk2, CVT313 (#238803), and purvalanol A (#P4484) were purchased from EMD/Calbiochem. The polyclonal antibodies anti-cyclin E (C-19), anti-cyclin D1(H-295), anti-Thr(P)-160-cdk2, anti-cdk2, anti-cdk4, anti-Rb (C-15), anti-Sp1 (PEP2), and anti-Sp1 (1C6)X), monoclonal antibody anti-β-actin, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit, and goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The polyclonal antibodies to Ser(P)-780-Rb and the phosphorylated C-terminal Rb fusion protein Ser(P)-795-Rb were acquired from Cell Signaling Inc. (Danvers, MA). Polyclonal antibody to tropoelastin was purchased from Elastin Products (Owensville, MI). Monoclonal anti-Rb antibody was purchased from BD Pharmingen. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen and 0.2% trypsin, 0.02% EDTA and other cell culture products were obtained from Multicell, Wisent Inc. (St. Bruno, QC, Canada). The One-step RT-PCR master solution kit and the RNeasy Mini Kit were purchased from Qiagen (Mississauga, ON). Tropoelastin and GAPDH RNA primers were ordered from The Centre for Applied Genomics at the SickKids Hospital (Toronto, ON). The genomic DNA isolation kit was purchased from Ambigen Life Science Technologies LTD. Methyl-[3H]thymidine and l-[3,4(n)]-3H]valine were purchased from Amersham Biosciences. Magnetic IgG beads for immunoprecipitation, Dynabeads G protein, were purchased from Invitrogen. The Dako-Cytomation LSAB2 System (HRP liquid, diaminobenzidine kit, and hematoxylin counterstain) were supplied by DakoCytomation (Carpinteria, CA). Permount histological mounting media was obtained from Fisher. A nuclear extraction kit for extracting nuclear proteins was obtained from Active Motif (Carlsbad, CA). Supersignal West Pico Chemiluminescent Substrate for the detection of chemiluminescence was acquired from Thermo Fisher Scientific Inc. (Rockwood, TN). The genomic sequence of the putative retinoblastoma control element (RCE) was ordered from Integrated DNA Technologies (Coralville, IA). Anti-streptavidin–HRP conjugate antibody was purchased from Immunoresearch Jackson Laboratory (Montreal, QC), and Biodyne B 0.45 μm membranes were purchased from Paul Gelman Laboratory (Ann Arbor, MI).

**Cell Cultures**—Dermal fibroblasts derived from normal human skin biopsy explants of three normal 18-, 26-, and 31-year-old females were generous gifts from Human Matrix Sciences, Inc. (Visalia, CA). Dermal fibroblasts were derived from three previously characterized children (20, 21) with Costello syndrome (a 9-month-old male (12195), a 24-month-old female (12196), and a 3-year-old male (7669)) bearing particular H-Ras mutations: G12Ahet, G12Shet, and G12Shet. CS cells were obtained from the Department of Genetics of Cell Culture Services at The Hospital for Sick Children (Toronto, ON) with the permission of the Institutional Ethics Committee. All described experiments were performed three times. In each experimental group quadruplicate cultures of fibroblasts (passages 2–5) derived from each normal subject or from each CS patient were maintained in the presence and absence of indicated reagents. All cultures were maintained in DMEM with 1% antibiotics/antimycotics and different concentrations of FBS. The details of particular experiments were described in the respective figure legends. In all our experiments we employed broadly used inhibitors of different kinases in concentrations recommended by their respective manufacturers. Results of our pilot experiments also indicated that cultures treated for 5 days with the chosen concentrations of these inhibitors did not demonstrate a significant increase in the number of dead cells (detected by trypan blue exclusion test) or apoptotic cells (detected by TUNEL test), as compared with untreated cultures. Moreover, their desirable effects due to the substrate- and site-specific phosphorylations were always reversible after transfer of treated cultures to the medium without inhibitors.
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[^H]Thymidine Incorporation—Cells were plated in 35-mm culture dishes (100,000 cells/dish) in quadruplicates and maintained in serum-free medium for synchronization of their cell cycle. Then they were treated as indicated with IGF-I (10 nM) and PDGF (1 nM) in the presence and absence of diverse reagents as indicated in the Fig. 3A legend. 1 μCi of[^H]thymidine/ml of media was added and maintained in 2% FBS for 72 h. Cells were lysed, and[^H]thymidine incorporated into DNA was counted using a Win Spectral 1414 Liquid Scintillation Counter.

K-67 Immunohistochemistry—5-Day-old cultures of normal and Costello syndrome fibroblasts, initially plated 50,000 cells/35-mm plate and maintained in DMEM with 10% FBS, were fixed in cold 100% methanol at −20 °C for 30 min. The methanol was then removed by washing with water and PBS. The DakoCytomation LSAB2 System (HRP liquid diaminobenzidine) kit was used as per the manufacturer’s instructions (DakoCytomation). The slides were examined under a Leica DMR microscope with Magnification. The specific localization of K-67 was evaluated by fluorescein-conjugated goat anti-rabbit secondary antibody, and nuclei were counterstained with propidium iodide as previously described (58–59).

Immunodetection of Elastic Fibers—Fibroblasts initially plated on coverslips (50,000 cells/culture) were cultured in 35-mm culture dishes in DMEM with 10% FBS. Cultures were grown to ~80% confluency and then pretreated first with the indicated inhibitors (for 1 h) before the addition of the growth factors. At the end of the indicated times all cultures were fixed and treated with polyclonal antibody to tropoelastin followed by fluorescein-conjugated goat anti-rabbit secondary antibody, and nuclei were counterstained with propidium iodide as described previously (17, 20). Cells were examined with a Nikon Eclipse E1000 microscope attached to a cooled CCD camera (Qimaging, Retiga EX), and the captured images were analyzed with a computer-generated video analysis system (Image-Pro Plus software, Media Cybernetics, Silver Springs, MD) as previously described (20).

One-step RT-PCR Analysis—Human dermal fibroblasts were cultured in 60-mm dishes in 10% FBS DMEM and treated as indicated in the figure legend for 18 h. Total RNA was extracted using the RNeasy Mini kit according to the manufacturer’s instructions, 0.5 μg of total RNA was added to each one-step RT-PCR (Qiagen One-Step RT-PCR kit), and reactions were set up according to the manufacturer’s instructions in a total volume of 25 μl. The reverse transcription step, the elastin, and GAPDH PCR reactions were performed as previously described (55). Samples of the elastin and GAPDH PCR products from each reaction were run on agarose gels stained with ethidium bromide. Levels of tropoelastin mRNA detected in particular cultures were quantitatively assessed by densitometry, and final results were normalized to the relative levels of GAPDH mRNA.

Preparation of Whole Cell Extracts—Cells were lysed in 60 μl of Nonidet P-40 lysis buffer (20 mM HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, phosphatase inhibitors (2.5 mM Na3O4P2, 1 mM Na2VO4, 1 mM β-glycerol phosphate)) and a protease inhibitor mixture (20 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF, 1 mM DTT). Then cells were scraped, collected into Eppendorf tubes, and incubated in 4 °C for 30 min on a shaking platform. The samples were then centrifuged at 16,000 rpm for 15 min. The supernatant containing both cellular and nuclear proteins was then isolated into separate tubes.

Preparation of Nuclear Extracts—Cells were extracted using the nuclear extraction kit (Active Motif) according to the manufacturer’s instructions.

Western Blots—Aliquots of the cellular or nuclear lysates containing 40–20 μg of proteins were resuspended in sample buffer (0.5 mM TrisHCl (pH 6.8), 10% SDS, 10% glycerol, 4% 2-β-mercaptoethanol, and 0.05% bromphenol blue), denatured, resolved on 8% SDS-PAGE gels, and transferred on to nitrocellulose membranes as previously described (58–59). Membranes were then blocked with bovine serum albumin (BSA) and probed with antibodies detecting the phosphorylated serine and threonine residues on Rb, cyclins, or other indicated proteins and their respective secondary antibodies. Immuno-detected proteins were visualized with the enhancement chemiluminescence kit (ECL) (Amersham Biosciences) according to the manufacturer’s instructions.

Co-immunoprecipitation—Cultures were treated for 1 h with and without IGF-I (10 nM) in the presence or absence of CVT313 (500 nM) or PD0332991 (200 nM) in serum-free conditions. Whole cell protein extracts were obtained as described above. The 300-μg aliquots (containing 50 μg protein) were then mixed with aliquots of Dynabeads (Invitrogen) conjugated with anti-Rb antibody (according to Manufacturer’s protocol) and incubated overnight at 4 °C on a shaking platform. The beads were washed extensively, and the beads-bound proteins were then subjected to the Western blotting with a rabbit polyclonal anti-Sp1 antibody.

Electromobility Shift Assay—Nuclear extracts were obtained from cell cultures treated with IGF-I (10 nM) with or without CVT313 (500 nM). In the annealing reaction, for complementary binding of the two RCE oligonucleotides (top, 5′-Biotin label TCCCCGGGGCGGGCTTCCCTCCCCCCTCITT-3′) and bottom, 5′-AGACGGGGAGGGCTGGGGAGGGGGCCGGGGGA-3′) (27), the equimolar aliquots of the two oligos were subjected to the routine annealing reaction in the buffer containing 1 mM EDTA (pH 8.0), 10 mM Tris (pH 8.0), and 50 mM NaCl immersed in 1 liter of boiling water and left to gradually cool to room temperature.

Binding Reaction—5 pmol of biotinylated RCE-DNA probe and 2 μg of nuclear extracts were mixed in a binding buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 mM KCl, and 12% glycerol) and incubated at room temperature for 30 min. The final products were then resuspended in bromphenol blue loading buffer and loaded into 6% non-denaturing polyacrylamide gels. The membranes were blocked in BSA for 1 h. To detect the possible interactions between Sp1 transcription factor and the RCE DNA probe, two sets of the final products of the binding reaction were run on a single native gel. To first detect total nuclear proteins bound to the RCE probe, the first set of binding reaction product was transferred to Biodyne B membrane, cross-linked by 0.12 J of UV light exposure, and then incubated with anti-streptavidin–HRP conjugated anti-
body. To further demonstrate that the same gel-shifted complex of RCE probe and total nuclear proteins bound the anticipated transcription factor, the second set of preparation was transferred to a nitrocellulose membrane and subjected to the Western blotting with anti-Sp1 antibody.

Isolation of RCE-bound Proteins—To further confirm that the human elastin gene promoter may bind the Sp1 transcription regulating factor and that such an interaction could be modified by IGF-I, the above-mentioned double-stranded DNA probe, reflecting a putative RCE domain present in the human elastin gene, was biotinylated and conjugated with 40 pmol of M-280 streptavidin-containing Dynabeads according the manufacturer’s instructions. Then 20-μl aliquots of beads were mixed with 200-μl samples of nuclear extracts (containing 50 μg of protein) and suspended in the binding buffer (50 mM KCl, 10% glycerol, 20 mM HEPES, pH 7.9, 1 mM MgCl2, 1 mM DTT, 0.1 μg of poly(dI-dC) and proteinase inhibitors) derived from cells that were maintained for 30 min in the presence and absence of 10 ng/ml IGF-I and/or 500 nM CVT313. All preparations were subsequently incubated for 2 h. Then the beads were washed 4 times in the same binding buffer without poly(dI-dC). The bound proteins were resolved on an 8% SDS-PAGE and probed by Western blot with the anti-Sp1 monoclonal antibody.

Statistical Evaluations—in all biochemical studies the individual cultures derived from three normal subjects or three CS patients were prepared and tested in three separate experiments. Quadruplicate cultures derived from each experimental group were assayed. The mean and standard errors (S.E.) were calculated for each experimental group, and statistical analyses were carried out by one-way analysis of variance followed by Bonferroni’s test comparing selected groups. A p value of less than 0.05 was considered significant. Densitometry measurements were recorded using Adobe Photoshop CS5 and Bio-Rad Multi-Analyst Version 1.0.2 (PPC).

RESULTS

To gain further insight into the link between heightened cellular proliferation and low elastogenesis, we explored cultures of dermal fibroblasts derived from skin biopsies of three previously characterized CS patients bearing H-Ras mutations (20, 21) as well as from fibroblasts obtained from three normal age-matched children. Results of immunohistochemistry with anti-K67 antibody (Fig. 1A) and radioactive thymidine incorporation (Fig. 1B) demonstrated that 5-day-old cultures of CS fibroblasts displayed heightened proliferation rate and did not deposit immuno-detected elastic fibers or retained metabolically labeled insoluble elastin compared with normal skin fibroblasts (Fig. 1, C and D). Although these results confirmed our previously published observations (20, 21), we now for the first time demonstrate that blocking hyperactive H-Ras with radicicol (56, 57) or inhibiting downstream proliferative signaling with Mek/Erk inhibitor, PD98059 (58), as well as inhibition of cdk4 with PD0332991 (59) (Fig. 1E) all resulted in a significant up-regulation in the synthesis of tropoelastin (Fig. 1F) and in the ultimate deposition of immuno-detectable elastic fibers by cultured CS fibroblasts (Fig. 1G). In contrast, inhibition of cdk2 activity with CVT313 (60) or with purvalanol A (61), which also attenuated the cellular proliferation rate (Fig. 1E), did not reverse the impaired elastogenesis observed in parallel cultures of CS fibroblasts (Fig. 1, F and G). The obtained data further confirmed that CS cells have the potential to produce elastic fibers only after inhibition of their hyper-proliferative phenotype.

We next investigated the Rb phosphorylation status in these hyperproliferative cells. The comparison of representative Western blots (Fig. 2A, left panel) and results of quantitative analysis of Western blots from cultures of skin fibroblasts derived from three normal individuals and from three different CS patients bearing H-Ras mutations (Fig. 1A, right panel) indicated that CS cells display heightened levels of cyclin D-cdk4-dependent Rb phosphorylation on Ser-780 and slightly lower levels of cyclin E-cdk2-dependent Rb phosphorylation on Thr-821 as compared with normal cells. Importantly, we demonstrate that blocking the proliferative H-Ras-induced signals in CS fibroblasts with radicicol not only caused a decrease in Rb phosphorylation on Ser-780 to the levels observed in normal dermal fibroblast but also led to the up-regulation of Rb phosphorylation on Thr-821 (Fig. 2A). Furthermore, we demonstrate that the treatment of CS fibroblasts with the cdk4 inhibitor PD0332991 that quenched the site-specific Rb phosphorylation on Ser-780 and subsequently arrested the cell cycle also promoted heightened phosphorylation of Rb on Thr-821. In contrast, treatment with the cdk2 inhibitor CVT313 attenuated Rb phosphorylation on Thr-821 (Fig. 2B).

To further explore the role and the mechanism by which Rb mediates the proliferative and elastogenic pathways, we then treated normal human dermal fibroblasts either with the pro-proliferative PDGF BB or pro-elastogenic IGF-I factors in the presence and absence of cdk4 (PD0332991) or cdk2 (CVT313) inhibitors. Representative Western blots (Fig. 2C, left panel) and results of quantitative analysis of Western blots derived from cultures of skin fibroblasts derived from three normal individuals (Fig. 2C, right panel) indicated that cells treated with IGF-I display lower levels of cyclin D and heightened levels of cyclin E, whereas cells treated with PDGF BB showed more cyclin D compared with untreated controls. Moreover, IGF-I-treated normal fibroblasts also displayed significantly more Rb phosphorylated on Thr-821 than untreated controls or fibroblasts treated with PDGF BB. The PDGF BB-treated cultures also contained more Rb phosphorylated on Ser-780 (Fig. 2D).

We then demonstrated that although the PDGF BB induced a significant increase in the proliferation rate of normal fibroblast cultures that could be eliminated in the presence of cdk4 inhibitor (PD0332991) or cdk2 inhibitors CVT313 and purvalanol A, IGF-I had no effect on the proliferation rate of these cells (Fig. 3A).

Furthermore, our results show that in contrast to PDGF BB, which did not induce elastogenesis, treatment with IGF-I significantly stimulated transcription of elastin mRNA level (Fig. 3B), tropoelastin protein production (Fig. 3C), deposition of metabolically labeled insoluble elastin (Fig. 3D and E), and ultimate assembly of the immuno-detectable elastic fibers (Fig. 3E). Furthermore, we also demonstrated that pretreatment with the cdk4 inhibitor (PD0332991) that inhibits the basal proliferation rate of fibroblasts induced by 2% serum also caused a

Proliferation versus Elastogenesis
5 Day-old cultures of Dermal Fibroblasts

A. Proliferative antigen Ki-67

Normal Fibroblasts | Costello Fibroblasts
---|---

B. Incorporation of[^3H]-Thymidine

C. Elastic Fibers

Normal Fibroblasts | Costello Fibroblasts
---|---

D. Incorporation of[^3H]-Valine Insoluble Elastin

E. Incorporation of[^3H]-Thymidine

F. Western Blots

G. Elastic Fibers

Control | Radicicol (10μM) | PD98059 (20μM) | PD032591 (400nM) | CTV313 (1.25μM) | Purvalanol A (35nM)
---|---|---|---|---|---

H. Morphometry

*P<0.001

FIGURE 1. A and B, representative micrographs demonstrate that 5-day-old cultures of Costello syndrome-derived dermal fibroblasts, maintained in medium with 5% FBS, contain more cells displaying the presence of immuno-detectable proliferative antigen, Ki-67, and incorporate significantly more[^3H]-thymidine than equally plated cultures of normal human skin fibroblasts. C and D, shown are representative micrographs of 5-day-old cultures immuno-stained with anti-tropoelastin antibody, and results of the quantitative assay of[^3H]-valine-labeled insoluble elastin demonstrate that Costello syndrome fibroblasts do not produce elastic fibers or deposit metabolically labeled insoluble elastin. E, results of the quantitative assay of[^3H]-thymidine incorporation to Costello syndrome fibroblasts cultured for 5 days in medium with 5% FBS indicate that daily treatments with the indicated doses of radicicol, PD98059, PD032591, CTV313, or purvalanol all induce a significant inhibition of their proliferation rate as compared with untreated counterparts. F, shown are the results of the quantitative evaluation of Western blots detecting tropoelastin levels (normalized for levels of β-actin) in the whole cell lysates of Costello syndrome fibroblasts that were maintained for 24 h in medium with 2% serum in the presence and absence of indicated reagents. These results demonstrate that in contrast to IGF-I, radicicol, PD98059, and PD032591 that induced a significant up-regulation of tropoelastin levels, treatments with cdk2 inhibitors, CTV313, or purvalanol radically inhibited production of tropoelastin. G, representative micrographs and results of quantitative morphometric evaluation of elastic fibers deposition in 5-day-old cultures of Costello syndrome fibroblasts confirm the ultimate pro-elastogenic effects of radicicol, PD98059, and PD032591 and the anti-elastogenic effect of cdk2 inhibitors, CTV313, and purvalanol. All graphs depicting the quantitative results are based on data obtained from three individual experiments in which individual cultures derived from three Costello syndrome patients were tested. Results from similar experimental groups (mean ± S.D.) were statistically evaluated and were finally expressed as a percentage of control. In the immunostained cells the nuclei were counterstained with propidium iodide. The scale bar = 15 μm.
remarkable increase in a net deposition of elastic fibers in both control and IGF-I-treated cultures. We also found that the inhibition of cdk2 with CVT313 or with purvalanol A completely inhibited basal and IGF-I-induced deposition of elastic fibers. These results indicate that inhibition of cell cycle initiation creates favorable conditions that promote elastogenic signals.

To study the involvement of the cyclinE-cdk2 complex on IGF-I-induced elastogenesis, we first examined the level and
FIGURE 3. A, results of the quantitative assay of 
$[^3]$H]thymidine incorporation demonstrate that in contrast to a significant proliferative effect of PDGF BB, treatment with IGF-I did not enhance proliferation of normal dermal fibroblasts in 3-day-old cultures maintained in media with 2% FBS. The additional treatments of parallel cultures either with cdk4 inhibitor (PD0332991) or with cdk2 inhibitors (CVT313 or purvalanol A) induced a significant inhibition of proliferation rates of fibroblasts from all three experimental groups. B and C, results of the quantitative one-step RT-PCR analysis of tropoelastin mRNA and results of densitometric analysis of immunoblotted tropoelastin detected in cell layers of cultures maintained for 18 h with 2% FBS show that treatment with cdk4 inhibitor (PD0332991) enhanced elastogenesis to the levels exceeding those observed in cultures treated with low doses of IGF-I. In contrast, the parallel cultures treated with cdk2 inhibitors (CVT313 or purvalanol A) demonstrate a significant inhibition of elastogenesis. D and E, results of the quantitative assay of 
$[^3]$H]valine-labeled insoluble elastin and results of quantitative morphometric evaluation of micrographs depicting immuno-detected elastic fibers in 5-day-old cultures of normal fibroblasts are shown. They consistently demonstrate that in contrast to control and IGF-I-treated cultures, fibroblasts treated with PDGF BB do not deposit elastin. They also demonstrate that inhibition of cdk4 with PD0332991 in control and both growth factor-treated cultures enhances the levels of their elastin deposition. In contrast, inhibition of cdk2 with CVT313 or purvalanol A eliminates elastic fiber deposition in all experimental groups. All graphs depict the mean ± S.D. of data obtained from three individual experiments involving triplicate cultures of fibroblasts derived from three individuals. In the immunostained cells the nuclei were counterstained with propidium iodide. Scale bar = 15 μm.
Our results demonstrate that IGF-I induced a quick (10 min) up-regulation of cyclin E levels and phosphorylation of cdk2 on threonine 160. This coincided with heightened phosphorylation of Rb on Thr-821 (Fig. 4A). In contrast, treatment with IGF-I did not induce changes in the levels of cyclin D and cdk4 nor cdk4-dependent Rb phosphorylation on Ser-780. Furthermore, the 1-h exposure of parallel cultures (maintained in serum-free medium) to cdk2 inhibitor, CVT313, significantly reduces levels of the IGF-I-induced phosphorylation of cdk2 on Thr-160 and the cdk2-dependent phosphorylation of Rb on Thr-821. Cultures of normal fibroblasts maintained in the presence of 2% FBS that were additionally exposed for 1 h to the cdk4 inhibitor, PD0332991, demonstrate enhanced levels of cyclin E and cdk2 proteins and the heightened levels of phosphorylated cdk2 on Thr-160. They also showed heightened levels of Rb phosphorylated on Thr-821. All graphs depict the mean ± S.D. of data obtained from three individual experiments involving quadruplicate cultures of fibroblasts derived from different individuals. Presented densitometric values of cyclins and cdks were normalized for β-actin, and values of phosphorylated Rb were normalized for total Rb.

Results of additional electromobility gel shift assays also demonstrate that the synthetic DNA probes reflecting RCEs of the elastin gene promoter immobilize proteins present in the cytoplasm of normal fibroblasts. Moreover, we demonstrate for the first time that the IGF-I-induced interaction between Rb and Sp1 is dependent on cdk2 activity (Fig. 5A). Thus, it does not occur in cells treated with CVT313. On the other hand, we showing that treatment of fibroblast with the cdk4 inhibitor PD0332991 promotes the intracellular pathway facilitating formation of the Rb-Sp1 complex and prevents the initiation of the cell cycle by PDGF BB that otherwise coincides with the inhibition of Rb/Sp1 interaction (Fig. 5B).

Results of additional electromobility gel shift assays also demonstrate that the synthetic DNA probes reflecting RCEs of the elastin gene promoter immobilize proteins present in the cytoplasm of normal fibroblasts.
nuclear extracts of cultured fibroblasts (Fig. 5C, left panel). Further probing of those proteins transferred to nitrocellulose membrane with anti-Sp1 antibody indicated that normal fibroblasts treated with IGF-I contained higher levels of RCE probe-bound Sp1 than their untreated counterparts. Interestingly, this IGF-I-induced enhancement of RCE/Sp1 association cannot be detected in fibroblasts in which activity of cdk2 has been inhibited with CVT313 (Fig. 5C, right panel). These results
were further confirmed in experiments in which nuclear extracts of IGF-I- and CVT313-treated cells were incubated with RCE DNA probe immobilized on Dynabeads (Fig. 5D).

**DISCUSSION**

The inverse relationship between heightened cellular proliferation and the exclusive cessation of elastic fibers deposition (but not other extracellular matrix components) has been frequently reported in occlusive neointima of the injured arteries and in hypertrophic dermal scars (6–15, 62). We also reported that this phenomenon contributes to the development of arterial stenoses in patients with Williams-Beuren syndrome and supravalvular aortic stenosis (17) as well as in patients with CS that develop severe hypertrophic cardiomyopathy (19). On the other hand, experimental induction of elastic fiber deposition in balloon-injured rabbit femoral arteries prevented formation of hyper-proliferative plaques (63). Also, we have established that the experimental induction of elastogenesis in astrocytoma cells cultures leads to decreased proliferation rate (64, 65). In the present study we show that pharmacological inhibition of pro-proliferative pathways including H-Ras activation, Mek/Erk signaling, or cyclin D-cdk4 complex formation coincides with the recovery of normal deposition of elastic fibers in cultures of CS fibroblasts. To inhibit the initial proliferative signals induced by the hyperactive mutated H-Ras in CS cells, we used the antibiotic radicicol that emerged as a potent inhibitor of heat shock protein 90, the ubiquitous chaperone of numerous oncogenic proteins that ensures their proper folding and activation (66). It has been shown that the radicicol-dependent inhibition of heat shock protein 90 results in degradation of Raf and the consecutive inhibition of the Ras/Raf-Mek/Erk signaling cascade (67–69).

Rb can be phosphorylated on serine and threonine residues by diverse cyclins-cdkks complexes enabling initiation or propagation of the cell cycle (34, 39, 40, 42, 43). On the other hand, hypo-phosphorylated Rb present in quiescent cells has been implicated in control of differentiation processes (46–49). We now demonstrated that the highly proliferative CS fibroblasts contain significantly more Rb phosphorylated on Ser-780 and less Rb phosphorylated on Thr-821 than fibroblasts derived from normal subjects. Importantly we also showed that inhibition of H-Ras with radicicol or inhibition of cdk4 activity with PD0332991 was able to reverse this phosphorylation pattern on Rb in CS fibroblasts (Fig. 2A) and at the same time induced elastin production. Thus, our data provide new insight into the role of Rb in cellular proliferation and elastogenesis.

We also demonstrated that inhibition of cyclin D-cdk4 complex in CS cells and consecutive elimination of Rb phosphorylation on Ser-780 in fibroblasts did not diminish activity of cyclin E-cdk2 complex that in quiescent cells causes Rb phosphorylation on Thr-821 (Fig. 2B).

Using normal fibroblast cultures, we also confirmed that the cyclin E-cdk2 complex, known for its role in propagation of cell-cycle through the S-phase, was also active in quiescent dermal fibroblasts. Importantly, we found that IGF-I treatment led to cdk2 activation and consequently up-regulated the levels of Rb phosphorylated on Thr-821, which occurred in the absence of Rb phosphorylation on Ser-780 (Fig. 4). The fact that heightened levels of cyclin E protein were detected just 10 min after IGF-I treatment suggested that IGF-I may halt the turnover of this kinase rather than induce new protein synthesis. Other investigations have shown that IGF-I is capable of reducing cyclin E turnover by blocking the actions of the cyclin E inhibitors p27/Kip and subsequently increasing Rb phosphorylation in differentiating macrophages (44). Interestingly, pretreatment with pharmacological inhibitors of cyclin E-cdk2 activity, which diminished the levels of Rb phosphorylation on Thr-821, did not inhibit the basic cellular proliferation rate in the IGF-I-treated cultures but abolished the IGF-I-induced increase in elastogenesis. These data indicate that IGF-I likely enforces the available cyclin E-cdk2 to preferentially participate in the propagation of pro-elastogenic signal.

Previous studies suggested that differential phosphorylation of Rb results in conformational changes allowing interactions with various transcription factors known to modulate a broad spectrum of genes (39, 40, 70–72). Although previous studies by Conn et al. (27) suggested that after IGF-I treatment Sp1 transcription factor regulates elastin gene transcription via RCE located within elastin gene promoter, the signaling pathways responsible for this effect had not been established.

Results presented in our study for the first time reveal that in IGF-I-treated cells the Rb could be co-immunoprecipitated with Sp1 transcription factor and that the pretreatment with cdk2 inhibitor, which halted the IGF-I-induced elastogenesis, also abolished the interaction between Rb and Sp1 (Fig. 5A). Importantly, we additionally found that inhibition of cdk4 activity with PD0332991, which results in the inhibition of cellular proliferation, also enhanced the associations between Rb and Sp1 (Fig. 5B). We then demonstrated that nuclear extracts of IGF-I-treated cells contained more Sp1 bound to the DNA probe reflecting RCE sequence than untreated counterparts and that such IGF-I-induced association could not be detected in cells in which cdk2 activity was inhibited (Fig. 5C). Thus, our results reveal a novel paradigm in which phosphorylation of Rb on Thr-821 by the cyclin E-cdk2 complex, in the absence of cyclin D-cdk4-dependent phosphorylation of Rb, recruits Sp1 to Rb, which in turn promotes the binding of Sp1 to the elastin RCE. On the other hand, we now demonstrate that signals leading to activation of the cyclin D-cdk4 complex to phosphorylate Rb on Ser-780 and eliminate elastogenesis.

Then we found that experimental inhibition of H-Ras activity with radicicol not only quenched the heightened proliferation of CS fibroblasts but also induced recovery of their normal elastogenesis. Our finding encourages the use of this non-toxic macrolidic antibiotic in the future therapy of CS patients that would combat tumorigenesis as well as phenotypic abnormalities resulting from impaired elastogenesis. This claim can be endorsed by the fact that radicicol, in addition to inhibition of cancer cells proliferation (73), has also been shown to quench proliferation of activated inflammatory leukocytes and endothelial cells, thereby reducing inflammatory responses in atherosclerosis (74) and experimental pneumonia (75). Radicicol has been also proposed as a potential drug for treating different angiogenesis-dependent diseases, such as solid tumors, psoriasis, rheumatoid arthritis, and diabetic retinopathy (76). Importantly, we have also established that treatment with PD0332991...
that inhibits the growth of solid tumors (59, 77) can also stimulate elastogenesis in cultures of normal and CS fibroblasts after Rb phosphorylation on Thr-821. Thus, this cdk4 inhibitor should also be considered in future therapies of CS patients.

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