Decorin-induced Growth Suppression Is Associated with Up-regulation of p21, an Inhibitor of Cyclin-dependent Kinases*

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Antonio De Luca†, Manoranjan Santra‡, Alfonso Baldi†, Antonio Giordano†, and Renato V. Iozzo†‡

From the §Kimmel Cancer Center, ‡Department of Microbiology and Immunology and †Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The secreted proteoglycan decorin has been implicated in the negative control of cell proliferation primarily by virtue of its ability to block transforming growth factor-β. Moreover, decorin expression is markedly up-regulated during quiescence but suppressed upon viral transformation, whereas de novo decorin expression in colon carcinoma cells abrogates the malignant phenotype by arresting the cells in the G1 phase of the cell cycle. Here we show that this decorin-induced growth arrest is associated with up-regulation of p21 mRNA and protein in a transforming growth factor-β- and p53-independent pathway. The augmented p21 protein is present as a multimeric complex with various cyclins and cyclin-dependent kinases in the nuclei of decorin-expressing cells, thereby leading to suppression of cyclin-dependent kinase activity and block of cell division. Through the usage of decorin-specific antisense oligodeoxynucleotide treatment, we demonstrate that the expression of decorin is closely linked to that of p21 and that abrogation of decorin leads to suppression of p21 and restoration of cell division. Collectively, our results provide a plausible mechanism by which decorin may contribute to retard and suppress the growth of tumor cells in vivo.

The genes that regulate the transition from a proliferative to a quiescent state are beginning to be understood at the molecular level. It has been recognized, however, that several key molecules in cancer development are proteins that operate outside the nucleus and often interact at the cell-matrix boundaries either by detecting changes in the extracellular environment or by relaying messages from receptor-transducer proteins to their control machineries. Under the appropriate circumstances, the latter genes may act as tumor suppressor genes. Several lines of evidence indicate that decorin (1), a member of the small leucine-rich proteoglycan gene family (2), is intimately associated with growth suppression activity (3, 4). For example, this proteoglycan is markedly up-regulated during quiescence in human diploid fibroblasts (5, 6) and vascular smooth muscle cells (7), and this effect is transcriptionally regulated and long lasting (6). Transcriptional activation of decorin gene expression also occurs when HeLa cervical carcinoma cells are rendered quiescent by serum deprivation (6). Moreover, decorin is rarely expressed by actively dividing normal cells as well as in SV40 transformed cells (2, 5), and its expression is totally abrogated in several tumor cell lines via methylation of the genomic control regions (2, 8). In contrast, both mRNA and protein levels are markedly augmented in the peritumorous struma of human colon cancer (8, 9), a process that may reflect a regional response of the host connective tissue cells to the invading neoplastic cells (10, 11). Using a gene transfer approach in human colon WiDrHT29 carcinoma cells, which do not constitutively express this gene (12), we discovered that the de novo expression of decorin reverted the cells to a “normal” phenotype: the cells lost anchorage-independent growth, failed to generate tumors in scid/ scid mice, and were arrested in the G1 phase of the cell cycle (13). The cells could reenter the cell cycle when decorin expression was abrogated by decorin-specific antisense oligodeoxynucleotide treatment (13). Of note, these effects were due to neither clonal selection nor integration site, indicating that decorin might be a component of a negative loop that controls cell growth.

In this paper, we show that the decorin-induced growth suppression is associated with an up-regulation of p21\(^{\text{WAF1/CIP1}}\) (p21)\(^{\text{WAF1/CIP1}}\) (14) and that this leads to a suppression of cyclin and cyclin-dependent kinase (CDK) activity in a TGF-β- and p53-independent pathway. Abrogation of decorin expression by decorin-specific antisense oligodeoxynucleotide treatment leads to suppression of expression of p21 and restoration of cellular growth. These results demonstrate for the first time that an extracellular matrix proteoglycan can regulate the cell cycle and that p21 is a downstream effector of this biological process.

EXPERIMENTAL PROCEDURES

Materials—All the reagents were of molecular biology grade; [α-\(^{32}\)P]ATP and [α-\(^{32}\)P]dCTP (~3000 Ci/mmol; 1 Ci = 37 GBq) were purchased from Amersham Corp.

Growth of Stably Transfected Cells in the Presence or Absence of TGF-β—To establish the growth characteristics and the effects of TGF-β (recombinant TGF-β1, Collaborative Research), 2–5 × 10\(^{-3}\) colon carcinoma cells, including wild-type and various decorin-expressing clones (13), were plated into 96-well plates and grown for 6 days in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. To assay that TGF-β was present at relatively constant levels, we added TGF-β (10 ng/ml) every 12 h, while feeding was every 48 h. At the end of each time point, the cells were washed and incubated for 1–1.5 h with an aqueous soluble tetrazolium/formazan compound and the chromogen was quantitated by determining its absorbance at 490 nm. This method,

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† To whom correspondence and reprint requests should be addressed: Dept. of Pathology, Anatomy and Cell Biology, Rm. 249, J efferson Alumni Hall, Thomas J efferson University, 1020 Locust St., Philadelphia, PA 19107. Tel.: 215-503-2208; Fax: 215-923-7969; E-mail: iozzo@acjd.tju.edu.

‡ The abbreviations used are: p21, the inhibitor of cyclin-dependent kinase activity p21\(^{\text{WAF1/CIP1}}\); CDK, cyclin-dependent kinase; TGF-β, transforming growth factor-β; PAGE, polyacrylamide gel electrophoresis.
the amount of formazan product is time-dependent and directly proportional to the number of viable cells (14).

Immunoblotting Detection of Cyclin, CDKs, and p21—Confluent cultures of wild-type or decorin-expressing colon carcinoma cells were extracted in 200 μl of lysis buffer (50 mM Tris, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na3VO4) plus the following protease inhibitors: 1 mM phenylmethylsulfonfyl fluoride, leupeptin (25 μg/ml), pepstatin (5 μg/ml), and aprotinin (25 μg/ml). About 50 μg of protein were separated by 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore) in CAPS buffer (10 mM CAPS, 20% methanol, pH 11), washed in TBS-T, and incubated for 1 h at ambient temperature with the following antibodies: rabbit polyclonal anti-CDK2 and anti-CDK2 antibodies raised against a peptide corresponding to amino acid residues mapping at the carboxyl terminus; for cyclin A and B antibodies, bacterialy expressed glutathione S-transferase-cyclin A and glutathione S-transferase-cyclin B fusion proteins were used to visualize rabbit. Each primary antibody was then incubated with the membrane in 3% milk and then washed in TBS-T three times (15). Goat anti-rabbit antibodies coupled to horseradish peroxidase were incubated with the membranes, washed in TBS-T, and visualized with ECL chemiluminescence (DuPont NEN). Exposure times was for 5–60 s.

Immunoprecipitation and Histone H1 Kinase Assays—Increasing amounts of protein (50–300 μg) from the various cell extracts were immunoprecipitated as described before (15) using anti-CDK2 antibodies and protein A-Sepharose beads. Equal amounts of protein from each fraction were immunoprecipitated with the polyclonal antibodies and tested for the presence of H1 kinase activity (15). Briefly, immunoprecipitates were incubated for 5 min at 30°C in a 50-μl reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 50 mM histone H1, 5 μCi [α-32P]ATP, and 1 mM cold ATP. The reactions were terminated with SDS-PAGE buffer containing 100 mM dithiothreitol and analyzed by 10% SDS-PAGE. The autoradiographic bands were cut and counted.

RNA Isolation and Northern Blotting—Total RNA was isolated as described before (13), separated on a 1% agarose gel containing 0.6M formaldehyde, blotted onto a nylon membrane, and hybridized at 65°C with full-length cDNAs harboring the human p21 (16) or decorin (1) genes, respectively. Thirty ng of each human insert, purified from vector sequence by low melting agarose gel electrophoresis, were labeled to high specific activity (1–2 × 10^6 cpm/μg DNA) using a random prime kit from Amersham. The blots were washed at 65°C down to 1 × SSC, 0.1% SDS and exposed to x-ray films using intensifying screens for 24 h at −70°C (17).

Sequential Immunoprecipitation and Immunoblotting—To detect p21 in immune complexes with cyclins and CDKs, equal amounts of proteins were immunoprecipitated with various antisera as detailed above. Following immunoprecipitation, the pellets were separated by 15% SDS-PAGE and immunoblotted with the 868 (Pharmigen) mouse monoclonal antibody (1 μg/ml) directed against the human p21 protein.

Immunohistochemistry and Antisense Oligodeoxynucleotide Treatment—For immunohistochemistry, cells were grown to subconfluence in slide chambers (Lab-Tek) in complete medium, washed, fixed for 10 min in 4% paraformaldehyde, and permeabilized for 3 min in 0.2% Triton X-100. The cells were reacted with the mouse monoclonal 6B6 (Jackson Laboratory) at 1:30 dilution (18). The cells were examined with a Nikon Optiphot epifluorescence microscope and photographs were taken from ten randomly-selected areas at ×200 magnification. The results were identical in two independent experiments.

For antisense oligodeoxynucleotide treatment we used a primer (5′-GTCATCAGGAATCTTGCG-3′) complementary to a sequence in the 5′ region of the decorin cDNA encoded by exon 2 (19), immediately following the glycosaminoglycan binding site. This treatment suppresses the expression of decorin by >95% (13) and, under these experimental conditions and oligodeoxynucleotide concentrations, the cells are viable and show no detectable toxic changes (13). The corresponding sense primer was used as control. Briefly, subconfluent cultures were washed and incubated in 0.5% serum with or without sense or antisense oligodeoxynucleotides (25 μM) for 22 h in the presence of polybrene (5 μM) to enhance uptake (13). After 22 h, media were changed, and cells were incubated with or without freshly added oligodeoxynucleotides for an additional 18 h. At the end of the incubation period (40 h total) RNA was extracted and subjected to Northern blotting hybridization. Parallel cultures were subjected to a nonradioactive cell proliferation assay (14).

RESULTS

Growth Kinetics of Wild-type and Decorin-expressing Colon Carcinoma Cells in the Presence or Absence of TGF-β—Because TGF-β has been implicated in decorin-induced growth suppression (3, 4), we tested whether this cytokine had any effect on the growth of wild-type or decorin-expressing colon carcinoma cells. TGF-β treatment had no effect on the growth of either the parental cell line or the various stably transfected clones (Fig. 1A). The wD1 and wD10 clones, which express relatively high levels of decorin driven by the cytomegalovirus promoter (13), had ~90% of the cells in the G0/G1 phase of the cell cycle as determined by propidium iodide staining and cell sorting (not shown). In contrast, the wild-type cells and the wC1 clone, which was transfected with the expression vector alone, contained 48% and 46% of the cells in G0/G1, respectively. These
results are consistent with previous studies which have shown that WiDr/HT29 colon carcinoma cells are totally insensitive to the cytotoxic effects of TGF-β (20). Although these cells synthesize significant levels of TGF-β, up to 110 pg/10^6 cells/day, they are not arrested in G1 nor do they exhibit radiation-induced growth arrest (21). Moreover, the WiDr/HT29 cells harbor a mutated (Arg-273 → His) p53 protein (22), thereby excluding p53 in the growth suppression associated with decorin.

To investigate further the role of decorin in growth regulation, we determined the levels of cyclins and CDKs in the parent and stably transfected clones. Immunoblotting of total cell lysates showed that the amounts of cyclins A and B, CDC2 and CDK2 (Fig. 1B), as well as those of cyclin D1 and CDK4 (not shown) remained unchanged in the decorin-expressing and growth-arrested tumor cells. Thus, cell cycle arrest could not be explained by changes in the protein levels of either cyclins or CDKs. Furthermore, immunoprecipitation of [35S]methionine-labeled cell extracts with anti-decorin antibodies did not show any additional band co-precipitating with decorin (not shown), indicating that decorin did not physically interact with intra-cellular cyclin-CDK complexes. This notion is also supported by the fact that nearly all the detectable decorin was rapidly secreted as a chondroitin sulfate proteoglycan in the wD1 and wD10 cells (13).

De Novo Expression of Decorin Is Associated with Inhibition of Cyclin and CDK Activity—Next, we explored whether decorin expression altered the functional activity of CDKs by measuring the kinase activity of total cell extracts following immunoprecipitation with anti-cyclin and anti-CDK antisera (15). Of note, the wD1 cells exhibited a ~60% reduction in the histone H1 kinase activity driven by CDC2 (Fig. 2A). These findings were obtained when increasing amounts of immunoprecipitated protein were used (Fig. 2B), and were reproduced in six independent experiments (Fig. 2C). When the same cellular extracts were immunoprecipitated with antibodies against cyclin A, cyclin B, or CDK2, a generalized and marked inhibition of kinase activity was observed (Fig. 2D). It should be pointed out that cyclin A can associate with both CDC2C and CDK2, and both of these complexes can phosphorylate histone H1 (23–25), with cyclin A-CDK2 complexes most prominent in S phase. On the other hand, cyclin B-CDK2 complexes are the primary kinases of mitosis (23).

De Novo Expression of Decorin Is Associated with Marked Up-regulation of p21 mRNA and Protein Levels—The results presented above suggested that the growth arrest could be mediated by a general inhibitor of cyclin and CDK activity. Accordingly, we hypothesized that the growth-suppressive properties of decorin could be mediated by p21, a potent inhibitor of G1, CDKs (26, 27), whose transcriptional activity is regulated by p53-dependent (26, 28) and p53-independent (29–31) pathways. This hypothesis was assessed by establishing the levels of p21 message and protein in the various colon carcinoma cells. In agreement with previous studies (21), the levels of p21 message in the colon wild-type cells were relatively low (Fig. 3A, lane 7; see also Fig. 4F) as compared with those detected in the wD1 and wD10 expressing cells (Fig. 3A, lanes 8 and 9). Note that, to better visualize the p21 transcript in the wild-type cells, lane 7 was exposed for 48 h, whereas all the other lanes were exposed for 20 h. Quantitation of the Northern blot data by scanning densitometry following normalization on ribosomal RNA or the housekeeping gene GAPDH gave identical results and showed a 6-fold increase ± 0.3 (mean ± S.E., n = 6) of the p21 message in the decorin-expressing cells. As additional controls, the p21 probe detected a similar transcript in human diploid skin fibroblasts, osteosarcoma and melanoma cells, and in lung and colon tissues (Fig. 3A). Corroborating the RNA data, immunoblotting analyses revealed a similar 5–6-fold increase in p21 levels in the wD1 cells (Fig. 3B). Of note, immunoblotting of same cell extracts with antibodies against either p16 or p27, two important inhibitors of CDKs (23, 25), showed no significant changes (not shown), suggesting that the induction of p21 was a specific effect.

Association of p21 in Multimeric Complexes with Various Cyclins and CDKs in the Nuclei of Decorin-expressing Cells—In the next series of experiments, we sought to determine whether the p21 was indeed associated in a multimeric complex with various cyclins and CDKs. We first immunoprecipitated equal amounts of cell lysates with antibodies directed against CDC2, cyclins A, B, or D1 and then performed immunoblotting analyses with anti-p21 monoclonal antibodies. The results (Fig. 4A) showed a significant increase of p21 in the wD1 and wD10 expressing cells when immunoprecipitated with either anti-CDC2 or anti-cyclin D1 antisera. Similar results were also obtained with antibodies against cyclin A or B (not shown). These findings, therefore, establish a direct connection between p21 protein levels and the inhibition of histone H1 kinase activities shown above. Furthermore, the enhanced expression of p21 demonstrated at both mRNA and protein levels correlated well with an increased nuclear staining in the decorin-expressing cells (Fig. 4, B–E). The two expressing clones, wD1 and wD10, showed a marked augmentation of p21 in the nuclei, with 25–40% of the nuclei positive for the p21 (Fig. 4, D and E), in contrast to <1% in the parental cells (Fig. 4D).
Decorin-expressing colon carcinoma cells exhibit marked up-regulation of p21 mRNA and protein levels. A, autoradiogram of a Northern blotting hybridized with a full-length p21 cDNA showing an increase of the p21-specific transcript in the two clones wD1 and wD10 (lanes 8 and 9, respectively) as compared with the wild-type cells (lane 7). To visualize the p21 message in the wild-type cells, lane 7 was exposed for 48 h, whereas all the other lanes were exposed for 20 h (see also Fig. 4F). Notice also the relatively high expression of p21 in fibroblasts (lane 1, 5 μg used) and the low levels in the osteosarcoma Saos2 cells (lane 4, ~30 μg of RNA used). The 18 S ribosomal RNA is shown in the bottom panel. B, immunoblotting detection of p21 protein using a mouse anti-human monoclonal antibody. Notice the marked up-regulation of p21 in the decorin expressing cells (lanes 2 and 4) as compared with the wild-type cells (lanes 1 and 3). As a positive control, equivalent amounts of cell extracts from human diploid fibroblasts were used (lane 5). In lanes 1 and 2, 30 μg of protein were used, whereas in lanes 3 and 4, 60 μg of total cellular proteins were analyzed.

4C). As expected all the fibroblast nuclei contained significant levels of p21 protein (Fig. 4B).

A Decorin/p21 Axis—To assess more directly the connection between decorin/p21 expression and growth suppression, we used sense and antisense oligodeoxynucleotides directed against the 5′ region of decorin to suppress its expression in the wD1 clones. The results showed that decorin antisense oligodeoxynucleotide treatment down-regulated not only decorin but also p21 expression by >95% in the decorin expressing clone wD1 (Fig. 4F, lane 4 versus lane 3); in contrast, the same treatment had no effect on the wild-type cells (Fig. 4F, lanes 1 and 2). The abrogation of decorin/p21 axis led to the entry of the cells into the cell cycle and enhanced growth rate (Fig. 4G). These results, thus, establish a direct link between decorin, p21, and growth suppression in the colon carcinoma cells and suggest that modulation of the decorin/p21 axis may bear biological significance in vivo.

DISCUSSION

An emerging concept in cancer biology is that the cyclin-CDK complexes are relatively well regulated in relation to the coordination of DNA replication and mitosis (23, 24, 32). However, transformed cells are unable to regulate these complexes vis-à-vis DNA damage or extracellular signals (23–25). Two independent lines of evidence point to decorin as a potential extracellular growth regulator. The first derives from the observation that decorin is up-regulated during quiescence in human diploid fibroblasts (5, 6) and in growth-arrested vascular smooth muscle cells (7). Decorin is one of several genes, named quiescins, whose expression is up-regulated 10–40-fold vis-à-vis rapidly proliferating cells (5). Of note, various types of collagen, such as types I, III, and VI, are members of the quiescins. Because, all these collagenous proteins interact specifically with the small leucine-rich proteoglycans (2), including decorin (33, 34), it is possible that part of the complex regulation of cell cycle arrest, e.g. during contact inhibition of normal diploid cells, may also include coordinately regulated extracellular matrix genes. The up-regulation of decorin is long lasting, up to 16 days after reaching confluence, and is transcriptionally suppressed by tumor necrosis factor-α (6). The second line of evidence is based on experiments employing gene transfer and forced expression of decorin in cells that do not otherwise express this proteoglycan. For example, ectopic expression of decorin in Chinese hamster ovary cells leads to growth sup-
pression via a block of TGF-β (3, 4), whereas denovo expression of decorin in human colon carcinoma cells abrogates the malignant phenotype by arresting the cells in G1 (13). In the present study, we extended our original observation and discovered a link between a secreted proteoglycan and the cell cycle machinery. Specifically, we discovered that decorin-induced cell cycle arrest of colon carcinoma cells was accompanied by a marked inhibition of cyclin and CDK activities without any significant change in their protein levels. The generalized reduction in kinase activity suggested the action of a potent inhibitory molecule acting at multiple cell cycle checkpoints. Our results demonstrate that this growth arrest was due to an induction of p21 message and protein levels via a mechanism that is independent of either TGF-β or p53 and that p21 exists in a multimeric complex with cyclins and CDKs in the nuclei of growth-arrested cells. Indeed, p21 is a potent inhibitor of CDK complexes (27), is induced in senescent cells (35), and is transcriptionally regulated by p53 (16). Recent evidence indicates that p21 acts as an inhibitory buffer whose levels dictate the threshold kinase activity required for cell cycle progression (36). Thus, the marked increase in p21 complexed with various cyclins and CDKs could provide a likely explanation for the action of decorin in the expressing cells: the induction of p21 would prevent phosphorylation of critical CDK substrates and block cell cycle progression. In the wild-type colon carcinoma cells with inactive p53 (22), this pathway would be defective, thereby permitting unregulated growth.

Through the use of antisense oligodeoxynucleotides to lower the expression of decorin, we provide evidence that the levels of decorin transcript are directly linked to those of p21 and that the expression of decorin, we provide evidence that the levels of p21 mRNA and a concomitant increase in the proportion of cells in the G1 phase of the cell cycle. Thus, decorin appears to be a general growth suppressor protein acting from the “outside” of the cell. Appearance of this gene around certain epithelial neoplasms (8) could represent a protective mechanism by which the normal stroma counteracts the invasive malignant cells.

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