Expression of the p16 and p15 Cyclin-dependent Kinase Inhibitors in Lymphocyte Activation and Neuronal Differentiation

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Abstract

The cyclin-dependent kinase inhibitors p16INK4a/MTSl and p15INK4b/MTS2 have been mapped to a region in chromosome 9 (p21) that is deleted frequently in acute lymphoblastic leukemias and malignant gliomas. To gain insight into the functions of these inhibitors in lymphocytes and neuronal cells, we studied the expression of p15 and p16 during lymphocyte mitogenesis and neuronal differentiation. Expression of p15 was extinguished during lymphocyte activation, concomitant with an increase in retinoblastoma kinase activity. The differentiation of the embryonic teratocarcinoma cell line NT2 into postmitotic neurons (hNT) was associated with enhanced expression of p15 and p16 proteins. These findings suggest that p15 and p16 play a role in maintaining cell quiescence in lymphocytes and neuronal cells, respectively. Deletions of these genes may thus promote unrestrained growth.

Introduction

During the development of multicellular organisms, cell division is strictly regulated, both spatially and temporally. The cell growth cycle depends upon the action of the CDKs, activities of which are regulated by cyclins and CDK inhibitors (reviewed in Refs. 1 and 2). The G1-specific cyclins (D type) promote phosphorylation of proteins of the Rb family and subsequent passage to the S phase of the cycle. Hypophosphorylated Rb protein in quiescent cells binds to transcription factors such as E2F, which are released upon Rb phosphorylation. The E2F family of transcription factors is required for DNA synthesis in S phase. When the developmental fate of a cell is attained, proliferation stops in early G1 (3). The precise mechanism(s) controlling cell arrest are largely unknown, but recent data suggest that the CDK inhibitors may play a major role in the process. p21 expression increases substantially in vivo during skeletal muscle (4, 5) and in vitro during hematopoietic (6) and hematoma (7) cell differentiation. The appearance of p21 is followed by cell arrest in G1. The mitogenic activation of quiescent human T lymphocytes is accompanied by elimination of the CDK inhibitor p27 (8, 9). Homozygous deletions on chromosome 9p21 of the gene for the CDK4 inhibitor p16 occur in a region of chromosome 9p21 of the gene for the CDK4 inhibitor p16 (8, 9). Homozygous deletions on chromosome 9p21 are linked CDK inhibitor, p15, are also common in acute lymphoblastic leukemias (11). In a normal situation, both lymphocytes and glial cells can remain in a quiescent state for prolonged periods. If the CDK inhibitors are responsible for maintenance of cell cycle arrest, their subnormal expression could lead to unrestrained growth and tumor formation. Therefore, it is important to assess the regulation of p15 and p16 during entry and exit from the cell growth cycle. In this study, we show that both p15 and p27 are eliminated during activation of resting lymphocytes, suggesting that they may contribute to the maintenance of the quiescent state. In a reciprocal manner, p16 expression increased markedly during the terminal differentiation of NT2 cells into neurons and in the transition from fetal to adult brain.

Materials and Methods

Lymphocyte Isolation and Activation. Peripheral blood lymphocytes were isolated from human blood by gradient centrifugation on Histopaque (Sigma Chemical Co., St. Louis, MO). Depletion of monocytes was carried out by two overnight cycles of adherence to plastic. The T lymphocytes were activated by incubation in RPMI 1640 supplemented with 20% homologous plasma and 100 units/ml interleukin 2 (R&D Systems, Minneapolis, MN) in plasticware previously coated with 10 μg/ml anti-CD3 antibodies (clone UCHT-1; Sigma). In some experiments, rapamycin was added at 2 ng/ml.

Kinase Assays and Western Blots. CDK2- and CDK4-associated kinase activities were determined in immune complexes as described (12). Briefly, 100 μg of protein lysate were immunoprecipitated with 5 μg of purified rabbit polyclonal antibody-protein A agarose complexes specific for CDK2 (Upstate Biotechnology, Lake Placid, NY) or CDK4 (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were incubated with 1 μg of histone H1 (CDK2) or 0.5 μg of recombinant glutathione S-transferase retinoblastoma fusion protein (kindly provided by J. Wang, Department of Biology, University of California, San Diego, CA) in the presence of [γ-32P] ATP (New England Nuclear) for 20 min at 30°C. After brief centrifugation, the pellet was washed, then the agarose-bound complexes were separated by electrophoresis, and the dried gel was exposed to X-ray film.

Western blots were carried out with the Western Light Protein Detection Kit (Tropix, Bedford, MA) by separating 50 μg of protein lysate/fane, blotting onto polyvinylidene difluoride membranes (Schleicher and Schuell, Keene, NH) and incubating with antibodies specific for p27 (Transduction Laboratories, Lexington, KY), p16 (PharMingen, San Diego, CA), and p15 (PharMingen). Autoradiograms were scanned using a laser densitometer.

Differentiation of NT2 Cells. NT2 cells were grown in DMEM supplemented with 10% FCS and were induced to differentiate to hNT cells with retinoic acid as described previously (13). Briefly, NT2 cells were treated with retinoic acid (0.2 mM) for 4 weeks and arrested cells (hNT) were purified by plating on selective media. Total RNA was isolated, and RT-PCR was carried out.

RT-PCR Analysis. Total RNA was isolated using RNA STAT-60 (Tel-test B, Inc., Friendswood, TX). cDNA was prepared from 5 μg of RNA with the Superscript Preamplification system (GIBCO-BRL, Gaithersburg, MD) in a 20 μl reaction. PCR was carried out in a 50-μl reaction containing 2 μl cDNA, 0.2 μg of each primer, 30 mm Tris (pH 9.0), 7.5 mm (NH4)2SO4, 1 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, and 1 unit AmpliTaq (Perkin-Elmer, Foster City, CA). Temperature conditions were: one cycle at 95°C for 3 min, 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and one cycle at 72°C for 7 min. Primers used were as follows: GAPDH, 5'-TCTCATGTGTTCAACACCATGGAGAACATG-3', 5'-AAGAACAGTGCCCTGACTGTGCAGCCACT-3'; p15, 5'-GTTAAAGAATAAAAGTGTTTG-3', 5'-GGAATGGCAGGAGGAAACAGGGCATG-3'; p16, 5'-GGATCCCCGATGAGCCGGCGGGGAGCAGCATGGAGCCTTC-3', 5'-GCACTGTGGTGGC-3'; and p27, 5'-AGTACCAAGTGGAGATGTTCAAGCG-3', 5'-TTACGTTTACGTCCTTCTGAGCGCCCA-3'. The figures depicted in "Results" represent typical assays obtained from triplicate experiments.
Ribonuclease Protection Assay. A 212-bp fragment of the p16 cDNA (nucleotides 251 to 465 from the 5' end) was inserted into pGEM3z at Avai sites. The resulting pYG10 plasmid was linearized with HindIII before producing antisense riboprobes. The vector used to express GAPDH RNA was purchased from Continental Scientific, Inc. (San Diego, CA). The radiolabeled antisense riboprobes were prepared by in vitro transcription with SP6 or T7 RNA polymerase (14). Total RNA (2–5 μg) isolated from NT2 and hNT cells was dissolved in 10 μl of H2O, mixed with 10 μl of 2× hybridization buffer (Continental Scientific) containing 1 × 10^5 cpm [α-³²P]UTP-labeled antisense p16 riboprobe, denatured at 90°C for 5 min, and then allowed to hybridize at 55°C for 14 h. Unhybridized RNA was digested with RNase T1 (GIBCO-BRL) and RNase A (Sigma) at 37°C for 1 h. The digestion reaction was terminated by the addition of stopping buffer (Continental Scientific, Inc.), and the protected RNA fragments were separated by electrophoresis on 6% polyacrylamide. The gels were dried and exposed to X-ray film.

Results

Lymphocyte Activation. Fig. 1A shows the results of a representative RT-PCR assay of total RNA isolated from normal resting human lymphocytes and from cells activated with anti-CD3 antibodies and interleukin 2. Whereas the expression of p15 and p27 decrease to undetectable levels following T-cell activation, the levels of GAPDH remain stable. In repeated experiments, p16 mRNA and protein were below the limit of detection in blood lymphocytes. The kinetics of p15 message elimination during lymphocyte mitogenesis is similar to that previously observed for p27 (Fig. 1B). Western blot analysis revealed that the amounts of p27 and p15 proteins generally corresponded to the levels of its mRNA (Fig. 2).

The p27 protein binds to and inhibits the kinase activity of CDK2/cyclin E complexes (8), while p15 inhibits mainly CDK6/cyclin D and CDK4/cyclin D complexes ((15). To determine if decreases in p15 and p27 message affected their respective CDK inhibitory activities, we assayed immune complexes of CDK2 and CDK4 from lymphocyte lysates at different times following anti-CD3 and interleukin 2 incubation. Histone kinase activity (CDK2) increased to a maximum at 24 h (Fig. 3) and was maintained for at least 72 h. Only a modest increase in CDK2-associated kinase activity is observed after 48 h of incubation with interleukin 2 in the presence of rapamycin. The CDK4-associated Rb kinase activity increased similarly following lymphoid activation and was partially inhibited by rapamycin. These results suggest that down-regulation of both p15 and p27 during lymphocyte activation allowed an increase in CDK activity that facilitated subsequent proliferation (8).

Neuronal Differentiation. To determine if expression of p15 and p16 change during neuronal development, RNA from total fetal and adult human brain were studied by RT-PCR. Fig. 4A shows that the overall levels of p16 mRNA were much higher in adult than fetal brain, while p15 and GAPDH mRNA levels were equivalent.

In vitro models of terminal differentiation are used widely to analyze developmentally regulated genes. The teratocarcinoma cell line NT2 differentiates into postmitotic hNT neurons after exposure to retinoic acid. The p16 mRNA levels in dividing and postmitotic NT2 cells were quantitated by RNase protection assay, and the results were comparable to those obtained by RT-PCR. Both p15 and p16 mRNA (Fig. 4, B and C) and protein (Fig. 2B) increase from undetectable levels in association with terminal neuronal differentiation of NT2 cells.

Discussion

To assess the possible roles in normal cells of the tumor suppressor p16 and the closely related CDK inhibitor p15, the expression of p15 and p16 were investigated during entry of lymphocytes...
into the growth cycle and during neuronal development. In lymphocytes, p15 protein disappeared during mitogenesis, while p16 transcripts and protein were below the limits of detection. In agreement with results reported previously (8), p27 message levels also fell during mitogenesis. CDK2 and CDK4 kinase activities increased during lymphocyte activation, while the levels of their specific CDK inhibitors p27 and p15 decreased. The inverse correlation suggests that both p27 and p15 are functionally important in T-lymphocyte development and may be responsible for G1 arrest of resting peripheral blood lymphocytes.

The immunosuppressant rapamycin is a potent inhibitor of T-lymphocyte proliferation. Rapamycin inhibits CDK activity in the early G1 phase of the cell cycle (16). Our data show that rapamycin inhibits both CDK2-associated histone and the CDK4-associated Rb kinase activities, suggesting that it acts both in early (CDK4) and late (CDK2) G1.

Considering that lymphocyte quiescence may be regulated by both p15 and p27, it is not surprising that homozygous deletions in p15 might occur at a high frequency in acute lymphocytic leukemia (11). The p15 gene on 9p21 is only 25 kilobases centromeric of p16 (17), and most deletions encompass both genes.4

Embryonic teratocarcinomas are pluripotent cell lines that can differentiate into several cell types. Retinoic acid induces teratocarcinoma cells to differentiate into neurons, glial cells, and fibroblasts (18). The expression of p15 and p16 increase from undetectable levels during differentiation of NT2 cells into postmitotic neurons. To show that the changes of CDK inhibitors observed during terminal differentiation of NT2 cells occurs in vivo, we compared RNA from fetal (16–23 weeks) and adult human brain. The results showed that the expression of p16 increased severalfold in adult brain tissue. The levels of p15 remained unchanged. However, it is possible that p15 began to accumulate earlier than 16 weeks of gestation and prior to p16 accumulation. The high rate of p16 deletions observed in gliomas and our results, which show increased levels in adult brain and in the differentiated hNT neurons, suggest that p16 has an important function in human brain development.

Several lines of evidence indicate that the Rb protein is a central component of the G1-to-S cell cycle transition (19). The level of hypophosphorylated Rb protein increases during terminal differentiation in a variety of cell types (3, 20). Rb can be phosphorylated by CDK4 or CDK2 in a complex with the D-type cyclins (reviewed in Ref. 19). The only known substrate of CDK4 is the Rb protein, which can be inhibited by both p15 and p16 (19). Therefore, it is likely that Rb phosphorylation, and thus the G1 to S transition, is regulated by the levels of both cyclins and CDK inhibitors.

Expression of the CDK inhibitor p21 correlates with G1 arrest during terminal differentiation of muscle and hematopoietic cells (4–7). It is conceivable that at least two inhibitors are required for complete cellular arrest. The activities of the CDK inhibitors may well be synergistic or at least additive.

Taken together, the results presented in this study strongly suggest that the CDK inhibitors p15 and p16 regulate the G1 phase-arrested state of human lymphocytes and postmitotic neurons. Deletions of the p16 and/or the p15 genes may interfere with terminal differentiation and promote tumorigenesis. Even in established malignancies, the loss of p15 and p16 may alter the balance between proliferation and exit from the growth cycle.

4 T. Nohori, unpublished data.
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References

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