CDK5 is a novel regulatory protein in PPARγ ligand-induced antiproliferation

EUGENE KIM, FEI CHEN, CHIA-CHI WANG and LAWRENCE E. HARRISON

Division of Surgical Oncology, UMDNJ-New Jersey Medical School, Newark, NJ 07103, USA

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Abstract. Cyclin-dependent kinase 5 (Cdk5) is a member of the cyclin-dependent kinase family and has been studied mainly in the differentiation of post-mitotic neurons. The purpose of this study was to determine the presence of cdk5 expression and activity in colon cancer cells and to investigate its role in the regulation of PPARγ ligand-induced antiproliferation. We observed that cdk5 protein levels and kinase activity were elevated in both HT-29 cells and human tumor tissue in comparison to decreased levels in normal colonic mucosa. To elucidate cdk5's role in PPARγ ligand-induced antiproliferation of colon cancer cells, HT-29 cells were treated with ciglitazone. A dose- and time-dependent decrease in cell proliferation were observed after ciglitazone exposure, which correlated with a decrease in cdk5 protein expression and kinase activity. Importantly, these ciglitazone-induced antiproliferative changes were reversed when cdk5 was overexpressed. Although present, p35, the regulatory protein of cdk5, showed no significant changes in protein expression with the introduction of ciglitazone. This is the first report of cdk5/p35 expression and kinase activity in colon cancer cells, which is associated with ciglitazone-induced antiproliferation in HT-29 cells.

Introduction

Peroxisome proliferator activated receptors (PPAR) are members of the nuclear receptor superfamily involved in cellular growth and differentiation. Three isoforms of the receptor have been identified which include α, ß, and γ. In particular, the PPARγ isoform has been shown to be overexpressed in various types of cancers, including colon cancer (1). Activation of PPARγ by ligands such as ciglitazone inhibits cell proliferation and induces differentiation or apoptosis (2). However, the mechanism through which these ligands cause the phenotypic changes remains unclear.

We have reported that ciglitazone induces early cell proliferation in HT-29 cells, while exposure to ciglitazone for more than 48 h inhibited cell growth (3). In addition to a decrease in cell proliferation, PPARγ ligands induce apoptosis in colon cancer cells and this induction of apoptosis may be activated by the mitogen-activated protein (MAP) kinase pathway (4). One novel regulator of the MAP kinase pathway is cyclin-dependent kinase 5 (cdk5) (5). Cdk5 is a 33 kDa protein which acts as a proline-directed serine/threonine kinase whose activity is not regulated by its interaction with cyclins, but rather by the catalytic subunits p39, p35 and its truncated product, p25 (6). In neurons, cdk5 does not directly regulate the cell cycle, but rather plays an active role in neurite outgrowth (7), synaptic transmission (8), neuronal migration and differentiation (9). Activated cdk5 is essential for normal neuronal development and abnormally expressed cdk5 has been implicated in neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (10,11). In addition, cdk5 activity has recently been discovered in other cell types and is associated with cell differentiation and apoptosis.

The purpose of this study was to determine whether colon cancer cells express functional cdk5, and to investigate its role in the regulation of PPARγ ligand-induced antiproliferation. Our results demonstrate that cdk5/p35 is expressed and is active in colon cancer cells with minimal expression in normal colonic mucosa. Moreover, ciglitazone inhibits cell growth after 48 h which is preceded by the inhibition of cdk5/p35. Conversely, overexpression of the cdk5/p35 complex reversed the inhibitory effects of ciglitazone and promoted cell growth. Our data suggest that cdk5 is overexpressed in colon cancer cells and may play an important role in PPARγ ligand-induced antiproliferation in HT-29 cells.

Materials and methods

Cell culture and biological reagents. HT-29 colon cancer cells (ATCC, Manassas, VA) were cultured in McCoy's media (Gibco BRL, Rockville, MD) supplemented with 10% complement-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37˚C, 5% CO_2. Experiments were carried out in reduced serum, 2% FBS. Ciglitazone (Biomol Research Laboratories, Plymouth Meeting, PA) was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO). Antibodies for

Correspondence to: Dr Lawrence E. Harrison, Department of Surgery, Division of Surgical Oncology, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, MSB G524, Newark, NJ 07103, USA
E-mail: l.harrison@UMDNJ.edu

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cdk5 and p35 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Whole cell extracts. HT-29 colon cancer cells were harvested and washed with cold 1X PBS. The extract was then suspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotonin, 25 μg/ml pepstatin A). Protein concentrations were determined by Bradford Protein Assay. Human colon tissue were placed in dry ice for 2 h and mechanically crushed. The crushed specimen was suspended in a protein lysis buffer and sonicated. The protein was then extracted as described above.

Western blotting, immunoprecipitation, and kinase assay. 3X SDS sample buffer [150 mM Tris (pH 6.8), 30% glycerol, 3% SDS, bromophenol blue dye 1.5 μg/100 ml, 100 mM DTT] was added to 30 μg protein extracts. The protein samples were then denatured by heating at 100˚C for 3 min and separated by a 12% SDS-PAGE (Bio-Rad Laboratories) gel and transferred to nitrocellulose membrane (Amersham, Piscataway, NJ) by electrophoresis. The membrane was subject to immunoblot analysis and the proteins were visualized by the enhanced chemiluminescence method of detection (ECL kit, Amersham, Pharmacia Biotech, Piscataway, NJ) as per the manufacturer's protocol.

For immunoprecipitation, the protein samples were incubated with the primary antibody at 4˚C overnight. Protein A plus A/G agarose beads were added and incubated for 1 h at 4˚C. The samples were washed in lysis buffer and 3X SDS sample buffer was added. The samples were heated for 3 min and separated in a 12% SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane and the proteins were detected using the aforementioned Western blotting technique.

For the kinase reaction assay, the protein extracts were immunoprecipitated with the primary antibody at 4˚C overnight. Protein A plus A/G agarose beads were added and incubated for 1 h at 4˚C. The beads were washed with a lysis buffer followed by a kinase buffer (Cell Signaling Technology, #9802). A kinase reaction buffer consisting of 10 μM ATP, 10 μCi [α-^{32}P]-ATP (specific activity 6,000 Ci/mmol, NEN, Boston, MA), and histone H1 were added to the protein samples and heated at 30˚C for 20 min. The kinase reaction was then terminated by the addition of 3X SDS buffer and the samples were stored overnight at -70˚C. A 12% SDS-PAGE gel was used to separate the proteins, which were visualized by autoradiography.

MTT assay. To measure cell mass, 1x10^4 HT-29 cells/well were plated in 96-well plates at 37˚C, 5% CO_2. Ciglitazone was added after 24 h of incubation. After completing ciglitazone treatment, 20 μl of an MTT reagent (0.5 mg/ml) was added to each well and the cells were placed in the incubator for 3 h. The media was aspirated and 2-propanol was added. Absorbance was then measured by an ELISA reader at 570 nm.

Transfection. HT-29 cells were cultured in 10% FBS McCoy's media 5A in a 96-well plate at a volume of 10^4 cells/well. After 24 h, 0.15 μg of pCMV-p35 and pCMV-cdk5 (gift from Dr Li-Huei Tsai, Harvard Medical School) diluted in 2% FBS McCoy's media 5A were transfected into the cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol.

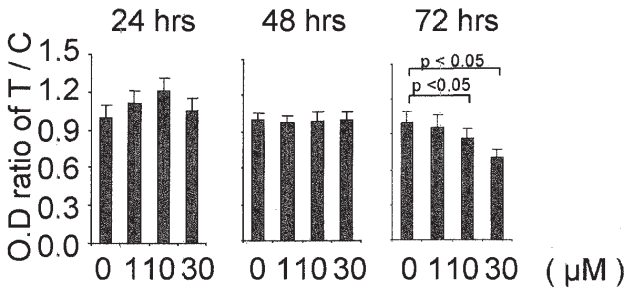

Figure 1. A dose-dependent decrease in cell growth when HT-29 cells are treated with ciglitazone for 72 h. HT-29 cells were treated with varying doses (1, 10, 30 μM) of ciglitazone and cell mass was measured by an MTT assay. After the first 24 h of exposure to ciglitazone, an increase in cell mass was observed with lower doses of ciglitazone (1, 10 μM). As opposed to the earlier time point, ciglitazone had a dose-dependent inhibitory effect on cell growth after 72 h of exposure.

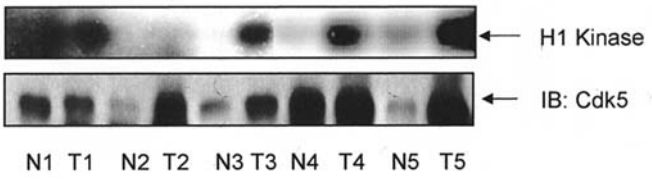

Figure 2. Cdk5 expression in human colon tumor tissue and adjunct normal mucosa (N-normal, T-tumor). Tumor tissue and normal colonic mucosa were obtained from five patients and evaluated for Cdk5 protein expression and histone H1 kinase activity. Four of five patients demonstrated significant cdk5 activity in tumor tissue compared to normal colonic mucosa by histone H1 kinase assay.

Results

We have previously demonstrated that ciglitazone induces proliferation of HT-29 cells transiently (<48 h) prior to a decrease in cell proliferation (3). We confirmed these findings and demonstrated in the first 24 h of exposure to ciglitazone, an increase in cell mass was observed with lower doses of ciglitazone (1,10 μM). As opposed to the 24 h time-point, ciglitazone exposure resulted in a dose-dependent inhibitory effect on cell growth after 72 h of exposure (Fig. 1).

Although cyclin-dependent kinase 5 has been studied predominantly in the process of differentiation in post-mitotic neurons, it has recently been reported to be expressed in other cell lines, including HL-60 and prostate cancer cells. To explore whether cdk5 plays a role in colon tumorigenesis, protein from tumor samples with corresponding normal colonic mucosa were obtained from five patients and assayed for cdk5 activity using histone H1 kinase activity assay. Four of five patients demonstrated significantly higher cdk5 activity in tumor tissue as compared to normal colonic mucosa (Fig. 2).

Based on the observation that colon cancers have cdk5 activity, the next set of experiments were designed to test whether ciglitazone-induced cell growth changes were

Figure 3. Ciglitazone exposure results in a dose and time-dependent decrease in cdk5 expression in HT-29 cells. (A), HT-29 colon cells were treated with varying doses (0, 1, 5, 15 μM) of ciglitazone. While there was no change in cdk5 protein levels within the first 24 h by Western blotting, a significant decrease in cdk5 protein levels were detected in a dose-dependent fashion after 48 h. No significant change was observed in p35 protein levels when HT-29 cells were treated with ciglitazone. (B), Cdk5 was immunoprecipitated with a cdk5 specific antibody, protein eluted and immunoblotted for cdk5 and p35. A dose-dependent decrease in both cdk5 and associated p35 protein expression was noted after 72 h of ciglitazone exposure.

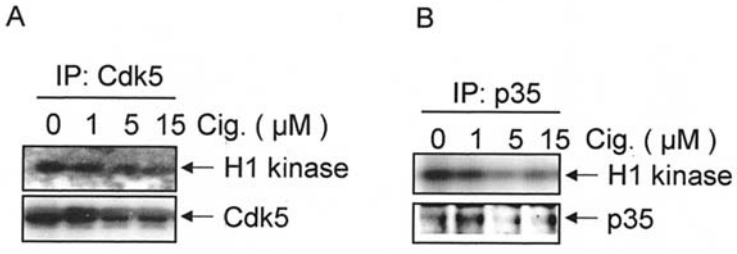

Figure 4. Cdk5 kinase activity decreases after ciglitazone exposure. Kinase activity for cdk5 was detected in HT-29 cells by either immunoprecipitating for cdk5 (A) or p35 (B), which decreased in a dose-dependent fashion after ciglitazone exposure. Immunoblotting for cdk5 demonstrated a decrease in protein expression, while minimal change was noted in p35 protein levels.

associated with changes in cdk5/p35 activity. HT-29 colon cells were treated with varying doses (0, 1, 5, 15 μM) of ciglitazone, cells were harvested at 24, 48 and 72 h, and the total cellular proteins were extracted. While there was no change in cdk5 protein levels within the first 24 h, a significant decrease in cdk5 protein levels were detected in a dose-dependent fashion after 48 h. However, no significant change was observed in protein levels of p35 when HT-29 cells were treated with ciglitazone (Fig. 3).

To confirm the results of the Western blot analysis and to demonstrate the physical association of cdk5/p35, cdk5 was immunoprecipitated with a cdk5 specific antibody, protein eluted and immunoblotted for cdk5 and p35. A dose-dependent decrease in both cdk5 and p35 protein expression was noted after 72 h of ciglitazone exposure (Fig. 4A). To determine whether cdk5 was functional in HT-29 cells, cdk5 activity was assessed using histone H1 as a substrate. Kinase activity for cdk5 was detected in HT-29 cells by either immunoprecipitating for cdk5 or p35, which decreased in a dose-dependent fashion after ciglitazone exposure (Fig. 4B). Immunoblotting for cdk5 demonstrated a decrease in protein expression, while minimal changes were noted in p35 protein levels.

To evaluate the relationship between cdk5/p35 and ciglitazone-induced anti-proliferation, cdk5/p35 expression plasmids were transiently transfected into HT-29 cells. These transfected cells were then treated with ciglitazone (15 μM) for 48 h and cell proliferation was measured by an MTT assay (Fig. 5). Cell proliferation was reduced in the control group after exposure to ciglitazone. No changes in cell proliferation were noted in untreated cells overexpressing cdk5/p35 as compared to untreated cells transfected with

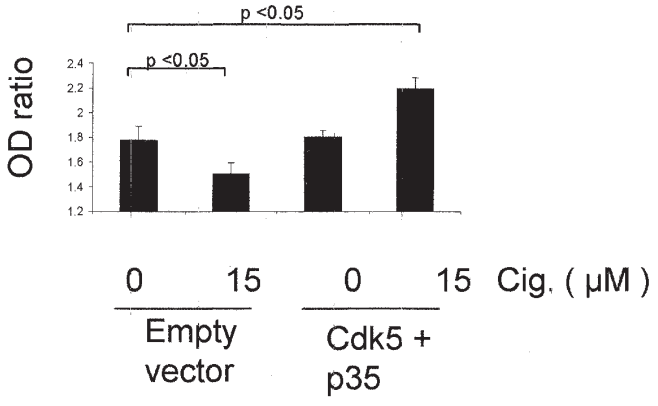

Figure 5. Cdk5/p35 overexpression reverses the antiproliferative effects of ciglitazone. Cdk5/p35 expression plasmids were transiently transfected into HT-29 cells. Cell proliferation was reduced after 48 h of ciglitazone exposure. No changes in cell proliferation were noted in untreated cells overexpressing cdk5/p35 as compared to untreated cells transfected with empty vector. However, ciglitazone exposure resulted in a significant increase in cell proliferation in cells overexpressing cdk5/p35.

an empty vector. However, ciglitazone exposure resulted in a significant increase in cell proliferation in cells overexpressing cdk5/p35 ($p<0.05$).

Discussion

Cyclin-dependent kinase 5 and its activator, p35, have been shown to be essential in neuronal development. In the nervous system, activated cdk5 is involved in neuronal cell maturation, differentiation and motility. Recent evidence suggests that cdk5 has an expanded role in other tumor types. For example, Lin and colleagues demonstrated the presence and activity of cdk5 in prostate cancer cells and reported that kinase activity is associated with digoxin-triggered apoptosis (12). Chen *et al* also reported that cdk5 plays a role in 1α, 25-dihydroxy-vitamin-induced monocytic differentiation in human leukemia HL-60 cells (13). Our data support the observation that cdk5 is involved in cellular functions outside of the neuronal cell system and this is the first report to demonstrate that cdk5 is overexpressed in human colon tumors and in an *in vitro* colon cancer cell line. Interestingly, while human tumor samples expressed functional cdk5, the adjacent normal mucosa had undetectable cdk5 protein levels and kinase activity, suggesting that cdk5 expression may play a role in colon carcinogenesis.

To further support the proliferative effects of cdk5, HT-29 cells were treated with ciglitazone, a PPARγ ligand shown to have antiproliferative effects on colon cancer cells. Treatment of HT-29 cells with ciglitazone resulted in decreased cell growth as well as decreased cdk5 protein expression and kinase activity. It is interesting to note that the decrease in cdk5 activity precedes the antiproliferative effects. In contrast, protein levels of its catalytic subunit, p35, were unchanged. We hypothesize that cdk5/p35 interacts with cell growth factors, which in turn triggers ciglitazone-induced antiproliferation. More than 15 types of substrates are phosphorylated by cdk/p35 under different situations such as cell maturation, migration or differentiation and further work is required to identify the specific substrates for cdk5/p35 in colon cancer cells (14).

While early investigations of PPARγ, a member of the steroid receptor superfamily, have centered on its effect on lipid metabolism, recent studies suggest that PPARγ may be involved in colon carcinogenesis. PPARγ has been shown to be aberrantly expressed in colon tumors and a growing number of PPAR ligands have been shown to have growth inhibitory effects in many cancer cell types, including colon cancer in both *in vitro* and *in vivo* models (1-3). Paradoxically, the administration of PPARγ ligands in APC^{Min} mice, an animal model for familial adenomatous polyposis, leads to increased polyp formation (4,5). Based on these conflicting data, PPARγ ligands can stimulate proliferation, as well as act as antiproliferative agents.

In this study, we observed that ciglitazone decreases HT-29 cell growth after 48 h of exposure. Conversely, ciglitazone exposure to HT-29 cells overexpressing cdk5 and p35 resulted in a significant increase in cell proliferation (Fig. 5). Although the exact mechanism through which ciglitazone affects cell proliferation remains unclear, recent evidence suggests that PPARγ ligands exert their pro-apoptotic effects, in part, through activation of the MAP kinase pathway (4). Cdk5 has been shown to be a novel regulator of the MAP kinase pathway. Sharma and colleagues examined the relationship between cdk5 and the MAP kinase pathway and reported that cdk5 regulates the MAP kinase pathway in postmitotic cells by phosphorylating MAP kinase kinase-1 (MEK1). They suggest that phosphorylation of MEK1 led to decreased kinase activity and subsequent inactivation of ERK 1/2 and the MAP kinase pathway (5). The observation that treatment of colon cancers cells with roscovitine (a non-specific cdk5 inhibitor) leads to activation of ERK 1/2 adds additional evidence to support participation of cdk5 regulation of the MAP kinase pathway in colon cells.

In conclusion, we demonstrate that cdk5 activity is not limited to the central nervous system but is also present in colon cancer. In colon cancer cells, cdk5 seems to play a role in proliferation which is unlike the effects seen in neurons. To further establish its involvement in cell growth, cdk5 was treated with ciglitazone and was associated with the down-regulation of cdk5 activity. Although, cdk5 may not be a direct substrate of ciglitazone, it is a potential novel target for future anticancer therapy.

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