Beclin 1 Gene Inhibits Tumor Growth in Colon Cancer Cell Lines

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Abstract. *Background: The Beclin 1 gene binds to the apoptosis-inhibiting protein Bcl-2. The expression and function of the Beclin 1 gene in four colorectal cancer cell lines (HT29, DLD-1, SW480, SW620) was investigated. Materials and Methods: The expression of Beclin 1 mRNA was examined by reverse transcription-polymerase chain reaction(RT-PCR), the cell growth rate of a stable cell line established after transfection of the Beclin 1 gene into low Beclin 1-expressing HT29 colon cancer cells. In addition, changes in cell cycle were investigated by flow cytometry, and the expression levels of cell cycle related proteins in the Beclin 1 transfected colon cancer cell line were assessed. Results: The expression level of Beclin 1 mRNA in the colon cancer cell lines was variable. Transfection of the low Beclin 1 gene-expressing colon cancer cell line with the Beclin 1 gene resulted in cell growth inhibition. The cell cycle analysis showed that the percentage of G1-phase cells was significantly higher in Beclin 1 transfectants than in mock transfected cells. The expression levels of cyclin E and phosphorylated Rb were decreased. Conclusion: These results indicate that Beclin 1 can inhibit the growth of colorectal cancer cells.*

The Beclin 1 gene was identified by Levine *et al.* in 1998 (1). It is located on chromosome 17q21 and codes for a protein that binds to the apoptosis-controlling protein Bcl-2. This molecule was shown to be a novel 60-kDa coiled-coil protein, Beclin 1, when the bcl 2-interacting gene was used for a two-hybrid screen in a study of lethal encephalitis due to central nervous system infection with Sindbis virus. A study with a recombinant Sindbis virus chimera suggested that Beclin 1 protein is involved in antiviral host defense (1, 2). Subsequently, it was shown that the expression of Beclin 1 was low in human breast cancer, that the transfection of a breast cancer cell line with the Beclin 1 gene resulted in autophagy as seen in budding yeast, *S. cerevisiae*, and that Beclin 1 had high structural similarity to the fission yeast autophagy gene apg6/vps30 (amino acid homology, 39.1%) (3, 4). Furthermore, heterozygous-deficient mice have an increased incidence of spontaneous tumors (5, 6) and transfection of MCF7 cells with the Beclin 1 gene resulted in a decrease in tumorigenesis in the absence of apoptosis (7). Recent studies have shown that the Beclin 1 gene is essential for the life-span extension of roundworms (8) and is associated with the autophagy of Purkinje cells in the nervous system (9). Colorectal cancer is the most common malignancy of the digestive tract in Europe, United States and Japan, and the prognosis of advanced colorectal cancer with distant metastasis is poor. In this study, we examined the expression of the Beclin 1 gene in colorectal cancer cell lines and the proliferation of HT29 colorectal cancer cells transfected with the Beclin 1 gene.

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Materials and Methods

Cell culture. The human colorectal cancer cell lines, SW620, HT29, DLD-1 and SW480 were cultured at 37˚C in 5% CO_2 in RPMI 1640 (HT29, DLD-1) or DMEM (SW620, SW480) medium containing 10% fetal bovine serum (10).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The total RNA was extracted from the colorectal cancer cells using guanidinium-thiocyanate. Single strand cDNA prepared from 3 mg of total RNA using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) with an oligo (dT) primer-14 was used as the template for the polymerase chain reaction (PCR). The primers for PCR to amplify the Beclin 1 gene-coding regions were as follows. The 5' primer, Beclin 1-AX, encompassed positions 64-81 of the published human Beclin 1 sequence (2), AGCCAGCC CCTGAAACTG. The 3' primer, Beclin 1-BX, encompassed positions 608-628, CTGCCACTATCTTGCGGTTCT. GAPDH amplification was used as internal PCR control with 5'-GGGGA GCCAAAAGGGTCATCATCT-3' as the sense primer and 5'-GAC GCCTGCTTCACCACCTTCTTG-3' as the antisense primer. Twenty-nine cycles of denaturation (94˚C, 1 min), annealing (50˚C, 1.5 min) and extension (72˚C, 2 min) were carried out in a thermal cycler (PTC-100, Programmable Thermal Controller, NJ Research Inc., MA, USA). Ten μl of the PCR products which showed the relevant bands in RT-PCR analysis were sequenced by

electrophoresis in 1.2% agarose gel. The sequencing was performed on PCR products that showed the bands in RT-PCR analysis.

Construction of Beclin 1 expression plasmid. PSG5/flag-Beclin 1 was obtained from Beth Levine (Columbia University College of Physicians & Surgeons) (7). Beclin 1 cDNA was amplified using primers as follows: Beclin 1-CX, the 5' primer, encompassed positions 1-27, ATGGAAGGGTCTAAGACGTCCAACAAC, and Beclin 1-DX, the 3' primer, encompassed positions 1327-1353, TCATTTGTTATAAAATTGTGAGGACAC. Using primers tagged with restriction enzyme sites (for 5'-BamHI and 3'-EcoRI), the BamHI and EcoRI site-tagged full length Beclin 1 fragments were amplified and cloned into a mammalian expression vector, (myc tag)pcDNA3.1 (Invitrogen, CA, USA) between the BamHI and EcoRI sites. The plasmid constructs were confirmed by DNA sequencing and constituted the DNAs for the constitutive expression vector of human Beclin 1 (pcDNA3.1-(myc)-Beclin 1).

Establishment of Beclin 1-transfected HT29 cell line. HT29 cells were transfected to overexpress Beclin 1, using methods as previously described (11). The HT29 cells were cultured in 6-well plates. DNA transfections were performed using Lipofectamine (Invitrogen) and 1 mg of pcDNA3.1-(myc tag)-Beclin 1 (Beclin 1 transfectant), or pcDNA3.1-(myc tag)-empty-vector alone (Mock transfected cells) as a control. After transfection, cells were selected for neomycin resistance by treatment with G418 sulfate (Promega, WI, USA). Individual G418 resistant clones were identified, expanded and analyzed for myc-Beclin 1 expression by immunoblotting of total cellular protein.

Western blot analysis. Total protein was extracted from the cultured cells using RIPA buffer [phosphate-buffered saline (PBS) containing 1% NP40, 0.1% sodium deoxycholate, 0.1% sodium docecyl-sulphate (SDS), and 10 μg/ml Leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride]. Protein concentrations were measured by a Bio-Rad protein assay kit (Bio-Rad Lab. Inc., CA, USA) using bovine serum albumin as a standard. One-hundred μg of protein was run on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. After the electrophoretic transfer, the membrane was blocked overnight at 4˚C and incubated with the primary antibody. The protein bands were incubated with α-myc mAb (Invitrogen) and GAPDH (Biogenesis, NH, USA). Density was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, NJ, USA).

Cell growth analysis. To examine the cell growth rate of the mock transfected cells and the Beclin 1 transfectants *in vitro*, $1x10^2$ cells were seeded into a 24-well plate and cell numbers were counted with a hemocytometer after 1, 3, 6 and 8 days.

Flow cytometric analysis. The cells were washed twice with PBS and fixed with 70% ethanol for 60 min at 4˚C. They were then washed with PBS once again and suspended in 0.5 ml of PI staining reagent (200 μg/ml propidium iodide, 200 μg/ml RNase A). The cells were left at room temperature for 30 min and analyzed on a FACScan (A Beckman Coulter Com., FL, USA).

Results

Expression level of Beclin 1 mRNA. RT-PCR assay showed that the expression level of Beclin 1 mRNA differed between the SW480, SW620, HT29 and DLD-1 cell lines.

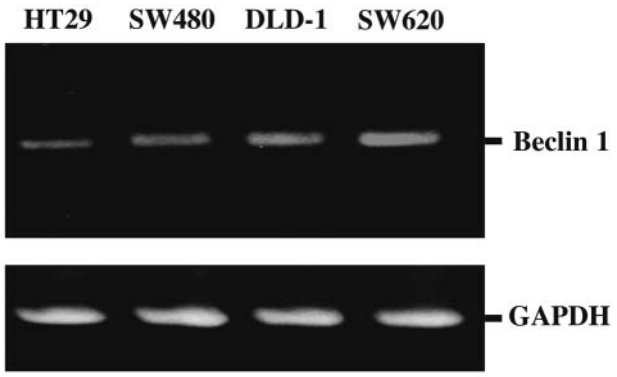

Figure 1. *Expression of Beclin 1 mRNA in colorectal cancer cell lines. The expression of Beclin 1 mRNA was lower in the HT29 cells than in the other colorectal cancer cell lines, SW480, SW620 and DLD-1.*

The Beclin 1 expression in HT29 was lower than that in the other cell lines (Figure 1).

Establishment of a stable colon cancer cell line transfected with the Beclin 1 gene. The transfectant clones expressing exogenous Beclin 1 protein were selected by Western blotting and that with the strongest expression was used as a stable cell line (Beclin 1 transfectant). The mock transfected cells were also a single clone. The results of Western blotting are shown in Figure 2. The 60 kDa band represents the exogenous Beclin 1 protein expressed by transfection.

Cell growth of the Beclin 1 gene-transfected colon cancer cell line. On the eighth day of culture, the density of the mock transfected cells was 38,500/ml, whereas that of the Beclin 1 transfectant was 8,500/ml, indicating a reduced cell growth rate (Figure 3).

Cell cycle of the Beclin 1 gene-transfected colon cancer cell line. The G1/S/G2-M ratio of the mock transfected cells was 32.3%/41.2%/25.3%, whereas that of Beclin 1 transfectants was 48.8%/39.0%/12.0%, indicating a significant increase in the percentage of Beclin 1 transfectant cells in the G1-phase and a significant decrease in the G2-M-phase. No significant difference was noted between the percentages of sub-G1 mock transfected cells and sub-G1 Beclin 1 transfectants (Figure 4).

Expression levels of cell cycle-related proteins in the Beclin 1 gene-transfected colon cancer cell line. The expression levels of the cell cycle-related proteins CDK2, CDK4, CDK6, CKI (p21, p27), cyclin A, cyclin D1, cyclin D3, cyclin E and Rb were examined. The expression levels of cyclin E and phosphorylated Rb were lower in the Beclin 1 transfectants than in the mock transfected cells. The expression levels of CDKs, CKIs, cyclin A, cyclin D1 and cyclin D3 remained unchanged (Figure 5).

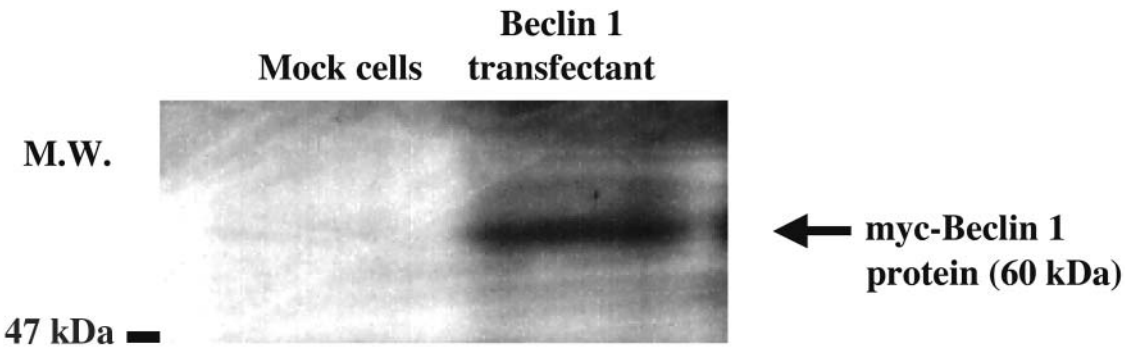

Figure 2. *Western blot of transfected HT29 cells. HT29 cells, either transfected with empty pcDNA3 vector (Mock transfected cells), or with pcDNA3 mammalian expression plasmid encoding myc-tagged Beclin 1(Beclin 1 transfectant), were lysed and equalized for protein content. The protein mixtures were separated by 10% SDS-PAGE and processed for immunoblotting with anti-myc antibody.*

Discussion

Colorectal cancer is the most common malignancy of the digestive tract and the main cause of cancer-related death in Europe, the United States and Japan. The molecular mechanisms involved in the proliferation and apoptosis of colorectal cancer cells are poorly understood.

The Beclin 1 gene, identified by Levine *et al.*, codes for a protein that binds to the apoptosis-controlling protein Bcl-2 (1). Recent studies reported that the expression of Beclin 1 was low in human breast cancer and that the transfection of MCF7 cells with the Beclin 1 gene resulted in a decrease of tumorigenesis (7). In this study, we examined the Beclin 1 gene in colorectal cancer cells. We found a reduced cell growth in the Beclin 1 overexpressing colorectal cancer cells when compared to the mock transfected cells. There are indications that the Beclin 1 gene plays an important role in tumor growth, although the mechanism is not known.

In the cell, certain molecules, called the cell cycle engine, phosphorylate the serine and threonine residues of target proteins, thereby inducing a group of genes involved in the progression to the next phase of the cell cycle. Cyclin Ds have been shown to form complexes with CDK4 and CDK6, and are considered to be induced in the mid-to-late G1-phase (12, 13). Cyclin E associates with CDK2 to induce the kinase activity of CDK2 (14), thereby accelerating the entry into the S-phase. Subsequently, cyclin E is degraded and CDK2 associates with cyclin A (15, 16). Although it has been reported that in cell lines overexpressing cyclin E, the percentages of S- and G2/M-phase cells increase markedly and the cell size decreases, the G1-phase cells were markedly increased in the Beclin 1 transfectants in the present study in contrast to cyclin E-transfected cell lines (17).

The target protein of the cyclin/CDK complex is retinoblastoma protein (pRb) (18). CDK/cyclin E phosphorylates the serine and threonine residues in pRb in the late G1-phase (19). Normally, pRb inhibits the transcription of the S-phase-related genes. We suggest that the decreased expression of cyclin E in Beclin 1 transfectants inhibited the phosphorylation of pRb, causing decreased release of pRb from the E2F/DP heterodimer, leading to a decrease in the expression levels of the S-phase-related genes, resulting in cell cycle arrest in the late G1-phase.

In conclusion, the Beclin 1 molecule, which inhibits the expression of cyclin E, plays an important role in the proliferation of colorectal cancer cells. Although it is necessary to investigate the detailed mechanism of the Beclin 1 effects, a therapy targeting the Beclin 1 molecule may control tumor growth.

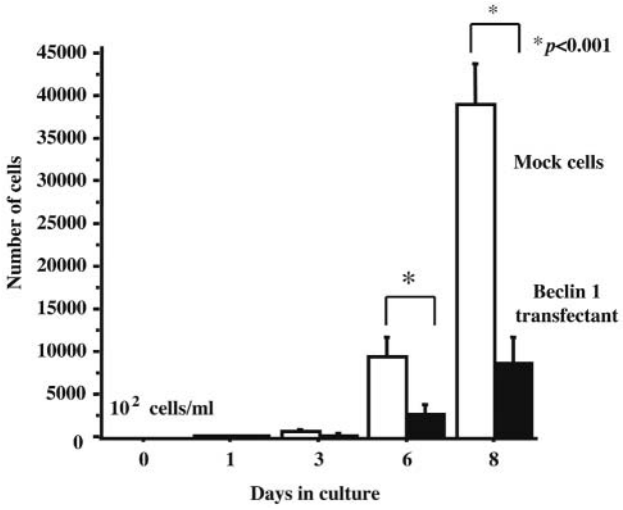

Figure 3. *Cell growth of mock transfected HT29 cells vs. Beclin 1 HT29 transfectants. Transfection with Beclin 1 gene resulted in cell growth inhibition (Days 6 and 8).*

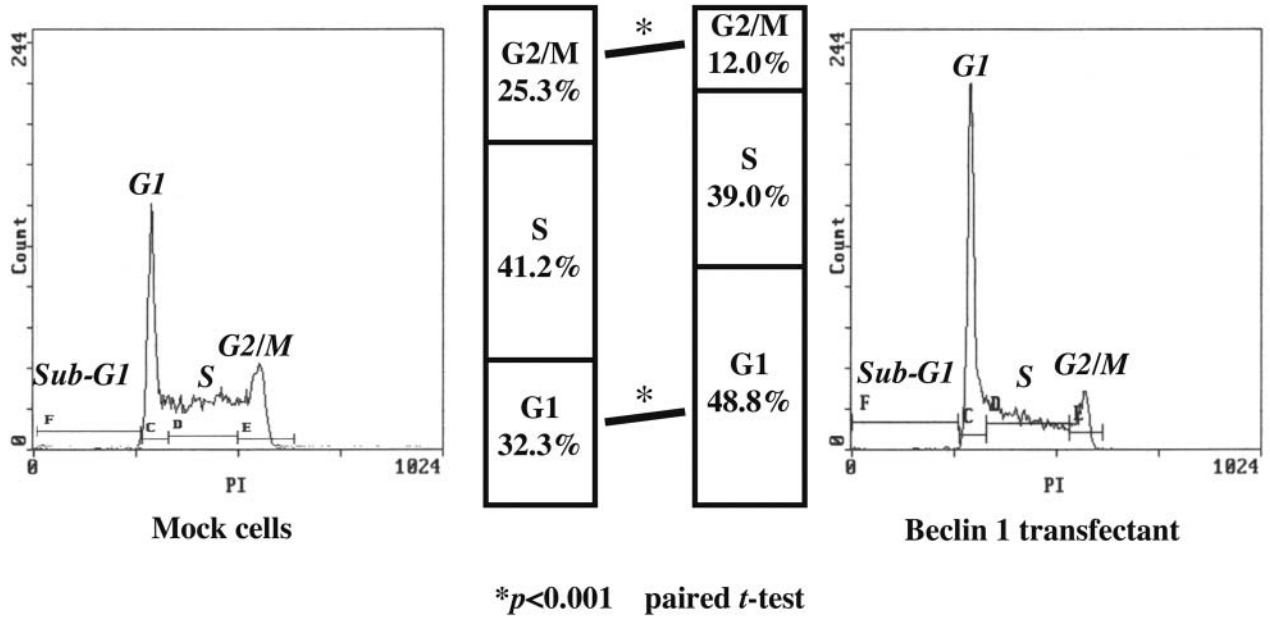

Figure 4. *Cell cycle parameters in mock transfected cells and Beclin 1 transfectants by FACS analysis(one day in culture). The percentage of G1-phase and G2-M cells were increased significantly in Beclin 1 transfectants.*

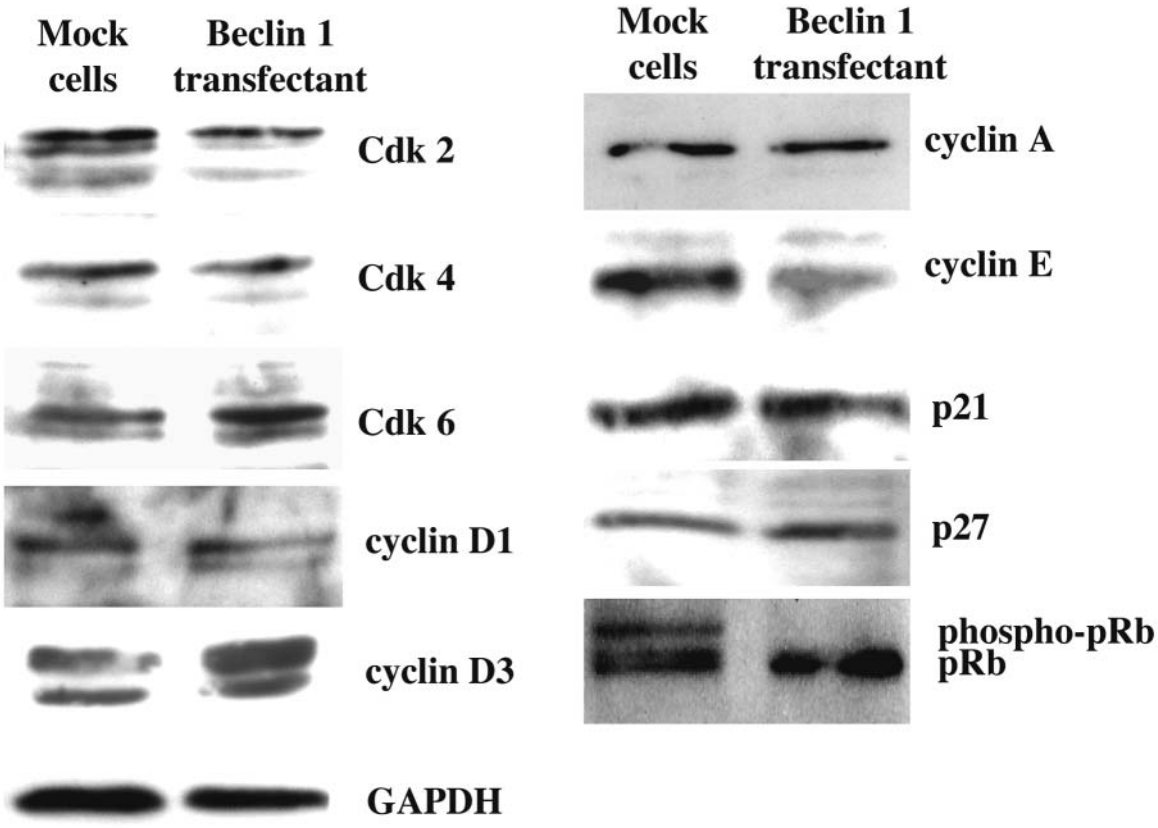

Figure 5. *Expression levels of cell cycle-related proteins in the Beclin 1 gene-transfected colon cancer cell line. The expression levels of cyclin E and phosphorylated Rb were lower in Beclin 1 transfectants than in mock transfected cells. The expression levels of cyclin-dependent kinases, cyclin-dependent kinase inhibitors, cyclin A, cyclin D1 and cyclin D3 remained unchanged.*

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