Adenovirus-mediated p53 Gene Transduction Inhibits Telomerase Activity Independent of Its Effects on Cell Cycle Arrest and Apoptosis in Human Pancreatic Cancer Cells

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ABSTRACT

Evidence for a relationship between overexpression of wild-type p53 and telomerase activity remains controversial. We investigated whether p53 gene transduction could cause telomerase inhibition in pancreatic cancer cell lines, focusing on the relation of transduction to growth arrest, cell cycle arrest, and apoptotic cell death. The cells were infected with recombinant adenovirus expressing wild-type p53 or p21WAF1 at a multiplicity of infection of 100 or were continuously exposed to 10 μM VP-16, which is well known to induce apoptosis. Adenovirus-mediated p53 gene transduction caused G1 cell cycle arrest, apoptosis, and resultant growth inhibition in MIA PaCa-2 cells; the cell number 2 days after infection was 50% of preinfection value, and 13% of the cells were dead. Moreover, the transduction resulted in complete depression of telomerase activity through down-regulation of hTERT mRNA expression. In contrast, p21WAF1 gene transduction only arrested cell growth and cell cycle at G1 phase, and VP-16 treatment inhibited cell growth with G2-M arrest and apoptosis; after treatment, the cell number was 73% of pretreatment, and 12% of the cells were dead. Neither p21WAF1 gene transduction nor VP-16 treatment caused telomerase inhibition. Similar results were obtained in two other pancreatic cancer cell lines, SUIT-2 and AsPC-1. Thus, our results demonstrate that the p53 gene transduction directly inhibits telomerase activity, independent of its effects on cell growth arrest, cell cycle arrest, and apoptosis.

INTRODUCTION

Telomerase is a ribonucleoprotein enzyme containing an integral short RNA template that directs the synthesis of telomeric repeats (5'-TTAGGG-3') at the end of replicating chromosomes called telomeres (1, 2). In most human somatic cells, telomerase activity is undetectable, and the telomere length is progressively shortened during cell proliferation (3, 4), leading to cellular senescence (5, 6). In contrast, telomerase is activated in the vast majority of cancer cells (7, 8). We previously detected telomerase activity in 80% of a series of surgically resected pancreatic cancers (9), and furthermore, we have used telomerase activity in the pancreatic juice as a useful diagnostic tool (10).

Reportedly, telomerase activity is regulated in a cell cycle-dependent manner (11). For example, maximal telomerase activity is detected in the S phase, whereas it is barely detectable in the G2-M phase (12). There are some reports that, although telomerase is active throughout the cell cycle in dividing, immortal cells, its activity is repressed in cells that exit the cell cycle (13, 14); therefore, its activity correlates with growth rate (15) and cellular proliferation (16). Telomerase activity is also apparently suppressed by apoptosis induced by anticancer chemotherapeutic agents (17).

p53 gene mutations and/or abnormal p53 protein accumulation are reported in ~60% of pancreatic cancers (18, 19) and are associated with its poor prognosis (20, 21). Wild-type p53 protein can transcriptionally transactivate the p21WAF1 gene, which mediates G1 cell cycle arrest by causing inhibition of the cyclin-dependent kinase activity required for progression from the G1 to the S phase (22, 23). Moreover, wild-type p53 protein up-regulates Bax expression, which, in turn, inhibits Bcl-2 expression, resulting in apoptosis induction as a strictly programmed mechanism for removal of damaged cells (24).

It was reported that induced expression of wild-type p53 resulted in the down-regulation of telomerase activity in immortalized fibroblasts (25) and that telomerase activity was significantly reduced in lung cancer cell lines transfected with sense p53 cDNA (26). In contrast, Maxwell et al. (27) proposed that telomerase activity is unaffected by overexpression of p53 in immortalized endothelial cells, suggesting that the evidence for a relationship between overexpression of p53 and telomerase activity remains controversial.

Here, we investigated whether adenovirus-mediated p53 gene transduction causes telomerase inhibition in human pancreatic cancer cells, focusing on the relation of transduction to cell growth arrest, cell cycle arrest, and apoptotic cell death.

MATERIALS AND METHODS

Cell Lines. Three human pancreatic cancer cell lines were used: MIA PaCa-2, generously provided by the Japanese...
Cancer Resource Bank (Tokyo, Japan), and SUIT-2 and AsPC-1, generously provided by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan). Point mutations of the p53 gene were found in two cell lines: a C→T transversion at the first base of codon 248 in MIA PaCa-2 cells and a G→A transversion at the second base of codon 273 in SUIT-2 cells (28). A single-base deletion between codons 134 and 135 (TTT transversion at the second base of codon 273 in SUIT-2 cells and 170 (ATG ACG→TGA CG; Ref. 28). The cell lines were cultured in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Preparation of Adenoviral Vectors. Replication-defective E1- and E3- adenoviral vectors expressing either human wild-type p53 (Ad-p53; Ref. 29), p21⁴⁷[WAF1] (Ad-p21; Ref. 30), or bacterial human β-galactosidase (Ad-lacZ; Ref. 31) were prepared as described previously (32–34). All cDNA were driven by a CA promoter composed of a cytomegalovirus enhancer and chicken β-actin promoter (35). The recombinant adenoviruses were purified by ultracentrifugation through a CsCl₂ gradient, followed by extensive dialysis. Contamination by wild-type adenovirus was excluded by PCR designed for E1 amplification. The virus titer was assessed by a plaque formation assay using 293 cells.

Drug. VP-16, a topoisomerase II inhibitor, was obtained in a powder form from Nippon Kayaku Co. (Tokyo, Japan), dissolved in DMSO (0.1% final concentration), and added to aliquots to a final concentration of 10 μM.

Cell Growth Assay. Exponentially growing cells were plated at an initial density of either 4 × 10³ cells (MIA PaCa-2 and SUIT-2 cells) or 6 × 10⁴ cells (AsPC-1 cells) per well in 24-well plates and incubated overnight at 37°C in a humidified atmosphere containing 10% CO₂ in DMEM containing 10% FBS. Then, the cells were infected with PBS, Ad-lacZ, Ad-p21, or Ad-p53 at a MOI₃ of 100 in 150 μl of PBS or were continuously exposed to 10 μM VP-16 at 37°C in a humidified atmosphere containing 10% CO₂. After 1 h, the culture supernatant was replaced with 1 ml of fresh culture medium. For 4 days, cells floating in the culture medium and attached cells collected by trypsinization (200 μl/well) were combined in a 1500-μl Eppendorf tube, and half were counted with a particle distribution analyzer, CDA500 (Sysmex, Kobe, Japan). The remaining cell pellets were stored at −80°C for the telomerase activity assay.

Cell Viability Assay. The percentage of dead cells was determined by staining with propidium iodide and performing an assay using a fluorescence multiple-well plate reader, Cytolinx II (PerSeptive Biosystems, Framingham, MA), as described previously (36). Briefly, cells were plated and treated as described above. Propidium iodide was added to aliquots (final concentration, 30 μM) to label nuclei of dead cells. Fluorescence was measured after incubation for 60 min at 530-nm (excitation) and 645-nm (emission) wavelengths. Subsequently, digitonin was added to aliquots (final concentration, 300 μM) to permeabilize all cells and label all nuclei with propidium iodide. Fluorescence was measured again after incubation for 30 min to obtain a value corresponding to 100% cell death. The proportion of the former fluorescence to the latter was defined as the percentage of dead cells.

Measurement of DNA Synthesis. Cells were plated and treated as described above. After 48 h, the cells were pulsed for 4 h with 1 μCi/ml [³H]thymidine. The incorporation of [³H]thymidine into the trichloroacetic acid-insoluble material was measured using a scintillation counter.

Morphological Analysis. Cells were plated on a coated coverslip in a 24-well plate and treated as described above. The cells adhered to the coverslip were stained with 10 μg of propidium iodide and observed under UV fluorescence microscopy.

Western Blot Analysis. Floating and trypsinized adherent cells were combined and washed with PBS. Cells were then lysed in SDS solubilization buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 2% SDS]. Fifteen μg of protein from the samples were boiled for 5 min, subjected to 12% SDS-PAGE, and transferred to a Hybond-ECL membrane (Amersham, Tokyo, Japan). The membrane was blocked in PBS containing 5% dried milk and 0.1% Tween 20 and probed with a primary antibody (mouse antihuman p53 monoclonal antibody OP9 or p21 monoclonal antibody OP76; Calbiochem, Cambridge, MA) and a secondary antibody (horseradish peroxidase-conjugated rabbit antigoat IgG1; ICN Pharmaceuticals, Inc., Aurora, OH). An Amersham ECL chemiluminescent Western system (Amersham) was used to detect secondary probes.

Flow Cytometric Analysis. Floating and trypsinized adherent cells were collected, washed with PBS, subjected to cold 70% ethanol for 4 h at 4°C, washed with PBS, suspended in phosphate-citrate buffer for 30 min at room temperature, and resuspended in PBS containing 1 mg/ml RNase A for 30 min at room temperature. Samples were then stained with propidium iodide at 50 μg/ml and analyzed in a cell sorter (FACScan;
Telomerase Activity Assay. Telomerase was assayed by the PCR-based telomeric repeat amplification protocol described previously (7), with minor modifications. The stored cell pellets obtained in the cell growth assay were suspended in 2 ml per 1000 cells of 3-[\((3\text{-cholamidopropyl})\text{dimethylammonio}\)]-1-propanesulfonic acid lysis buffer [10 mM Tris (pH 7.5), 1 mM MgCl$_2$, 1 mM EGTA, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 5 mM \(\beta\)-mercaptoethanol, 0.5% 3-[\((3\text{-cholamidopropyl})\text{dimethylammonio}\)]-1-propanesulfonic acid, 10% glycerol, and 1 mg/ml each protease inhibitor (antipain, leupeptin, phosphoramidon, elastatinal, pepstatin A, and chymostatin; Peptide Institute, Osaka, Japan)] and incubated for 30 min on ice. The lysate were centrifuged at 15,000 \(\times\) g for 20 min at 4°C. Two \(\mu\)l of the extracts, equivalent to 1000 cells, were incubated in a solution of 20 mM Tris (pH 8.3), 1 mM MgCl$_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 \(\mu\)M dNTP, 0.3 \(\mu\)Ci \([\alpha^32P]dCTP, 2\) units of Taq DNA polymerase (Promega, Madison, WI), and 0.1 \(\mu\)g of TS primer (5’-AATC-CGTCGAGCAGAGAGTT-3’) at 20°C for 30 min and then heated to 90°C for 3 min to inactivate telomerase activity. After the inactivation of telomerase, 0.1 \(\mu\)g of CX primer (5’-CCCT-TACCCTTACCCTTACCCTTAA-3’) was added, and the reaction mixture was subjected to 31 PCR cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s (2 min for the final step). Half of the PCR products were analyzed by electrophoresis in 0.5% Tris-borate EDTA buffer on 12% polyacrylamide nondenaturing gels. Signal intensity was measured by NIH Image (Version 1.60; NTIS, Springfield, VA). The relative densities of the telomeric ladders in each sample were expressed as percentages of pretreated cells after subtraction from background. A 36-bp internal standard (Oncor, Inc., Gaithersburg, MD) was used as internal control.

Table 1  Effects of p53 and p21\(^{WAF1}\) gene transduction on SUIT-2 and AsPC-1 cells\(^a\)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pretreatment</th>
<th>PBS</th>
<th>Ad-lacZ</th>
<th>Ad-p21</th>
<th>Ad-p53</th>
<th>VP-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUIT-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell no. ((\times 10^4))</td>
<td>8.4 ± 0.2</td>
<td>49.9 ± 0.7</td>
<td>37.9 ± 1.2</td>
<td>14.6 ± 0.5</td>
<td>7.3 ± 0.4</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>Dead cells (%)</td>
<td>2.4 ± 1.0</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>2.2 ± 0.5</td>
<td>6.6 ± 0.2</td>
<td>7.1 ± 1.1</td>
</tr>
<tr>
<td>Telomerase activity (%)</td>
<td>100 ± 2</td>
<td>105 ± 3</td>
<td>112 ± 1</td>
<td>100 ± 4</td>
<td>22 ± 7</td>
<td>108 ± 0</td>
</tr>
<tr>
<td>AsPC-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell no. ((\times 10^4))</td>
<td>8.3 ± 0.2</td>
<td>34.8 ± 1.0</td>
<td>30.0 ± 0.7</td>
<td>12.1 ± 1.1</td>
<td>10.8 ± 1.2</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>Dead cells (%)</td>
<td>0.7 ± 0.9</td>
<td>1.8 ± 0.4</td>
<td>1.9 ± 0.9</td>
<td>3.6 ± 1.4</td>
<td>12.2 ± 0.8</td>
<td>10.5 ± 2.4</td>
</tr>
<tr>
<td>Telomerase activity (%)</td>
<td>100 ± 1</td>
<td>104 ± 2</td>
<td>100 ± 3</td>
<td>97 ± 4</td>
<td>14 ± 2</td>
<td>105 ± 1</td>
</tr>
</tbody>
</table>

\(\text{a}\) Values represent mean ± SD of three independent wells.
RT-PCR Analysis. RNA was isolated from MIA PaCa-2 cells treated as described above. Two μg of total RNA were reverse-transcribed. The resulting cDNA was subjected to PCR with the primers as follows: hTERT, 5'-CGGAAGAGT-GTCCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCT-GGA-3; and β-actin (as an internal control), 5'-GTGGGGCGGCCCAGGCACCA-3' and 5'-TGTAGCGGCGCTCGTGAG- GA-3'. The amplification reactions were performed with an initial incubation step at 94°C for 3 min, followed by 33 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 90 s. Ten μl of the reaction products were subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

Expression of p53 and p21WAF1 Proteins in MIA PaCa-2 Cells 2 Days after Treatment. The expression of p53 and p21WAF1 proteins was confirmed by Western blotting (Fig. 1). Although MIA PaCa-2 cells infected with PBS or Ad-lacZ expressed p53 slightly, p21WAF1 protein was hardly detected in the cells. The cells transduced with the p21WAF1 gene showed low levels of p53 and high levels of p21WAF1 protein, and those transduced with the p53 gene showed overexpression of both p53 and p21WAF1 proteins. In contrast, the cells treated with VP-16 showed high levels of p53 protein without induction of p21WAF1, which indicates overexpression of mutated p53, as suggested previously (37).

Effect of p53 and p21WAF1 Gene Transduction and VP-16 Treatment on Telomerase Activity. In MIA PaCa-2 cells infected with either PBS or Ad-lacZ, the telomerase activity was not altered for 4 days (Fig. 2). In MIA PaCa-2 cells transduced with the p21WAF1 gene, the telomerase activity was only slightly reduced 3 days after infection; however, in those transduced with p53 gene, activity was completely absent 2 days after infection. Telomerase activity was not affected by the mixing of extract from control cells with that from p53-transduced cells (data not shown), suggesting that the lack of telomerase activity is not due to a trans-acting inhibitor of activity. In contrast, telomerase activity increased in the VP-16-treated cells 2 days after treatment and was the same as that in the pretreated cells 3 days after treatment. In both SUIT-2 and AsPC-1 cells, 3 days after treatment, p21WAF1 gene transduction and VP-16 treatment did not decrease telomerase activity, but p53 gene transduction caused a significant depression of this activity (Table 1). The telomerase inhibition by p53 gene transduction was also observed in other cell types such as a breast cancer cell line MCF-7 and a gallbladder cancer cell line GB-d1 after 3 days (data not shown).

Effect of p53 and p21WAF1 Gene Transduction and VP-16 Treatment on hTERT mRNA Expression 2 Days after Treatment. Down-regulation of hTERT mRNA expression was clearly demonstrated in MIA PaCa-2 cells 2 days after infection with Ad-p53 (Fig. 3). In contrast, the expression levels of hTERT mRNA on RT-PCR were almost unchanged in the cells infected with PBS, Ad-lacZ, and Ad-p21 and were slightly increased in those treated with VP-16.

Effect of p53 and p21WAF1 Gene Transduction and VP-16 Treatment on Cell Growth in Vitro. The time course of MIA PaCa-2 cell growth in vitro after treatment is shown in Fig. 4. Cells infected with Ad-lacZ had growth rates similar to those of mock-infected cells, which grew exponentially, whereas p21WAF1 gene transduction and VP-16 treatment completely inhibited cell growth. A more severe loss of cell numbers was observed in the cells transduced with p53 gene. Changes in numbers of SUIT-2 and AsPC-1 cells 3 days after treatment are summarized in Table 1. Although treatment with VP-16 significantly inhibited cell growth of all cell lines, p53 and p21WAF1 gene transduction had less effect on inhibition of cell growth in SUIT-2 and AsPC-1 cells than it did in MIA PaCa-2 cells.

Effect of p53 and p21WAF1 Gene Transduction and VP-16 Treatment on Apoptotic Cell Death. Cell viability assays were performed based on the binding of propidium
iodide to the nuclei of cells whose plasma membranes have become permeable due to cell death (Fig. 5, a–e). After addition of digitonin, the nuclei of all cells were labeled with propidium iodide (Fig. 5f). Nuclei of dead cells were stained with propidium iodide (red). Arrows, apoptotic changes. After addition of digitonin, nuclei of all mock-infected cells were labeled with propidium iodide (f).

Fig. 5 Propidium iodide staining of MIA PaCa-2 cells 2 days after infection with PBS (a), Ad-lacZ (b), Ad-p21 (c), or Ad-p53 (d) or treatment with VP-16 (e). Nuclei of dead cells were stained with propidium iodide (red). Arrows, apoptotic changes. After addition of digitonin, nuclei of all mock-infected cells were labeled with propidium iodide (f).

the time course of the percentage of MIA PaCa-2 dead cells after treatment. Degraded cells or cells that had disappeared were not counted in the cell growth assay and are not reflected in the percentage of dead cells. Cells infected with Ad-lacZ showed a percentage of dead cells similar to that of parental cells and had a similar morphology as parental cells (Fig. 5, a
and b). In the cells transduced with the p21WAF1 gene, the percentage of dead cells was slightly increased, and the morphology was altered (e.g., an increased cytoplasmic-to-nuclear ratio), but apoptotic changes were not prominent (Fig. 5c). In contrast, both p53 gene transduction and treatment with VP-16 caused a marked time-dependent increase in the percentage of dead cells and induced specific morphological changes in some cells such as shrunken or fragmented nuclei, which are characteristics of apoptotic cell death (Fig. 5, d and e). In both SUIT-2 and AsPC-1 cells, 3 days after treatment, p53 gene transduction and treatment with VP-16 increased the percentage of dead cells, but the effect was less than that on MIA PaCa-2 cells (Table 1).

DNA Synthesis and Cell Cycle Transitions in MIA PaCa-2 Cells 2 Days after Treatment. [3H]Thymidine incorporation of mock-infected MIA PaCa-2 cells was assigned a value of 100 ± 2 (mean ± SD). The relative incorporations of Ad-lacZ-, Ad-p21-, and Ad-p53-infected cells and that of VP-16-treated cells were 90 ± 3, 40 ± 4, 24 ± 2, and 30 ± 4, respectively. Cell cycle analysis of MIA PaCa-2 cells 2 days after treatment is depicted in Fig. 7. Neither mock-infected nor Ad-lacZ-infected cells showed a specific pattern of cell cycle distribution. In contrast, slow cytometry of cells infected with either Ad-p21 or Ad-p53 demonstrated a decrease of S phase and an increase of G1/G0 phase, thus indicating G1 arrest. Moreover, cells transduced with the p53 gene exhibited a sub-G1 area, indicative of apoptotic cell death. VP-16 caused G2-M arrest with apoptosis.

DISCUSSION

Here, we demonstrated that adenovirus-mediated p53 gene transduction caused a marked inhibition of the pancreatic cancer cell growth with apoptotic cell death and G1 arrest and that p21WAF1 gene transduction arrested the cell growth and the cell cycle at the G1 phase without prominent apoptotic changes. These results suggest that recombinant adenoviral vectors containing tumor suppressor genes can be used effectively for gene therapy of pancreatic cancer and that p53 gene therapy is more effective than the p21WAF1 gene therapy, probably due to induction of apoptosis.

Reportedly, telomeres on chromosome 17p, where the p53 gene is encoded, are shortened more rapidly than others during successive cell divisions (38). The loss of telomeric repeats would cause additional genetic changes as a consequence of chromosome instability (39). Although a direct relationship between p53 alteration and telomerase activation has not been observed, it is possible that telomerase activation is promoted by alterations in the p53 gene. Some investigators have reported a close relationship between overexpressed p53 and telomerase activity. Induced expression of wild-type p53 in immortalized fibroblasts resulted in the down-regulation of telomerase activity after 48 h (25), and telomerase activity was significantly reduced in lung cancer cell lines transfected with sense p53 cDNA and increased in those transfected with antisense p53 cDNA (26). Here, we demonstrated that p53 gene transduction caused complete inhibition of telomerase activity in all three pancreatic cancer cell lines. In addition, we demonstrated that MIA PaCa-2 cells treated with VP-16 showed high levels of mutant p53, and we detected significantly high levels of telomerase activity in this cell line. These findings may indicate that there is a linkage between mutant p53 gene expression and telomerase activation, a supposition that is consistent with a previous report that telomerase is activated in many cells with p53 mutations, which induces telomerase in normal human fibroblasts and mammary epithelial cells (40). These results seem to be supported by the fact that mutant p53 can transactivate c-Myc (41), which induces telomerase in normal human fibroblasts and mammary epithelial cells (42). In contrast, Maxwell et al. (27) proposed that telomerase activity is unaffected by overexpression of p53 and apoptosis in immortalized endothelial cells; however, they measured telomerase activity 18 h postinfection. We also demonstrated that telomerase activity was unaffected by p53 gene transduction 24 h postinfection.

The p53 tumor suppressor gene plays important roles in regulating the cell cycle and in apoptosis; however, our results showed that both cell growth inhibition and cell cycle arrest had little influence on telomerase activity of pancreatic cancer cells. We observed just a slight decrease or no change in telomerase activity of the cells infected with recombinant adenovirus harboring the p21WAF1 gene compared to the preinfectious, mock- or Ad-lacZ-infected cells in all three pancreatic cancer cell lines. Kallassy et al. (43) reported that retrovirus-mediated p21WAF1 gene transduction led to the suppression of telomerase activity in p53-mutated immortalized human keratinocytes 14 days after infection. The discrepancy between their findings and ours may be based on differences in conditions such as different viral vectors, cell lines, and/or duration from viral infection to meas-
measurement of telomerase activity. In addition, our results showed that there was no relation between induction of apoptosis and inactivation of telomerase in pancreatic cancer cells because the treatment with VP-16, which induced the G2-M cell cycle arrest and apoptotic cell death, did not repress telomerase activity.

Here, we further showed that telomerase inhibition by p53 gene transduction correlates with decline in hTERT mRNA expression, suggesting that p53 regulates telomerase at the transcriptional level in MIA PaCa-2 cells. Currently, we are examining the effect of p53 gene transduction on hTERT expression in various types of cells with different p53 status. This would provide a clue to understand a signaling pathway for telomerase-inhibitory effect by overexpressed p53. Although the precise mechanism remains to be elucidated, our results suggest that the p53 gene transduction directly inhibits telomerase activity in pancreatic cancer cells through down-regulation of hTERT mRNA expression.

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