Cytostatic and Cytotoxic Properties of Chimeric Peptides Containing Cyclin-Inhibiting Fragments

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Properties of chimeric peptides containing cell-penetrating sequences and p16INK4a and E2F fragments were studied *in vitro* on immurtal cultures of human cells. Both sequences exhibit cytostatic activity. The peptide containing fragment p16INK4a inhibited proliferation during the G0/G1 stage of the cell cycle, while sequence E2F suppressed proliferation in S phase. Both sequences exhibit cytotoxic properties and can induce apoptosis.

Key Words: *cell-penetrating peptides; cyclin-dependent kinase inhibitors; apoptosis*

Dysregulation of proliferation and impaired activation of apoptosis are the major pathogenetic mechanisms of malignant tumors [1]. Such malignant tumors as lung cancer, pancreatic cancer, and malignant lymphomas are characterized by high incidence of inactivation of proliferation inhibitor *p16INK4a*. These changes are accompanied by increased expression of D-type cyclin-dependent kinases [10]. Hence, *p16INK4a* gene and its product are the targets for targeted therapy of tumors. *p16INK4a* function can be restored by methods of gene therapy [11]; low-molecular-weight inhibitors of cyclin-dependent kinases are also intensively studied [3,5]. The use of vectors constructed on the basis of cell-penetrating peptides is a new method for the transport of high-molecular weight compounds into the cell [6,7]. This method provides transport of peptide molecules, DNA, or RNA into the intracellular space [9] and synthesis of the target peptide with affinity for a certain target [4]. The peptides with a vector sequence of Antennapedia protein and cyclin-inhibiting fragments were synthesized from natural cyclin-dependent kinase inhibitors p16INK4a and cyclin-binding fragment *E2F*. Introduction of these sequences into the cell inhibits proliferation and activates apoptosis [2,10,13].

Here we studied inhibition of proliferation and activation of apoptosis in various cultured cells after treatment with these peptides.

MATERIALS AND METHODS

The following cell cultures were used: 293 (embryonic kidney, transformed DNA of type 5 adenovirus), A549 (lung carcinoma), and MCF-7 (breast cancer). The cells were cultured in DMEM (PanEko) containing 10% fetal bovine serum (FBS, PanEko), 10 μg/ml gentamicin (PanEko) and 2 mM L-glutamine (PanEko) at 37°C and 5% CO_2. These cells were synchronized in the G0/G1 phase of the cell cycle using an FBS-depleted medium (0.1%). The mononuclear leukocyte fraction was obtained in a Ficoll gradient [2].

We studied the effects of 2 chimeric peptides. Peptide Antp_p16 contains N-terminal p16INK4a and penetrating sequence Antp (NH_2-DAAREGFL DTLVVLHRAGAR-RQIKIWFQNRRMKWKK-COOH). Peptide Tat-E2F contains N-terminal penetrating sequence Tat and C-terminal cyclin-binding fragment E2F (NH_2-YGRKKRRQRRRG-PVKRRL DL-COOH).

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Chimeric peptides were dissolved in the culture medium to a concentration of 40 μM. The study was performed by the method of flow cytofluorometry. Cells were fixed with 75% ethanol at -20°C and stained. For evaluation of the cytotoxic effect, we used double staining with annexin and propidium iodide. The cytostatic effect was evaluated by cell distribution in various phases of the cell cycle after staining of fixed samples with propidium iodide.

The results are expressed as the mean values and standard errors.

RESULTS

The antiproliferative effect of peptides depended on the cell culture. Cultures of 293 and A549 cells were most sensitive to peptides. Culture MCF-7 derived from breast cancer cells exhibited the lowest sensitivity to both peptides. A correlation was found between the cytostatic and cytotoxic effects. Spontaneous apoptosis in A549 cells was 11.2±2.4%. Apoptosis in A549 cells increased to 43.3±5.6% after incubation with Antp_p16. Spontaneous apoptosis in the 293 culture was 5.1±1.5%. Apoptosis in these cells increased to 46.1±8.4% after incubation with the peptide.

Cytotoxic activity of peptide Tat_E2F was studied on the most sensitive culture, A549 cells. Apoptosis in these cells reached 30.1±3.7% after 24-h incubation with Tat_E2F. The cytotoxic effect of peptide Tat_E2F was minimum in cultures of 293 and MCF-7 cells. The cytotoxic effect of peptides did not depend on the expression of intrinsic *p16* gene by cells. High level of apoptosis was typical of 293 ($p16^+$) and A549 ($p16^-$) cells. Moreover, the peptides had little effect on the mononuclear leukocyte fraction of human peripheral blood. Therefore, they had a target effect on proliferating cells.

Study of antiproliferative activity showed that the peptide with active site p16INK4a stops cells in the G0/G1-phase of the cell cycle (Fig. 1). The decrease in the number of G0/G1-phase cells and increase in the number of S-phase cells after addition of the peptide with active site p16INK4a occurred later than in the control. The number of G0/G1-phase cells decreased after 6-h incubation and the number of S-phase cells increased after incubation for 8 h. In the control, the number of G0/G1-phase cells decreased by the 2nd hour, while the number of S-phase cells increased after 4-h incubation.

Experiments with chimeric peptide containing fragment E2F showed that changes in G0/G1 phase did not differ from those in the control, but the number of S-phase cells in the peptide-treated sample remained high (65-70%) by the 14th hour of incubation. The number of S-phase cells in the control sample decreased after 10-h incubation and corresponded to 45% by the 14th hour (Fig. 2). The number of G2/M-phase cells in the peptide-treated and control sample reached maximum after incubation for 18-20 and 10-12 h, respectively. It can be hypothesized that antiproliferative activity of the peptide with active site E2F is related to suppression of cell transition from the S-phase to the G2/M phase.

We compared the cytotoxic effects of peptides with various functional sites. The peptide with functional sequence p16INK4a exhibited higher cytotoxic activity than E2F-containing peptide. Sponta-

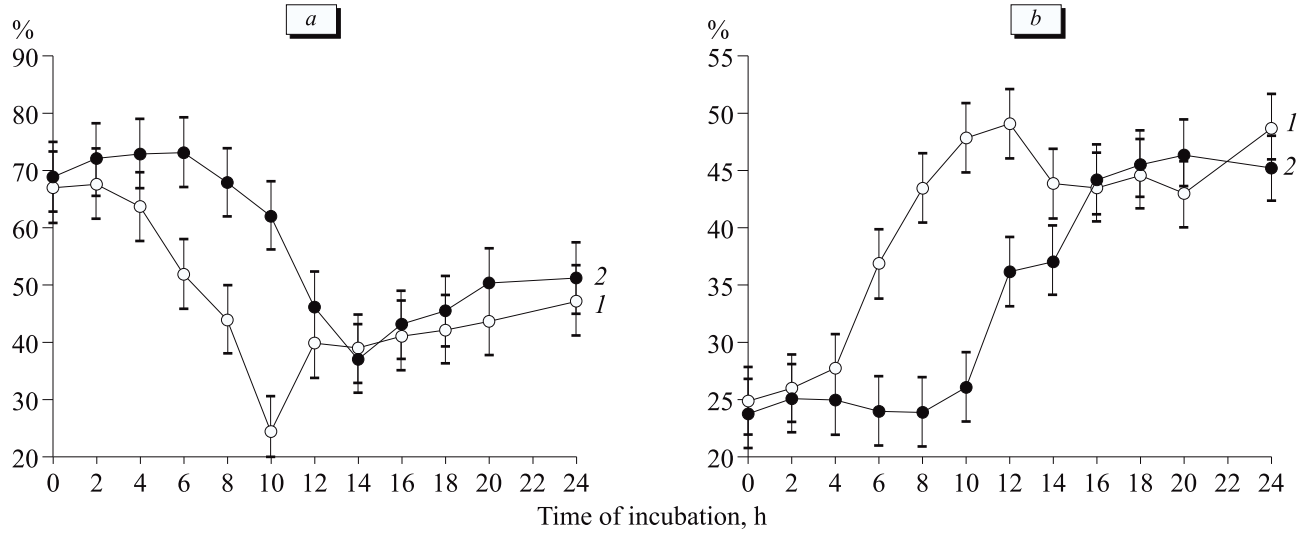

Fig. 1. Effect of chimeric peptide with fragment p16INK4a and penetrating sequence Antp on cell cycle transition in synchronous culture of 293 cells. Change in the number of cells in the G0/G1 phase (*a*) and S phase of the cell cycle (*b*). Control (*1*) and 40 μM Antp_p16 (*2*).

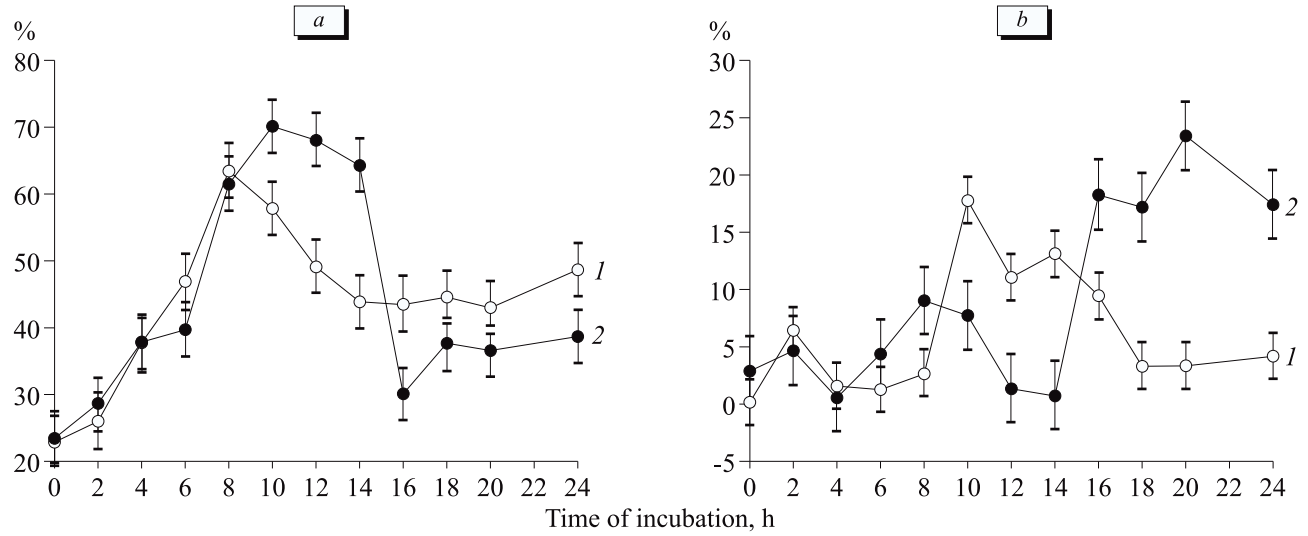

Fig. 2. Effect of chimeric peptide with fragment E2F and penetrating sequence Tat on cell cycle transition in a synchronous culture of 293 cells. Change in the number of cells in the S phase (*a*) and G2/M phase of the cell cycle (*b*). Control (*1*) and 40 μM Tat_E2F (*2*).

neous apoptosis in the culture of A549 cells was 12.3%. Apoptosis in these cells increased to 43.8 and 30.1% after 24-h incubation with peptides Antp_p16 (40 μM) and Tat_E2F, respectively. The cytotoxic effect was highest in the culture of A549 cells. The culture of MCF-7 cells was least sensitive to these peptides.

Since the chimeric peptide with fragment p16INK4a exhibited maximum cytotoxic activity, we studied the dependence of apoptosis on the time of cell incubation in the presence of Antp_p16. The degree of apoptosis in samples was maximum after incubation with the chimeric peptide for 24 h. However, the increase in the number of apoptotic particles was irregular. Incubation of samples with chimeric peptide Antp_p16 was accompanied by 2 local maxima of the formation of apoptotic bodies (Fig. 3). The local maximum of apoptosis in cultured 293 cells was observed after 2 and 10 h. The increase in apoptosis after 2-h incubation with this peptide coincided with cell transition from the G0 phase to the G1 phase. The changes after incubation for 10 h were associated with the delay of transition from the G1 phase to the S phase of the cell cycle. It may be suggested that exogenous p16INK4a initiate apoptosis in cells, which was blocked in the control point of G1/S.

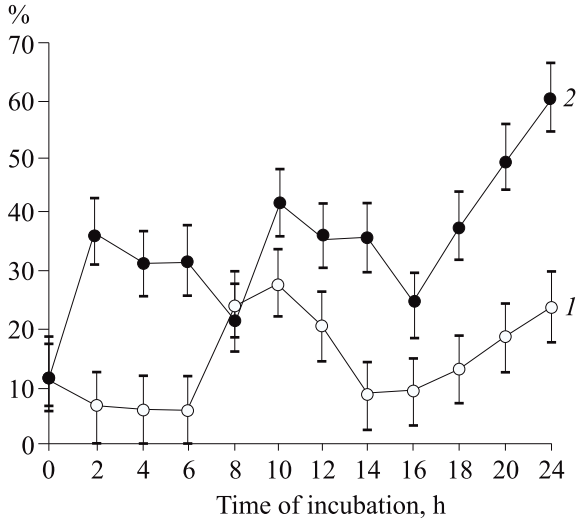

Fig. 3. Dependence of apoptosis in control samples and samples with chimeric peptide Antp_p16 on the time of incubation. Control (*1*) and 40 μM Antp_p16 (*2*).

The search for potent inhibitors of proliferation and tumor-specific activators of apoptosis is an urgent problem of molecular biology [10]. Malignant transformation is most often accompanied by damage to the molecular region of p16INK4a-cyclin D-pRB-E2F, which regulates proliferation of normal cells [1,8]. Hence, target modulation of components in this region of the molecular network holds much promise for the synthesis of new antitumor drugs. The main cytostatic effect of peptide Antp_p16 is manifested in suppression of proliferation in the G0/G1 phase of the cell cycle. This effect is associated with the ability of protein p16 to inhibit cyclin D. p16 inhibits binding of cyclin D to cyclin-dependent kinases of types 4 and 6, which prevents E2F release from the complex with hypophosphorylated protein pRb. These changes are followed by inhibition of transcription and cell arrest in the G1 phase of the cell cycle [12].

The inhibition of proliferation and activation of apoptosis by chimeric peptides depend on the type of cells, but not on p16 expression. Our results suggest that the intrinsic mechanism for regulation

of proliferation in the G1—S stage involves p16INK4a and does not modulate the antiproliferative effects of exogenous peptides, which contain cyclin-inhibiting fragments of this protein.

Chimeric peptide E2F inhibited cell proliferation in the S phase. Our results are consistent with published data that amino acid sequence PVK RRLDL binds and inhibits cyclin A and cyclin A-dependent kinases [3,8,12]. This motif is present in all proteins of the E2F family and cyclin-dependent kinase inhibitor p21Cip/Kip. Hence, addition of the cyclin A-dependent kinase inhibitor should be followed by suppression of the S phase and inhibition of S—G2 transition.

It should be emphasized that the test peptides can stimulate apoptosis in cells of several lines. In this respect, Antp_p16 is more potent than E2F. Moreover, this process depends on the time of incubation with the peptide. The increase in cell apoptosis after addition of peptide p16 is probably related to cell arrest in the G1 phase and impaired transition into the S phase. The observed changes contribute to cell death. The maximum of apoptosis was revealed 2 and 10 h after the end of the G0/G1 phase, which probably resulted from the inability of cells to "overcome" the limit points.

Another mechanism of activation of apoptosis suggests the increase in E2F activity. E2F overexpression in cells also induces apoptosis. It is important that cell death can be mediated by the p53-dependent and p53-independent mechanisms [8].

Our results indicate that activity of these sequences is related to a direct effect of peptides on the molecular mechanism, which regulates transition from the G1 phase to the S phase. The cytotoxic and cytostatic effects of peptides with cyclin-dependent kinase inhibitors should be studied to perform a search for new antitumor drugs.

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