CROATICA CHEMICA ACTA CCACAA 81 (1) 81-87 (2008) ISSN-0011-1643 CCA-3215 Original Scientific Paper

Transduction of p27 to Induce Apoptosis in Tumor Cells

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RECEIVED MARCH 2, 2007; REVISED JULY 4, 2007; ACCEPTED JULY 13, 2007

Keywords apoptosis cell lines B-CLL

Transduction is a biochemical technique for the introduction of full-length proteins into the cells. It has the potential to be used in the development of a new therapeutic strategy for cancer therapy. Different forms of p27 (TAT-p27, TAT-Mp27, TAT-p23) were transduced into tumor cell lines, lymphocytes and B-CLL cells, and their influence on proliferation and apoptosis was investigated. The metabolism of transduced proteins differed between the cell types. TAT-p27 protein is metabolized faster than the mutated form. Furthermore, the half-life of TAT-p27 depended on the type of cells. All forms of TAT-p27 fusion protein moderately decreased the proliferation of different types of the cells and induced apoptosis. The cells from some B-CLL patients were sensitive to TAT fusion proteins, and the sensitivity was increased with the addition of Fluda. This study provides valuable results for further development of TAT technology p27 as the potential tool for a specially targeted therapy of tumors.

INTRODUCTION

The progression of the mammalian cell cycle is regulated by a combination of positive and negative regulators.¹ It is activated by the family of cyclins and cyclin dependent kinases (CDKs). During the G1 phase, cyclin D1 and other D-type cyclins accumulate in nuclei and assemble with their catalytic partners, CDK4 and CDK6. The progression of the cell cycle through the G1 to S phase occurs after phosphorylation of retinoblastoma protein (pRb) with cyclin D1-CDK4 complex and sequestration of CDK inhibitors. On the other hand, Cip/Kip family members of CDK inhibitors (p21, p27) act as negative regulators of the cell cycle progression. They inhibit the activity of cyclin-CDK complexes (cyclin A/CDK2, cyclin E/CDK2) and mediate the exit from the cell cycle. The level of p27 increases in quiescent non-proliferating cells and decreases prior to entry into the cell cycle. The rate of increase is controlled by the rate of degradation in both cytoplasm and nuclei. Degradation of p27 in cytoplasm is associated with the transition of cells from the G0 to the G1 phase, whereas nuclear degradation occurs during the S and G2 phases.

The hyperproliferation of cancer cells is associated with deregulation of the cell cycle progression, driven by the activities of CDKs.^{1,2} Therefore, inhibition of CDK activity is a realistic goal in the clinical treatment of cancer. CDK inhibitors are selective small molecules, which halt G1/S transition and induce cell death. One of them, p27, has a significant role in cancer progression and antitumor drug response, and stems from the correlation between low p27 expression and either high tumor grades or poor survival. Conversely, its overexpression in cancer cells inhibits tumor cell growth by enforcing cell cycle arrest and apoptosis, and this has contributed to p27 being referred to as a tumor suppressor.

B-CLL (chronic lymphocitic leukemia) is a currently incurable disease, characterized by accumulation of

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neoplastic B-cells, arrested in the early phases (G0/G1) of the cell cycle.^{3,4} The pathogenesis of the disease is largely unknown. Defective apoptosis and deregulation of some cell cycle regulatory genes/proteins may contribute to the malignant process of B-CLL.^{5,6} An elevated level of p27 has been found in B-CLL. This protein plays an important role in the arrest of the cell cycle and in communication between the extracellular signals and cell cycle controlling mechanism. Because of the pivotal role in the regulation of the cell cycle p27 could be a suitable target for therapeutic drugs.

In order to examine the influence of p27 on tumor cell proliferation and apoptosis TAT-p27 (wild type), a mutated form of p27 (TAT-Mp27) and a truncated form (TAT-p23) were transduced into different cell types and their metabolism and half-lives were investigated. By the mutated form we wanted to investigate whether the cleaving of p27 by caspase is a necessary event in apoptosis, because mutation was in a place where caspase cleaves to p27. That cleaving results in p23 protein and we would like to show whether it stimulates or prevents apoptosis.

EXPERIMENTAL

Cell Culture

Human cell lines (Raji and Ramos) were maintained in RPMI medium plus 10 % fetal bovine serum (FBS) (Gibco, Austria). B-CLL cells were isolated from the peripheral blood of B-CLL patients. Their diagnosis, besides appropriate clinical and cytological features, relied on a characteristic phenotype with CD5 positivity and weak expression of surface immunoglobulines. Samples were obtained by the addition of anticoagulant (heparin) and processed within a few hours after collection. Mononuclear cells (> 90 % B-lymphocytes) were isolated with a lymphocyte separation medium (Eurobio Biotechnology, Les Ulis, France) from peripheral blood of B-CLL patients. After washing, the cells were incubated at a final concentration of 2×10^6 cells/ml in RPMI 1640 (Life Technologies Ltd, Paisley, Scotland) containing 10 % heat-inactivated fetal bovine serum (FBS), 2 mmol dm⁻³ glutamine, 50 U/ml penicillin (1 U/ml \approx 1 mg/ml) and 50 µg/ml streptomycin at 37 °C in a 5 % CO2 humidified atmosphere.

Cells were transduced by addition of purified TAT fusion proteins (TAT-p27, TAT-Mp27, TAT-p23) at a concentration of 50–300 nmol dm⁻³, directly to cell culture medium. Transduction efficiency was verified by immunoblotting for the HA epitope contained in all TAT fusion proteins. The number of live cells expressed in percent was determined by MTT test.

Purification of TAT-fusion Proteins

Purification of TAT fusion proteins was performed according to slightly modified method previously described.⁷ Briefly, bacterial lysates containing recombinant TAT fusion proteins were sonicated in 8 mol dm⁻³ urea, 100 mmol dm⁻³ NaCl, 20 mmol dm⁻³ Hepes, pH = 8.0, and passed over a Ni-NTA resin (Amersham-Pharmacia Biotech AB, Uppsala, Sweden). All TAT fusion proteins contain the N-terminal 6x-His, which specifically binds to Ni-NTA resin. Proteins were eluted with imidazole. The fractions with TAT fusion proteins were pooled, diluted with 20 mmol dm^{-3} Hepes pH = 8.0 and desalted on a PD-10 column in phosphate-buffered saline (PBS) (Amersham-Pharmacia Biotech AB, Uppsala, Sweden). All TAT fusion proteins were sterile filtered and stored in 10 % glycerol at -80 °C. In the experiments three forms of protein p27 were used: (i) TAT-p27 fusion protein of transduction domain and p27 wild type (30-35 kDa), (ii) TAT-Mp27 fusion protein of TAT transduction domain and p27 with point mutation where Thr-187 is replaced by Ala (30-35 kDa), and (iii) TAT-p23 fusion protein of transduction domain and p27 truncated at the C-terminal end (20-23 kDa).

Cell Viability

The influence of TAT-fusion proteins, with or without 5 μ mol dm⁻³ Fluda [9- β -D-arabinosil-2-fluoroadenine-monophosphate] (Schering AG, Berlin, Germany) on the viability of cells, was determined by MTT assay⁸ or by Trypan blue exclusion. This method is based on reduction of MTT (yellow) with a mitochondrial dehydrogenase that is active only in living cells to yield a DMSO-soluble formazan product (red). Absorbance was measured at 570 nm. The number of living cells is linearly proportional to the amount of reduced MTT. Results represent a mean value from four parallel samples in two independent experiments.

In situ Cell Death Detection

After the transduction of TAT-p27 fusion proteins into B-CLL cells, the number of apoptotic cells was determined by using "*In Situ* Cell Death Detection Kit AP" (Boehringer Mannheim, Germany), which allows simultaneous TUNEL-based apoptosis detection and morphological localization under a light microscope. The procedure prescribed by the Manufacturer was followed.

Cell Lysates

Cells in culture $(2-5 \times 10^6 \text{ per sample})$ or B-cells $(2-4 \times 10^6 \text{ per sample})$ were harvested and cell lysates prepared at time points. Cells were washed with PBS and lysed on ice for 30 min in a buffer containing: 20 mmol dm⁻³ Tris-HCl pH = 7.6, 150 mmol dm⁻³ NaCl, 1 % Triton X-100, 5 mmol dm⁻³ EDTA, 1 mmol dm⁻³ sodium orthovanadate, 10 mmol dm⁻³ NaF, 10 µg/ml aprotinin, 1 mmol dm⁻³ PMSF. After centrifugation (15 min at 13000 rpm), supernatants were collected and used, either immediately or after freezing at -80 °C, for Western blot analysis. The concentration of protein in each sample was determined by the Bio-Rad assay method (Bio-Rad Laboratories, Richmond, CA).



Figure 1. (a) Purified TAT-fusion proteins after SDS-PAGE and Western blot with specific antibody to p27. rp27 was used as a control for p27. Metabolism of TAT-p27: (b) in Raji cell line, (c) in B-cells from CLL patient, and (d) its influence on the level of expression of intracellular p27.

Western Blot Analysis

To examine the effects of transduced proteins on the expression of cell cycle regulatory proteins, cell lysates were separated on SDS-PAGE and analyzed by Western blot. Briefly, 50 µg of each lysate was separated on gradient gel (5-10 %) SDS-PAGE9 and transferred to Immobilon-P nitrocellulose membranes (Millipore, Bedford, MA). As a loading control, the transferred proteins were stained with Naphthol Blue Black (Sigma, St. Louis, MO). Membranes were cut up and after blocking with 5 % non-fat milk in phosphate-buffered saline (PBS) for 30 min, the resulting subsections were incubated with the specific antibodies for p27 (N-20, 0.5 µg/ml) and cyclin E (cyc E; HE-12, 0.5 µg/ml) (Santa Cruz Biotechnology Inc. CA, USA). Subsequently, the membranes were washed twice in TBS-Tween buffer $(10 \text{ mmol dm}^{-3} \text{ Tris-HCl pH} = 8.0, 200 \text{ mmol dm}^{-3} \text{ NaCl},$ 1 % Tween 20) and incubated for 1 hour with the appropriate horseradish peroxidase-linked secondary antibody (Dako, Glostrup, Denmark). After the incubation, membranes were washed three times with TBS-Tween buffer, and the proteins were detected using chemiluminescence (ECLTM reagents, Amersham Pharmacia Biotech, Buckingamshire, UK) using X-ray film (BiomaxTM film, Kodak). For quantitation, films were scanned on a densitometer with BioCapt software and analyzed with Bio-Profil Bio-1D software.

RESULTS

Transduction of TAT-fusion Proteins and Their Metabolism

Purified TAT fusion proteins on SDS PAGE corresponded to the proteins about 35 kDa (Figure 1a). Preparation of TAT-p27 had a very good purity, but TAT-Mp27 and TAT-p23 contained some cleaved forms. Rp27 was loaded as a control. The proteins were transduced into different

cell types in the range from 50 to 300 nmol dm⁻³. According to the references, after addition of TAT fusion proteins into the medium and their penetration into the cells, TAT-domain is cleaved and degraded by cell proteolytic system. Released proteins are refolded by chaperons and exhibit their original, physiological function. The behavior of TAT-p27 after entering into the cells was followed by monitoring the time of degradation. As is shown in Figure 1b, in the Raji cell line, the amount of TAT-p27 decreased with the time of incubation. In first 24 h extracellular p27 did not have influence on the level of expression of intracellular protein. However, 24 and 48 h after TAT-p27 transduction, increased levels with an additional metabolite were detected. In the cells from B-CLL patients, the metabolism of TAT-p27 protein was different (Figure 1c). After 3 h the majority of TAT-p27 was degraded and after 6 h it almost disappeared. After 20 h TAT-p27 was added again, and its concentration increased in the cells. During the incubation of 20 h, extracellular p27 did not have any influence on the level of intracellular protein, but after longer incubation (up to 72 h), TAT-p27 increased the level of intacellular p27 (Figure 1d). On the other hand, the presence of TAT-p23 decreased the level of intracellular p27 in B-CLL, which might be related to the induction of apoptosis. It seems that metabolism of TAT fusion protein differs between the types of cells.

Viability of Cells after Treatment with TAT-fusion Proteins

The viability of the cells in the culture, lymphocytes or cells from B-CLL patients, was measured by MTT test or by Trypan blue exclusion (Figure 2). Inhibition of proliferation did not depend on the concentration of added TAT-fusion protein. The effect of TAT-fusion protein



Figure 2. Viability of different cell types after treatment with TAT-fusion proteins, alone or in combination with Fluda (a) Trypan blue; (b) MTT-test. Cells were treated with TAT-fusion proteins for 48 h (details in Experimental). Each number represents a mean from four parallel samples in two separate experiments.

depended on the type of cells as well as the form of p27 protein. Transduced TAT-p27 inhibited proliferation of the Ramos cell line by about 20 %.

However, the samples from B-CLL patients exhibited different sensitivity on TAT-fusion proteins (Figure 2b). In combination with Fluda, a known cytotoxic agent, the effect of TAT-fusion protein was remarkable, but not better than Fluda itself.

Expression of Intracellular p27 and Cyclin E after Transduction of TAT-fusion Proteins

The influence of TAT-fusion proteins on the expression of intracellular p27 and cyclin E was investigated on the blood samples from B-CLL patients and lymphocytes. The results are shown in Figures 3 and 4. Different metabolism of TAT-fusion proteins was also noticed in these samples. The level of intracellular p27 in lymphocytes was not changed in the presence of TAT-p23 or TAT-Mp27, but incubation with TAT-p27 resulted in a remarkable decrease of intracellular p27 after 68 h. A similar effect was noticed in the expression of cyclin E. In comparison with the results obtained on the blood samples from B-CLL patients, transduction of TAT-fusion proteins had a different effect. There were some differences in the expression of intracellular p27 after transduction of TAT-p27 or TAT-p23, but TAT-Mp27 had low or no influence. A better effect was achieved when Fluda was added together with TAT-fusion proteins. The level of the expression of cyclin E was changed depending on the type of TAT-fusion protein and time of incubation.

Induction of Apoptosis with TAT-p27

Besides the viability, the induction of apoptosis was determined using the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) method. The results are shown in Table I. A small amount of spontaneous apoptosis was detected in control cells, but after treatment of the lymphocytes or cells from B-CLL patients with TAT fusion proteins, the number of apoptotic cells increased. Among TAT-fusion protein, TAT-p23 induced apoptosis to the highest extent. Addition of Fluda remarkably increased the number of apoptotic cells.

DISCUSSION

The expression of cell cycle regulatory proteins is determined by a number of signaling pathways that are activated by various growth factors and other mitogenic stimuli.^{1,10} In order to efficiently control cell cycle progression, p27 has to be expressed at a high level in quiescent cells and decrease in response to mitogenic stimulation, allowing time activation of cyclin E and cdk2 complexes. B-CLL cells are arrested in the early G1 phase by the up-regulation of p27 and down-regulation of cyclin D3 and E.^{6,11}

Molecular analysis of human tumors has demonstrated that p27 is functionally inactivated by different means in a majority of neoplasia. This foundation suggests that p27 level represents an important determinant in cell transformation and cancer development. Abundance of p27 is regulated by multiple extracellular stimuli and it acts as a sensor of external signals to cell cycle regulation.¹² Because of its pivotal role in the regulation of the cell cycle, p27 could be a good candidate for targeted therapy. For that purpose we transduced three forms of p27, the wild type, point mutated p27 (TAT-Mp27) and the truncated form of p27 (TAT-p23) into tumor cell lines and B-cells from CLL patients and examined their behavior and metabolism. The results in Figure 1 show different metabolic pathways of TAT-p27 in Raji cells and B-CLL cells. It seems that in B-CLL cells the metabolism is faster than in Raji cells. It seems that transduced

TAT-p27 had some influence on the expression of intracellular p27, depending on the time of incubation. On the other hand, transduction of TAT-p23, a truncated form of protein p27,¹³ decreased intracellular p27 after 48 h.

The survival (Figure 2) and the expression of intracellular p27 and cyclin E (Figures 3 and 4), after transduction of TAT- p27 proteins in the lymphocytes and B-cells from CLL patients, were investigated. TAT-fusion proteins had an influence on the survival of cells to different extents, according to the type of cells and the pa-



Figure 3. Influence of TAT-fusion proteins on the level of expression of intracellular p27 and cyc E, investigated on the lymphocytes from a healthy donor and the B-cells from two CLL patients (Pt 1 and Pt 2). Cells were treated with TAT-fusion proteins and the samples were withdrawn at different time points (details in Experimental). The lysates were analyzed by SDS-PAGE and Western blot.



Figure 4. Influence of TAT-fusion proteins alone or in combination with Fluda on the level of expression of intracellular p27 and cyc E, investigated on the B-cells from two CLL patients (Pt 3 and Pt 4). Cells were treated with TAT-fusion proteins and the samples were withdrawn at different time points (details in Experimental). The lysates were analyzed by SDS-PAGE and Western blot.

Sample	Number of dead cells / %						
	Control	TAT-p27	TAT-p23	TAT-Mp27	TAT-p27	TAT-p23	TAT-Mp27
		$(100 \text{ nmol } dm^{-3})$	$(50 \text{ nmol } dm^{-3})$	$(50 \text{ nmol } dm^{-3})$	$(100 \text{ nmol } dm^{-3})$	$(50 \text{ nmol } dm^{-3})$	$(50 \text{ nmol } dm^{-3})$
					+ Fluda	+ Fluda	+ Fluda
Lymph	18	n.d.	n.d.	n.d.	32	43	n.d.
Pt 3	32	41	57	n.d.	n.d.	57	n.d.
Pt 4	20	19	26	9	65	58	53
Pt 6	30	34	55	n.d.	n.d.	n.d.	n.d.
Pt 7	36	46	69	n.d.	n.d.	n.d.	n.d.

TABLE I. Analysis of apoptotic cells by TUNEL method^(a)

^(a)The cells were incubated with TAT-p27, TAT-Mp27 or TAT-p23 for 48 h and the number of apoptotic cells was determined.

tients. When TAT-p27 protein was added together with Fluda, a well known cytotoxic agent,¹⁴ a better effect was achieved. In a patient (Pt 1) insensitive to Fluda,¹⁵ a cytotoxic effect of TAT-fusion proteins was remarkable. Cytotoxic effect of TAT fusion proteins in some cases was in concordance with a number of apoptotic cells, determined by TUNEL (Table I). Differences in metabolism between the forms of TAT-p27 were also seen in the experiments with B-cells from CLL patients. TAT-Mp27 and TAT-p23 were metabolized slower in comparison with TAT-p27. In examined blood samples transduced proteins had no or very small influence on the expression of intracellular p27. However, transduced p27 remarkably decreased intracellular p27 in lymphocytes, after 68 h incubation. As to cyclin E, TAT-p27 proteins moderately influenced its expression. There is some indication that its expression is increasing, which could point to the activation of cell cycle progression. A similar effect was seen after the treatment of B-CLL with immunostimulatory CpG-oligonucleotides¹⁶ and with CD40L.⁹

The use of TAT-technology for intracellular delivery of therapeutic molecules has already been reported.^{17,18,19} The described biologically active TAT-fusion proteins function in either the cytosolic or nuclear compartments of the cell^{8,20,21} or are localized on the mitochondrial membrane.²² The feasibility of using TAT-mediated recombinant protein-based therapy was shown in some cases, such as neonatal hypoxic-ischemic (H-I) injury.²³ It was shown that expression of Bcl-xL protein correlates with the survival of neurons after ishemia or other acute brain insults. Peripheral delivery of Bcl-xL protein with TATvector could inhibit both caspase- and non-caspase-mediated apoptotic pathways, and therefore combats H-I induced cell death. TAT-technology could also be applicable in the treatment of tumors. It was shown that dendritic cells, transduced with TAT-Her2/neu inhibited a development of breast cancer in mice and increased the survival of animals with tumor.24

These results shed some light on the potential treatment of tumor cells by direct introduction of physiologically active proteins into the cells. Maybe some forms of TAT-p27 proteins could be useful in the treatment of tumors. However the antitumor effect needs to be additionally confirmed.

Acknowledgments. – This work was supported by Grant 0098093 from the Ministry of Science, Education, and Sports, Republic of Croatia. Part of this study was carried out in the Laboratory de cinetique cellulaire, Hopital Hotel Dieu, Paris, France. Sincere thanks to all who supported this investigation.

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SAŽETAK

Indukcija apoptoze u tumorskim stanicama transdukcijom proteina p27

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Transdukcija je biokemijska metoda za unos proteina u stanice. Postoje naznake da bi se metoda mogla koristiti za razvoj novih strategija u liječenju tumora. Različite forme proteina p27 (TAT-p27, TAT-Mp27, TAT-p23) transducirane su u tumorske stanične linije, limfocite iz humane periferne krvi i u B-stanice pacijenata s CLL-om te je praćen njihov utjecaj na proliferaciju i apoptozu. Metabolizam transduciranih proteina je različit u pojedi-nom tipu stanica. TAT-p27 je metaboliziran brže od mutiranog oblika. Također i vrijeme poluraspada TAT-p27 ovisi o tipu stanica. Sve tri forme TAT-fuzijskih proteina utječu na preživljavanje ispitivanih stanica i apoptozu, ovisno o vrsti stanica. B-stanice izolirane iz periferne krvi pacijenata s CLL-om pokazale su osjetljivost na TAT-fuzijske proteine, a u kombinaciji s Fluda ta osjetljivost je povećana. Ova studija dala je preliminarne rezultate vrijedne za razvoj TAT-tehnologije kao pogodne metode za ciljanu terapiju tumora.