TAT-Mediated Delivery of p27 in Tumor Cell Lines as a Potential Therapeutic Peptide

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Abstract: TAT-fusion proteins (TAT-wt-p27, TAT-pt-p27, TAT-N'-p27) were introduced into the cells by protein transduction method. The mechanism by which transduced 27 influences on the regulation of cell cycle and apoptosis, were explored.

TAT-p27-fusion proteins affected the proliferation of examined cell lines depending on type of the cells and protein. Transduced p27 induced accumulation of cyclin D1 and D3, with slight differences among the form of protein. Expression of cyclin D2 and E was mainly unchanged. Furthermore, TAT fusion proteins promoted apoptosis, which resulted in activation of caspase 3, appearance of poly (ADP-ribose) polymerase and DNA fragments, as well as the activation of apoptosis-inducing factor.

The results pointed that transduced p27 activates apoptosis through activation of different signal transduction pathways. Thus, the molecule of p27 could be appropriate for treatment of tumors with deregulated its function. Also, the protein transduction method could find the application in specifically targeted cancer therapy.

Keywords: Apoptosis, cell cycle, p27, TAT fusion proteins.

INTRODUCTION

Many malignant diseases are characterized by disrupted proliferation, differentiation and apoptosis, which can be a consequence of malfunction in cell cycle control. Molecular analysis of human tumors has widely demonstrated that p27 is functionally inactivated by different means in a majority of neoplasias of several different origins. This suggests that p27 levels represent an important determinant in cell transformation and cancer development. Regulation of p27 is achieved through many signal transduction pathways, and it integrates diverse signals into a final decision between proliferation and cell cycle exit. The cdk-inhibitory activity of p27 is controlled by its concentration, subcellular localization and phosphorylation status. The level and activity of p27 increase in response to cell density [1], differentiation signals [2,3], loss of adhesion to cellular matrix [4], and in response to growth inhibitory signaling [1,2]. In addition to its effects on the cell cycle, p27 also regulates cell migration [4]. Thus, p27 is not a classic tumor suppressor. It is rarely mutated or deleted in human cancers [5], but frequently it is deregulated with reduced level (increased degradation) or mislocalization.

The abundance of p27 is controlled in a cell-type specific and signal-specific manner by the integration of mitogenic and antimitogenic signal pathways that affect its translation, stability and localization. p27 mRNA levels remain constant throughout the cell cycle, while its protein levels are controlled by ubiquitin-mediated proteolysis [6].

It appears that p27 activity could be directly targeted by its mislocalization to the cytoplasm in some tumors [7-9]. Mislocalization of p27 effectively inactivates its inhibitory activity, because cytoplasmic p27 is portioned from its nuclear cyclin-cdk targets.

Sequestration of p27 in the cytoplasm by cyclin D-cdk4/6 complexes is another way of p27 inactivation in human tumors. Overexpression of cyclin D3 [7] or inhibition of cyclin D1 degradation [10] could be involved in sequestration of p27, and prevent association with its targets.

Apoptosis is triggered through activation of one or more signal transduction pathways, which activates a family of aspartic acid-specific cysteine proteases, referred to as caspases [11] or other death effectors such as apoptosis-inducing factor (AIF) and endonuclease G [12]. Activation of caspases is central in caspase-dependent pathway and can be initiated by either of three distinct mechanisms: ligation of death receptors, release of cytochrom c from mitochondria and stress to ER (endoplasmatic reticulum) [13, 14]. In response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effectors, AIF and endonuclease G. Triggering of caspase-independent pathways could be an attractive approach to eradicating tumor cells. It has also been demonstrated that caspase-independent processes

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ISSN: 1929-2260 / E-ISSN: 1929-2279/12

play a prominent role in mediating cell death following acute injuries (stroke, myocardial infarction) and neurodegenaerative diseases [15]. AIF is a mitochondrial intermembrane flavoprotein located in the intracellular membrane space (IMS). It has a dual role in controlling cellular life and death; in normal mitochondria, it functions as an antiapoptotic factor *via* its oxidoreductase activity [16]. On the other hand, upon induction of apoptosis, AIF is translocated from the mitochondria to the nucleus to function as a proapoptotic factor in a caspase-independent pathway [17]. It is believed that AIF is synthesized as 67-kDa apo-protein with an N-terminal extension and imported into mitochondria, where it is processed to the 62-kDa mature form. During apoptosis, processing of mature AIF to a 57-kDa form occurred caspase-independently in the intermembrane space, which leads to releasing the processed form into the cytoplasm and translocation in the nucleus, where it functions as a proapoptotic factor that causes large scale DNA fragmentation [18, 19]. The mitochondrial protease responsible for apoptosis-dependent AIF processing has not been identified yet.

In this study p27 was delivered into the cells by protein transduction and its influence on the induction of the apoptotic signal transduction pathways in cancer cell lines was examined. The molecular mechanism pointed out that p27 is involved in caspase dependent and independent apoptotic pathways, depending on the type of cancer cell line.

MATERIAL AND METHODS

Cell Lines

Human cell lines (Raji, RKO and MCF7) were maintained in RPMI-1640 or DMEM (Gibco, Grand Island, N.Y. USA) medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 µg/mL streptomycin and 100 units/mL penicillin, at 37°C in a humidified 5% CO_2 atmosphere. Plasmids encoding TAT-wt-p27 (wild type), TAT-pt-p27 (point mutation in the region responsible for cdk binding) and TAT-N'-p27 (truncated form on C-terminal end) were obtained from Dr. S. Dowdy, St. Louis, MO, USA [20, 21]. Each of the constructs was expressed in E. coli and the proteins were produced and purified as described previously [20]. Cells were transduced by adding the appropriate TAT-protein to the medium at the final concentration of 150 nM. Transduction efficiency was verified by immunoblotting for the HA region contained in all TAT fusion proteins. Percentage of live cells was determined by WST-1 (for cell lines growing in suspension) or MTT (for cell lines growing attached on surface) test. WST-1 is a water-soluble tetrazolium salt based proliferating reagent. After completing the experiments (72 h), 10 µL/well of WST-1 reagent per 100 µL culture volume was added and incubated for additional 3 h. The absorbance was measured using microplate reader at 450 nm. In MTT test, yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl] tetrazolium bromide is reduced to purple formazan with mitochondrial reductase from living cells. After solubilisation in DMSO, the absorbance at 570 nm was measured. Each experiment was performed in quadruplicates and repeated 3-5 times.

Western Blot

Procedure was described earlier [22]. Briefly, the whole cell lysates were prepared in RIPA buffer (10 mM Tris-HCl pH 7.5; 1% Na-deoxycholate; 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with protease inhibitors (1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, 50 µg/ml aprotinine, 1 mM DTT). Each lysate (50 µg) was separated on gradient gel (5-10 %) SDS-PAGE and transferred to Immobilon-P nitrocellulose membranes (Bio-Rad Trans-Blot® Transfer Medium, 0.2 µm). Loading of proteins was routinely assessed by staining the transferred proteins with Naphthol Blue Black (Sigma-Aldrich, St. Louis, MO). After blocking with 5% non-fat milk in TBST for 30 min, membranes were cut and the resulting subsections were incubated with the indicated specific antibodies: p27 (N-20, 0.5 µg/mL), cyclin D1 (HD11, 0.5 µg/mL), cyclin D3 (C-16, 0.5 µg/mL), cyclin E (HE -12, 0.5 µg/mL), AIF (H-300, 0.5 µg/mL), cytochrome c (7H8, 1 µg/mL) and caspase-3 (H-277, 0.5 µg/mL) (Santa Cruz Biotechnology Inc. CA, USA) and cyclin D2 (2 µg/mL) and poly (ADP-ribose) polymerase (PARP) (dilution 1:2000) (Pharmingen, San Diego, CA, USA). Subsequently, the membranes were washed twice in TBS-Tween buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 % Tween 20) and incubated for 1 hr with the appropriate horseradish peroxidase-linked secondary antibody (Dako A/S, Denmark). The membranes were then washed again three times with TBST buffer, and the proteins were detected by chemiluminescence (Perkin Elmer™ Western Lightning Chemiluminescence Reagent Plus or Thermo Scientific SuperSignal® West Pico/Femto Chemiluminiscent Substrate), using X-ray film (Biomax™ film, Kodak). Images were acquired using Agfa Curix 60 (Agfa Gevaert N.V.).

FACS-Analysis

a) Cell Cycle Phase

The cells were seeded at 1×10^5 cells per well into a 6-well plate and treated with 150 nM TAT-p27 fusion proteins. Cell cycle analysis was performed 24 h after the protein treatment by assessment of DNA content after staining with propidium iodide. On the day of the analysis the cells ($2.5x10^5$) were fixed with 70% ethanol at -20°C. On the day of the analysis the cells were washed 3 times in cold PBS and then incubated with propidium iodide (2.5 µg/mL) and ribonuclease A (50 µg/mL) (Sigma-Aldrich, St. Louis, MO) for 15 min at 37^o C in dark. Analysis was performed using FACSCalibur™ (Becton Dickinson) and the data was analyzed using ModFit LT software.

b) Annexin-V Binding Assay

Detection and quantification of apoptotic cells at single cell level, was performed using Annexin-V–FLUOS staining kit (Roche, USA), according to the manufacturer's recommendations. The cells were seeded at 1×10^5 cells per well into a 6-well plate and treated with 150 nM TAT-p27 fusion proteins. After 24 or 48 h both floating and the attached cells were collected, washed in Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) and dyed with FITC conjugated Annexin-V and PI. The stained cells were then analyzed with FACSCalibur™ (Becton Dickinson) and the results were processed with FlowJo 7.2.5 software.

Caspase 3 Activity

The activity of caspase 3 was measured by Colorimetric Caspase 3 Assay kit (Sigma-Aldrich, Co), according to manufacture procedure. The cells ($1x10^5$ RKO and MCF7, $1x10^6$ Raji) were treated with 150 nM TAT-p27 fusion proteins for 24 h. Both, floating and the attached cells were collected and lysed. Each lysate (30 µL) was incubated for 4 h with 60 µL of the reaction buffer plus 10 µL of the Ac-DEVD-pNA substrate (Thermo Labsystems MS). Upon cleavage by caspase-3, released p-NA was quantified using a microtiter plate reader at 405 nm (yellow). Enzyme activity was calculated using a standard curve generated with free p-NA, and expressed as µmol p-NA/min.mg proteins.

DNA Fragmentation

Apoptotic DNA fragments were isolated according to slightly adjusted previously described method [23]. Briefly, after harvesting the cells were washed with PBS and the pellets were lysed for 10 sec in 1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5 (10 µL per 10^6 cells, minimum 50 µL). After centrifugation (5 min, 1600xg) the supernatants were collected and the extraction was repeated with the same amount of the lysis buffer. The supernatants were brought to 1% SDS and treated for 2 h with RNase A (1 µg/ µL) at 56°C, followed by over night digestion with proteinase K (2.5 µg/ µL) at 37°C. After addition of 1 M ammonium acetate (1/2 vol.), DNA was precipitated over night at -80°C with ethanol (2.5 vol.). Next day samples were centrifugated for 30 min (+4°C, 16 000xg) and the precipitated DNA was washed with 70% ethanol and dissolved in miliQ H_2O. Fragments were separated on 1% agarose (Sigma-Aldrich, St. Louis, MO) and visualized with ethidium-bromide, using ImageMaster VDS (Pharmacia Biotech).

RESULTS

Influence of TAT Fusion Proteins on Proliferation

TAT-fusion proteins were introduced into the cells by protein transduction method, and found in the cell lysates shortly after exposure by Western blot (Figure **1A**) A time course of the proteins has revealed that extracellular (transduced) p27 does not influence the levels of the host protein. The presence of TAT-fusion proteins was detected inside the cells 90 min after exposure by immuno-fluorescence method, using antibodies against p27 and HA region (*Suppl. 1).*

Influence of TAT-fusion proteins on the proliferation of examined cells was measured by MTT or WST-1 test (Figure **1B**). The results from 3-5 individual experiments performed in quadruplicates are expressed as a mean ± SD. The inhibition of cell proliferation depended on the form of proteins and type of the cells. TAT-wt-p27 slightly inhibited the proliferation of the examined cell lines (RKO 15.7±9.1%; MCF7 6±1.7%; Raji 14.3±8.5%). Similar effect was achieved with TAT-N'-p27 (RKO 14.1±8.3%; MCF7 6.7±3.0%; Raji 26.9±10.6%). The most pronounced inhibitory effect was seen with TAT-pt-p27 protein (RKO 22.5±7.9%; MCF7 10±2.6%; Raji 28.3±11.3%). TAT protein itself has not affected the proliferation of examined cell lines (0-2%).

To investigate the molecular mechanisms by which TAT-p27 influences on the regulation of cell cycle and apoptosis, the expression of molecules involved in these events were determined.

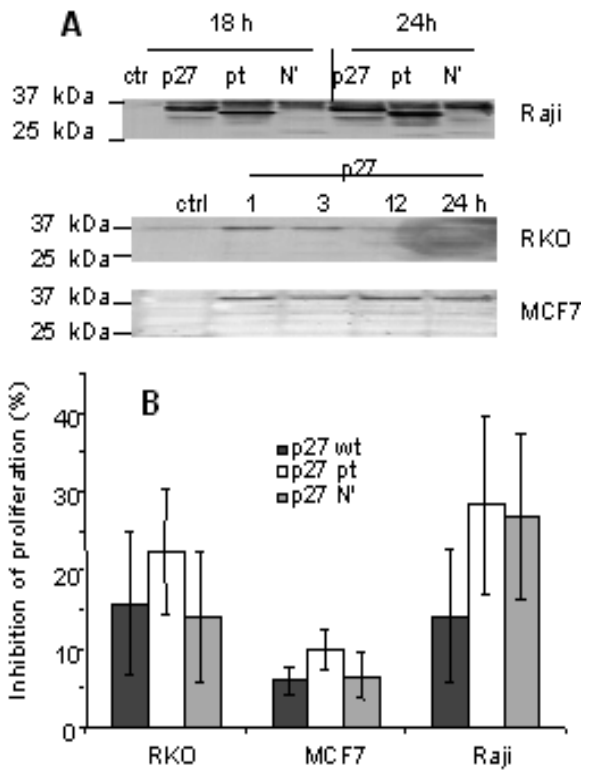

Figure 1: Time course (**A**) and the percentage of inhibition (**B**).

A) Presence of TAT-fusion proteins (~35 kDa) was detected by Western blot using specific antibody to p27.

B) Growth inhibition of RKO, MCF7 and Raji cells after the treatment with TAT-p27 fusion proteins (wt, pt, N'). The results are expressed in percentage of treated and control cells. Mean values are calculated from the results obtained in 3-5 separate experiments, performed in quadruplicates. Error bars represent standard deviation.

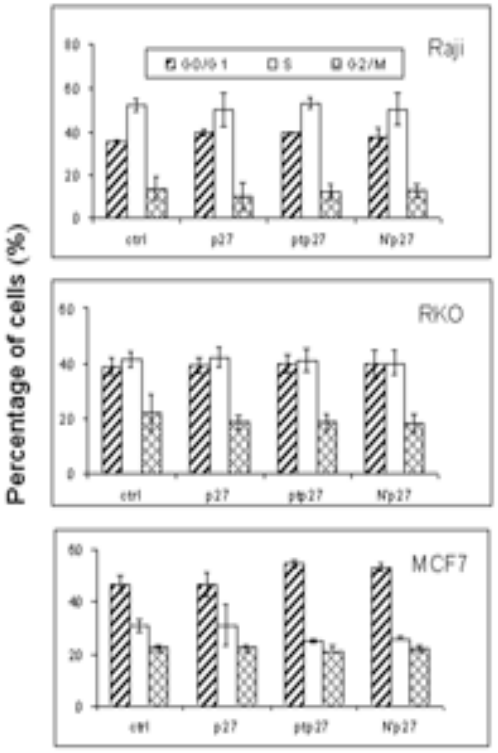

Figure 2: Flow cytometry analysis performed on Raji, RKO and MCF7 cell lines, after 24 h treatment with TAT-p27 proteins (wt, pt, N'). Analysis was performed using FACSCalibur™ (Becton Dickinson) and the data was analyzed using ModFit LT software. The results are expressed in percentage of cells in particular phase as a mean from three independent experiments. Error bars indicate standard deviation.

Influence of TAT-p27 Proteins on Cell Cycle

The results of flow cytometry analysis on RKO, MCF7 and Raji cell lines are shown in Figure **2**. Flow cytometry analysis of the RKO cells treated with TAT-p27 wt, pt and N' fusion proteins revealed slight increase of cells in G0/G1 phase (39.05±3.19%; 39.86±3.20%; 39.59±5.44%, respectively) in comparison with control cells (37.01±3.19%). On the other, hand the number of cells in S-phase slightly decreased with N' form (40.07±4.34%), while pt form and wt protein did not affect the amount of cells in S-phase, in comparison to control cells (41.30±4.24%; 42.12±3.63%, 41.36±2.96, respectively). Percentage of the cells in G2/M phase in decreased after all the treatments (wt 18.84±0.38%, pt 18.84±2.88% and N' 20.35±3.46%) in comparison with the control (22.23±6.74%).

Treatment of MCF7 cells with p27 variants resulted in some increase of the cells in G0/G1 phase, treated with pt- and N'-p27 protein (54.48±1.5%, N' 53.10±1.3%, respectively) in comparison with control (46.6±3.3%) or wt-p27 (46.3±4.4%). Similar observation was noticed with the number of cells in S phase. The same percentage of cells in S phase was found in the control cells (30.8±2.2%) and treated with wt-p27 (30.9±7.9%). On the other hand, the treatment with pt and N'-p27 led to a slight decrease in the number of cells (24.88±0.8%, 25.83±0.6%, respectively). The number of cells in G2/M phase was not affected with any treatment (control 22.88±1.8%; wt 22.5±1.7%; pt 20.74±0.7%; N' 21.70±0.6%).

Treatment of Raji cells with TAT-p27 fusion proteins (wt, pt, N') resulted in increased amount of cells in G0/G1 phase (39.62±1.48%, 39.27±0.36%, 36.97±4.19%, respectively), in comparison with control cells (35.76±0.19%). Slight decrease of S phase was visible after treatment of Raji cells with TAT-p27, wt and N' (50.62±7.76% and 50.31±7.30% respectively), but pt form caused some increase (52.93±7.30%) in relation to the control (51.95±2.89%). The reduction of the cells in G2/M phase was also observed (ctrl

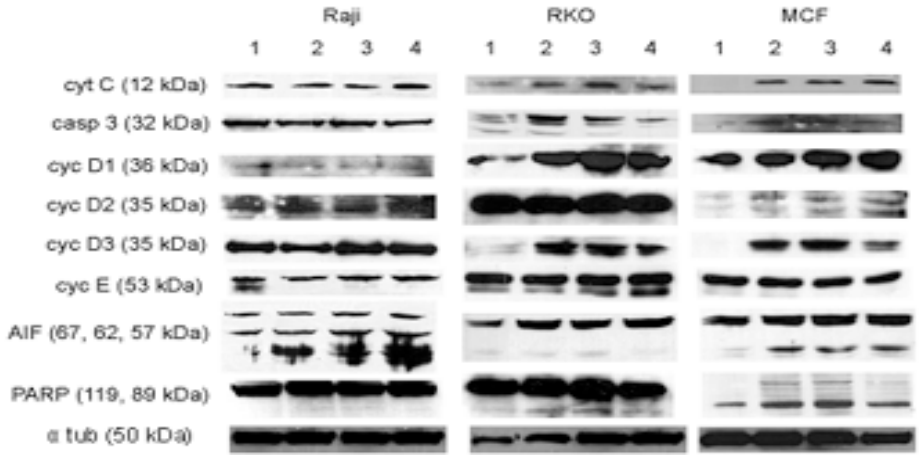

Figure 3: Western blot analysis of the cell lysates obtained after 24 h treatment of Raji, RKO and MCF7 cell lines with TAT-p27 proteins (*line 2* - wt, *line 3* - pt, *line 4* - N') and control cells (*line 1*) (see the details in Methods). Loading of proteins was routinely normalized by staining of transferred proteins with Naphthol Blue Black (*suppl. 2*). After separation of the proteins on PAGE, the incubation with indicated specific antibodies was performed. The bands were visualized by chemiluminescence's method, using X-ray film (Biomax™ film, Kodak). Images were acquired using Agfa Curix 60 (Agfa Gevaert N.V.). One representative of 5 individual experiments is shown.

13.80±5.20%, wt 10.32±6.28%, pt 13.31±3.49, N'12.73±3.10%).

Influence of TAT-p27 on the Regulatory Proteins of Cell Cycle and Apoptosis

To further evaluate the mechanism by which transduced p27 influenced the expression of cell cycle and apoptosis regulatory proteins, Western blot was used. Following the treatment of cancer cell lines (RKO, MCF7 and Raji) with TAT-p27 fusion proteins (wt, pt, N'), cell lysates were prepared and analyzed. Results are summarized in Figure **3** (control of loading in *Suppl. 2*). Cell cycle regulatory proteins were moderately affected by transduced proteins. Some differences were noticed among the cancer cells, as well as among the forms of p27 protein. Cyc D1 and D3 were accumulated after the treatment with TAT-p27 proteins (wt, pt, N'), but the expression of cyclin D2 was not affected. Slight differences were also visible in expression of cyclin E, depending on the cell line and the form of TAT-p27 protein.

Expression of characteristic proteins for the particular apoptotic signal transduction pathway, varied among the cancer cells. The expression of procaspase 3 as well as caspase 3 was changed in RKO and Raji cells, pointing out the involvement of p27 in apoptotic pathway. These findings were supported with activation of caspase 3, determined by colorimetric method (Figure **4**). Furthermore, the presence of cleaved PARP and increased expression of cyt c was noticed. Unexpectedly, in MCF 7 cells truncated form of PARP (78 kDa) was visible, despite unchangeable expression of 110 kDa form.

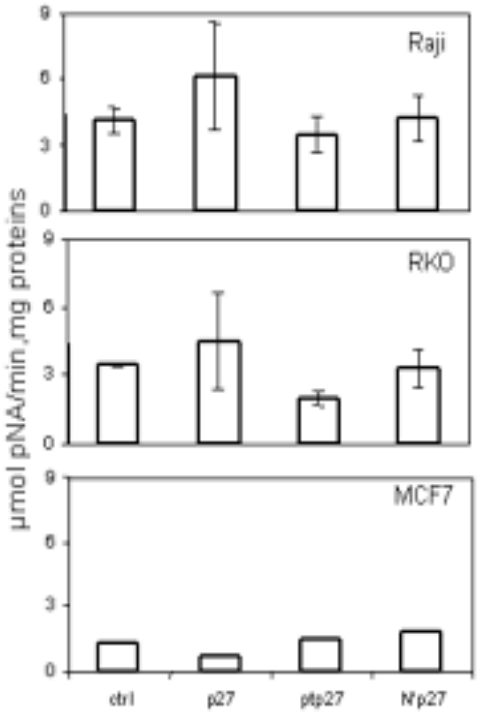

Figure 4: Activity of caspase 3 determined by colorimetric method, using Ac-DEVD-pNA as substrate. Released p-NA was quantified using a microtiter plate reader at 405 nm (yellow). Enzyme activity was calculated using a standard curve generated with free p-NA, and expressed as µmol p-NA/min.mg proteins. The results are shown as mean ± SD from 3 separate experiments, each performed in triplicate.

Expression of apoptosis linked 57 kDa form of AIF was found to be changed in Raji and MCF7 cell line

after the treatment with all three TAT fusion proteins while expression of 67 kDa and 62 kDa forms remained unaffected. On the other hand, these changes were not visible in treated RKO cells.

To support the above results, the presence of apoptotic cells was determined by Annexin-V binding. The attached cells, RKO and MCF7 were not suitable for that kind of experiment. Thus, the results are shown only for Raji cells (Table **1**, *Suppl. 3*). FACS analysis revealed an increase in the Annexin-V positive cells (apoptotic), after the treatment of Raji cells with TAT p27 (wt, pt, N'), in comparison with control cells.

Table 1: Percentage of Apoptotic Cells After the Treatment of Raji Cells with TAT- wt-p27 During 24 h. The Cells were Determined by Using Annexin-V–FLUOS Staining kit. Stained Cells were then Analyzed with FACSCalibur™ (Becton Dickinson) and the Results were Processed with FlowJo 7.2.5 Software (Image in *Suppl. 3*)

Treatment	% Annexin-V positive Raji cells
Control	12.09
TAT-p27, wt	16.99
TAT-p27, pt	16.61
TAT-p27, N'	20.35

DNA Fragmentation

Presence of small fragments of DNA, after the treatment of carcinoma cell lines with TAT-p27 proteins (wt, pt, N'), was examined by agarose gel electrophoresis. The results are shown in Figure **5**. Small DNA fragments were noticed in RKO and Raji cell lines, but not in MCF 7 cell line, where they are not expected. All three forms of p27 induced the fragmentation DNA in Raji cells at the similar extent. In RKO cell line TAT-p27 (wt) induced stronger DNA fragmentation then other forms of p27.

DISCUSSION

The introduction of specific targeted drugs which interfere with signal transduction pathways to block tumor cell proliferation or to induce apoptosis is the beginning of a fundamental change in the treatment of cancer. Cell division is regulated by a highly conserved group of proteins which together constitute the basic cell division machinery that controls the cell cycle [24]. Changes in the expression or activity of these proteins are usually detected in cancer cells. In many cases these changes are targets of genetic alterations which lead to cancer formation. Molecular analysis of human tumors has pointed out that p27 is functionally inactivated by different means in a majority of neoplasias, suggesting that it represents an important determinant in cell transformation, cancer development, as well as a target for specific therapy. Moreover, enforced expression of p27 in tumor cells could block the cell proliferation and that may result in apoptotic cell death.

In the present study it was shown that p27, introduced into cells as TAT-fusion protein, activated the apoptotic signal transduction pathways in tumor cell lines, depending on the type of cells and the form of protein. Protein transduction method was used for introduction of TAT- p27 into the cells. In these experiments three different forms of p27 proteins were

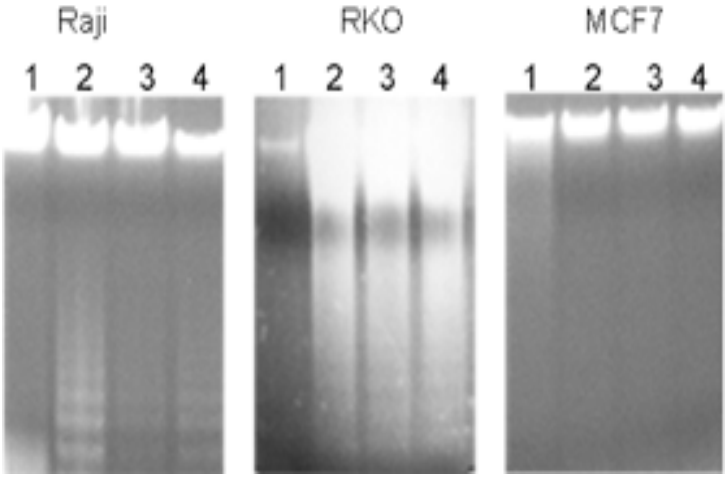

Figure 5: DNA fragmentation in Raji, RKO and MCF7 cell lines 24 h after treatment with TAT-p27 proteins: *line 1*-control, *line 2*- wt, *line 3*- pt, *line 4*- N'. Isolated DNA's were separated on agarose gel and visualized with EtBr, using ImageMaster VDS. One representative of 3 independent experiments is shown.

used: TAT-wt-p27, TAT-pt-p27 with a point mutation in the region responsible for cdk binding and TAT-N'-p27 truncated form on C-terminal end (region responsible for nuclear localization of p27) [20]. Also the cell lines have been selected to represent solid tumors (RKO, MCF7) and leukemia (Raji). Numerous approaches have been tested to introduce CKIs in different cell types and it seems that using recombinant proteins are the most promising. As a complementary antitumor approach several groups tried the delivery of CKIs in cancer cells by Ad vectors [25-30]. But, introduction of functionally active proteins into the cells using transduction method has advantage because the recombinant proteins are produced in bacteria with low cost and apparently no necessity of further refolding process.

Entering of TAT-fusion proteins into the cells was very fast and 1.5 h after the treatment the proteins were detected in cytosol (Figure **1A**, *Suppl. 1*). Using different forms of p27, with distinctive functions, some differences in effect were noticed. That could be consequence of different potential localization of particular form of p27. Also the metabolism (half life) of different forms TAT-27 proteins differed among the cells and the extracellulary added p27 did not have influence on the level of intracellular protein (Figure **1A**). Extracellular p27 decreased proliferation of examined cell lines, depending on the type of cells and form of p27 protein. Similar results were observed earlier on variety of tumor cell lines [31] as well as on rabbit endothelial cells [32].

Influence of extracellular p27 on cell cycle (Figure **2**) differed among the type of cells as well as the protein. Accumulation of cells in G0/G1 (up to 10%) phase was noticed in all examined cell lines, with some variation among the proteins, which is in accordance with the role of p27 [1, 2]. In addition, the cells in S and G2/M phases were decreased in almost all treatments and examined cell lines. The only exception was noticed in Raji cells treated with TAT-pt-p27, where the cells in S phase were increased for around 10%. That could be the consequence of mutation in cdk binding region [33]. Also some discrepancy was noticed in MCF7 cells treated with TAT-wt-p27, which induced increase in the cells in G2/M phase (14%). The most pronounced effect of transduced p27 proteins on cell cycle was detected in RKO cell line. The effect was visible in all phases of cell cycle, especially on G2/M phase where the decrease was up to 32% for wt and pt and 49% for N' forms. These results indicate p27 involvement in variety of signal transduction pathways not only at restriction point but also at G2/M transition.

Regarding the cell cycle regulatory proteins, accumulation of cyclin D1 and moderate decrease of cyclin E was noticed (Figure **3**). As the cell enters S phase, cyclin D1 level declines [34], and hence accumulation of cyclin D1 would explain the cell cycle arrest. Similar observation was reported earlier [35, 36]. Recently, it has been demonstrated that aberrant cyclin D1 expression acts as a p27 trap in B lymphocytes but does not induced relocation of p27 from nucleus to cytoplasm and does not modulate the G1/S transition [37]. Cyc E is an unstable protein and in normal cell cycle its level is low during G1 phase [38]. In our experimental systems it was altered, depending on the cell line. That could be the consequence of p27 ability to induce different signal transduction pathways.

To assess whether apoptotic signaling pathways were activated, FACS analysis after Annexin-V/PI binding was performed on Raji cell line (Table **1**, *Suppl. 3*). All three forms of p27 led to increase in Annexin-V binding, but at different extent (12.09%, 16.99%, 16.62%, 20.35%; ctrl, wt, pt and N', respectively). Furthermore, activation of caspase 3 as well as the cleavage of AIF and PARP was examined. Activation of caspase 3 after the treatment with TAT-wt-p27 in Raji (174%) and RKO (168%) cell lines (Figure **4**) and the presence of cleaved PARP (Figure **3**) demonstrated that caspase-dependent pathway was induced. Although the MCF7 cells are caspase 3 deficient [39], the cleavage of PARP might be induced by caspase 7 [40]. Meanwhile, the presence of apoptosis related 57 kDa form of AIF [18, 19] was detected in MCF7 and Raji cell lines without the difference regarding the form of TAT-27 protein. Together, those findings indicate that p27 could be responsible for the induction of caspase-dependent as well as caspase-independent programmed cell death.

Induction of apoptosis in examined cell lines with transduced p27 was corroborated with the presence of DNA fragments (Figure **5**). In caspase-dependent apoptotic pathway, caspase-activated nuclease induced degradation of DNA in small fragments [41], which were detected after the treatment of Raji and RKO cells with all three forms of the proteins. Small DNA fragments were not detected in MCF7 cells, where perhaps some other apoptotic pathway through AIF is activated. Activated AIF can induce cell death by binding to DNA, stimulating DNase activity and triggering chromatin condensation [42] and large scale

DNA fragmentation [43]. Extracellular p27 probably activates multiple signal transduction pathways with cross-talk among them. To solve this, the additional investigations would be necessary.

Over expression of p27 has been shown to trigger apoptosis in mammalian cells [36, 44-47]. However, the relationship between p27 and induction of apoptosis is not clear yet. p27 may be indirectly associated with apoptosis through CDK inhibition, and may be able to regulate the cell cycle or apoptosis to protect cells from over-growth and apoptosis-inducing stimulation. Thus, p27 could be suitable target for specific treatment of tumor cells. Many additional proofs would be necessary before using p27 as specific molecule for treatment of cancer.

ACKNOWLEDGEMENT

This work was supported form Ministry of Science, Education and Sports, Republic of Croatia, Grant No. 098-0982464-2514.

SUPPLEMENTAL MATERIALS

The supplemental figures can be downloaded from the journal website along with the article.

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Received on 28-04-2012 Accepted on 01-06-2012 Published on 21-08-2012

DOI: http://dx.doi.org/10.6000/1929-2279.2012.01.01.12