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Chapter 4

Proteasomal insensitivity of apoptin in tumor cells

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Abstract

The small viral protein apoptin is capable of inducing apoptosis selectively in human tumor cells. In normal cells apoptin localizes in the cytoplasm where it forms aggregates, becomes epitope-shielded and eventually degraded. By inhibiting the proteasome activity with the chemical inhibitors bortezomib and Ada-Ahx₃L₃VS apoptin levels can be stabilized in normal cells similar to the tumor suppressor p53 protein. In contrast, proteasome inhibition in tumor cells did not affect the apoptin stability while it still stabilized p53 levels. Apparently, apoptin is degraded by proteasomal activity in normal human cells, a process that no longer takes place in tumor cells. This loss of proteasomal susceptibility appears to be specific for apoptin.

Introduction

Apoptin is a small protein produced by Chicken Anemia Virus (CAV) that is capable of inducing apoptosis in human tumor cells while leaving normal cells intact (Danen-van Oorschot et al., 1997). In tumor cells apoptin is phosphorylated on T108 and translocates to the nucleus whereas in normal cells it stays in the cytoplasm in a non-phosphorylated fashion (Rohn et al., 2002; Backendorf et al., 2008). In normal cells, microinjected bacterially expressed MBP-apoptin forms in time large aggregates that after 24 hours can no longer be detected in an immune-fluorescence assay. The protein can still be detected by the use of stringent buffers and Western blot, showing that it becomes epitope-shielded. At later time points the protein could no longer be detected in normal cells and was eventually degraded (Zhang et al., 2003). This particular behavior was not observed in tumor cells.

Zhang et al. (2003) showed that the disappearance of MBP-apoptin was not caused by lysosomal degradation prompting us to investigate the role of the proteasome in the degradation of apoptin in both normal and tumor cells. The proteasome is a multi-subunit protease, which is composed of a proteolytic 20S core with regulatory 19S caps. The proteolytic activity is contained in the β 1, β 2 and β 5 subunits, all with their own substrate specificity (Hershko and Ciechanover, 1998; Murata et al., 2009).

To study the role of the proteasome in the degradation of apoptin we used the proteasome inhibitors bortezomib (Cusack et al., 2001) and Ada-Ahx₃L₃VS (Kessler et al., 2001) to block proteasomal degradation in cells expressing apoptin. We show that inhibition of the proteasome in normal cells leads to stabilization of apoptin and p53 levels, while in tumor cells both proteasome inhibitors affect p53 protein levels but have no influence on the amount of apoptin protein.

Results

Apoptin is degraded in normal human fibroblasts

In order to analyze a possible role of proteasomal degradation of apoptin we first examined the fate of apoptin levels in normal human fibroblasts ectopically expressing apoptin. After transfection of normal human fibroblasts with plasmid encoding flag-tagged apoptin, cells were fixed at various time-points and stained with antibody against the flag-tag. At 8 hours post transfection a diffuse apoptin distribution is observed throughout the cytoplasm. At later time-points apoptin starts to form granules and eventually can no longer be detected

(Figure 4.1A). Transfected flag-apoptin shows the same cytoplasmic localization as microinjected MBP-apoptin (Zhang et al., 2003). Apoptin protein levels were also analyzed by Western blot. Already 40 hours after transfection a drop in protein can be clearly observed, with hardly any to be detected three days after transfection (Figure 4.1B). This result indicates that apoptin likely gets degraded.

To analyze whether this reduction in protein was due to degradation of the apoptin protein, the proteasome was inhibited with the proteasome inhibitor bortezomib. Stabilization and an actual increase in apoptin protein (1.5x) could be observed after several days (Figure 4.1B/C) suggesting that the decrease in apoptin levels in normal cells is due to proteasomal degradation.

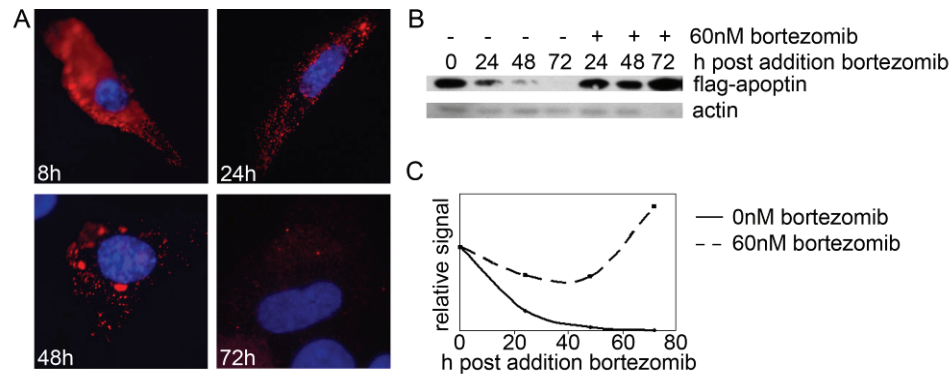


Figure 4. 1: Apoptin is degraded in time in normal cells, a process that can be blocked by bortezomib. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin. (A) Cells were fixed 8, 24, 48 and 72 hours post transfection. Flag-tagged apoptin was stained with a flag-specific antibody conjugated to rhodamine (red) and DNA was detected with Hoechst (blue). Apoptin appears in the cytoplasm as fine particles (8h), gradual clusters (24h), increased aggregates (48h) and eventually becomes degraded (72h). (B) Sixteen hours post transfection 0 or 60nM bortezomib was added to the cells and at three time points after addition cells were lysed. Total apoptin levels were determined by Western blot analysis with anti-flag antibody. (C) The relative amount of apoptin (as compared to $t=0$) with and without bortezomib was quantified and plotted.

Bortezomib and Ada-Ahx₃L₃VS inhibit apoptin and p53 degradation equally in normal human fibroblasts

Next, we analyzed whether besides apoptin a protein known to be degraded by the proteasome, i.e., tumor suppressor protein p53, can be stabilized by bortezomib with similar kinetics as apoptin in normal human fibroblasts. To that end, 16 hours after transfection increasing amounts of bortezomib were added to human normal fibroblasts expressing ectopic apoptin and endogenous p53. Twenty-four hours after the addition of bortezomib the cells were lysed and analyzed for apoptin and p53 levels by means of Western blot. A

gradual raise of apoptin protein levels could be observed as the concentration of bortezomib increased (Figure 4.2A, up to ~7.5x increase compared to control). The increase in the amount of apoptin was mimicked by the level of p53 (Figure 4.2B, up to ~7.5x increase compared to control).

As bortezomib mainly inhibits the $\beta 5$ subunit of the proteasome (Kisselev et al., 2006) we also examined the broad-spectrum proteasome inhibitor Ada-Ahx₃L₃VS (Kessler et al., 2001) in a similar experiment. This led to a stabilization of both apoptin and p53 levels (Figure 4.2), however, not to higher levels than observed with bortezomib (compare Figure 4.2A/B, ~7.5x increase with bortezomib compared to a ~4x increase of flag-apoptin signal with Ada-Ahx₃L₃VS).

Our results based on two different proteasomal inhibitors indicate that apoptin is degraded in normal human cells via the proteasomal pathway.

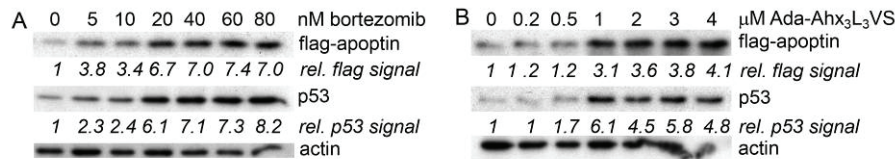


Figure 4.2: Proteasome inhibitors stabilize apoptin and p53 in normal cells. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin. (A) 16 hours post transfection increasing concentrations of bortezomib or (B) Ada-Ahx₃L₃VS were added and 24 hours later cells were lysed. Total levels of apoptin and p53 were determined by Western blot analysis. The relative amounts of apoptin and p53 (relative to the untreated signal) were quantified and are indicated below the corresponding panels.

Bortezomib inhibits phosphorylation-deficient apoptin mutant protein with similar kinetics as p53 in normal human cells

Apoptin induces apoptosis in tumor cells. This complicates the study of proteasomal breakdown in tumor cells. The phosphorylation of apoptin in cancer cells is closely linked to its apoptotic potential. Removal of the phosphorylation site T108 and the adjoining threonines greatly reduces the cell death potential of apoptin (Rohn et al., 2005). Therefore, we used the flag-apoptin(5Ala)106 mutant in which the stretch from position 106 to 110 has been replaced by alanines (Danen-van Oorschot et al., 2004).

Normal human fibroblasts transfected with plasmid encoding this apoptin(5Ala)106 mutant showed high protein expression at early time points, but as for the wild-type apoptin (Figure 4.1B/C), the apoptin(5Ala)106 mutant protein level diminished in time (Figure 4.3A). On the contrary, upon addition of bortezomib the amount of apoptin(5Ala)106 stayed high

(Figure 4.3A). Increasing concentrations of bortezomib resulted in more apoptin(5Ala)106 protein (Figure 3B, almost 3x increase).

The similar kinetics of degradation of the phosphorylation-deficient mutant compared to wild-type apoptin in normal cells makes it an appropriate construct to study the degradation characteristics of apoptin in human tumor cells. P53 stabilization showed similar kinetics as in the wild-type apoptin experiment.

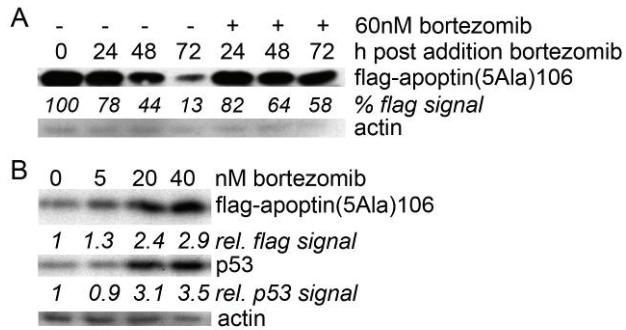


Figure 4.3: Apoptin(5Ala)106 is degraded in normal cells in a similar fashion as p53 and apoptin. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin(5Ala)106 mutant. 16 hours post transfection bortezomib was added. (A) At various time points cells were lysed and total apoptin was determined by Western blot analysis. (B) 24 hours after addition of increasing concentrations of bortezomib total apoptin and p53 were determined by Western blot. The relative amounts of apoptin and p53 (relative to the untreated signal) were quantified and are indicated below the corresponding panels.

Bortezomib and Ada-Ahx₃L₃VS stabilize p53 but not apoptin(5Ala)106 in human osteosarcoma cells

To examine the behavior of apoptin protein upon a bortezomib or Ada-Ahx₃L₃VS treatment in tumor cells, human osteosarcoma U2Os cells were used because they express wild-type p53 and are sensitive for apoptin-induced apoptosis (Diller et al., 1990; Zhuang et al., 1995). U2Os cells were transfected with plasmids encoding flag-tagged apoptin(5Ala)106 protein. Sixteen hours after transfection, the p53-positive human osteosarcoma cells were treated with increasing concentrations of bortezomib or Ada-Ahx₃L₃VS and the cells were lysed 24 hours later.

Increasing amounts of both bortezomib and Ada-Ahx₃L₃VS led to an increase in total p53 (Figure 4.4A/B). The p53 levels changed similarly as in normal cells as they increased 5x with bortezomib and 4x with Ada-Ahx₃L₃VS. This shows that the proteasome and the proteasomal inhibitors function in the U2Os tumor cell line. Remarkably, the amount of apoptin(5Ala)106 protein was not influenced by inhibition of the proteasome by increasing the concentrations of both bortezomib and Ada-Ahx₃L₃VS (Figure 4.4). In accordance with this finding is the observation that the basal level of apoptin protein in the

U2Os cells without addition of inhibitor is 15x higher than in normal cells (Figure 4.4C). Altogether, these results show that apoptin(5Ala)106 can not become degraded by the proteasome in human U2Os tumor cells.

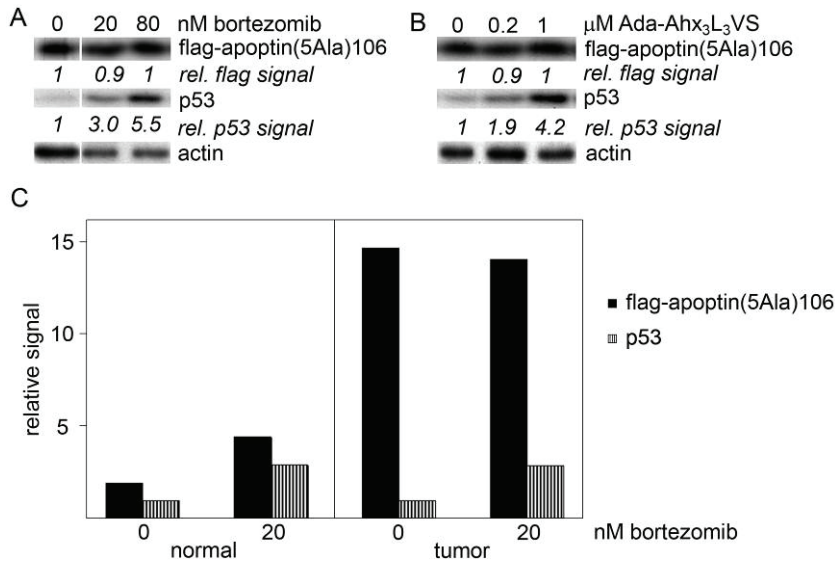


Figure 4.4: Proteasome inhibitors influence p53 but not apoptin(5Ala)106 stability in tumor cells. Human osteosarcoma cells U2Os were transfected with plasmid encoding flag-tagged apoptin(5Ala)106. (A) 16 hours post transfection cells were treated with increasing concentrations of bortezomib or (B) Ada-Ahx₃L₃VS and 24 hours later cells were lysed. Total amounts of apoptin and p53 were determined by Western blot analysis. The relative amounts of apoptin and p53 (relative to the 0nM bortezomib signal) were quantified and are indicated below the corresponding panels. (C) In order to make a direct comparison between the apoptin protein level in normal and tumor cells two representative experiments, one in normal and one in tumor cells, are visualized in the same chart. The relative amounts of apoptin and p53 in fig. 3B and fig. 4A were quantified relative to the untreated p53 levels and are plotted.

Discussion

By inhibiting the proteasome activity with the known proteasome inhibitors bortezomib and Ada-Ahx₃L₃VS, which both clearly positively affected the tumor suppressor p53 protein stability in normal and cancer cells, we revealed that apoptin protein is degraded in normal but not in tumor cells through the process of proteasomal degradation.

Apoptin is a potential therapeutic agent with very distinct behavioral differences between normal and tumor cells. The differential proteasomal degradation of apoptin between normal and tumor cells will likely contribute to its selectivity.

The tumor selective induction of apoptosis is the main feature of apoptin. This characteristic is preceded by the nuclear localization in cancer cells (Backendorf et al., 2008). In tumor cells apoptin is phosphorylated on T108, a modification that is not observed in normal cells (Rohn et al., 2002) To circumvent that apoptin-induced apoptosis interferes

with our protein degradation or stabilization measurements we developed a flag-tagged phosphorylation-deficient apoptin(5Ala)106 mutant that has a greatly reduced cell death inducing property (Rohn et al., 2005). In normal cells this mutant showed the same reaction to inhibition of the proteasome as did wild-type apoptin. In tumor cells, however, both the proteasome inhibitor bortezomib and Ada-Ahx₃L₃VS had no influence on the level of apoptin(5Ala)106, whereas the positive control p53 was clearly stabilized by the inhibition of the proteasome with similar kinetics as in normal cells.

Nuclear translocation is a characteristic of apoptin in tumor cells. P53 can also localize to both the nucleus and cytoplasm and is degraded in both compartments (Yuan et al., 2010). This raises the question if the difference in degradation of apoptin is due to a different proteasomal activity in the nucleus. Although several different catalytic subunit compositions are known between cell types as described above, no distinction has yet been established between the subunit composition of the proteasome in the nucleus and the cytoplasm.

In normal cells the highest levels of apoptin and p53 are reached with the inhibitor bortezomib compared to Ada-Ahx₃L₃VS. Bortezomib has a high affinity for the $\beta 5$ enzymatic subunit of the proteasome relative to Ada-Ahx₃L₃VS which has an equal effect on all three β subunits. Apparently it is the $\beta 5$ subunit that plays a major role in both apoptin and p53 degradation.

Although elevated levels of proteasome subunits have been found in some cancers, and proteasome inhibitors are used in the treatment of certain cancer types, a clear molecular difference between tumor and normal proteasome subunits has not yet been reported (Hoeller and Dikic, 2009; Bousquet-Dubouch et al., 2011).

Possibly, it is not a difference in proteasome assembly that leads to the higher stability of apoptin in tumor cells. Some viral proteins are known to modulate (viral) protein stability through the proteasome (Hu et al., 1999). In this respect it is interesting to mention that apoptin influences the anaphase promoting complex or cyclosome (APC/C) complex. Teodoro et al. (2004) showed that interaction of apoptin with the APC1 subunit results in destabilisation of the APC/C complex. The APC/C is an upstream effector of the proteasome that is responsible for correct progression of the cell cycle by targeting cell cycle related proteins for degradation (Garcia-Higuera et al., 2008). APC/C is the main E3 ligase in the nucleus targeting proteins for degradation. Subunits of the APC/C are mutated in

several cancers and are a popular target for viral proteins (Smolders and Teodoro, 2011). It is tempting to hypothesize that by disrupting the APC/C E3 ligase activity apoptin itself creates the situation that enables its stabilization. More research will, however, be needed to fully comprehend the molecular basis of the stable apoptin protein levels in tumor cells.

The tumor-selective apoptosis induction by apoptin makes it a very promising anti-cancer agent. Here, we show a novel characteristic of apoptin. The differential proteasomal sensitivity likely will contribute to the application of apoptin as a safe and efficient therapeutic agent.

Materials & Methods

Cell culture

The normal human diploid foreskin F44 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980's from Dr. M. Ponc (Dept. Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. F44 cells were used below passage 15 and cultured in 1:1 Dulbecco's Modified Eagle's Medium : Ham's F12 (DMEM/F12) (PAA, Colbe, Germany) containing 10% fetal calf serum (Thermo Scientific, Geel, Belgium), 100µg/mL penicillin, 100µg/mL streptomycin (Duchefa, Biochemie, Haarlem, The Netherlands) and 2mM glutaMAX (PAA). The human osteosarcoma cell line U2Os was selected as it expresses wild-type p53 (Diller et al., 1990). U2Os cells were purchased from the American Type Culture Collection (ATCC, Wesel, Germany) and cultured in DMEM (PAA) containing 10% newborn calf serum (Thermo Scientific), 100µg/mL penicillin, 100µg/mL streptomycin (Duchefa) and 2mM glutaMAX (PAA). Cells were cultured at 37°C in a humidified 5% CO₂ incubator. Cell morphology was regularly monitored to control the absence of cross-contamination.

Plasmids

The construction and expression of pcDNA3.1(+)-flag-apoptin was previously described (Zimmerman et al., 2012). In short, the DNA sequence encoding apoptin was synthesized by Baseclear (Leiden, The Netherlands) according to the apoptin sequence published by

Noteborn et al. (1991) and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Breda, The Netherlands). The oligonucleotide fragment encoding the flag-tag (Invitrogen) was inserted to create the pcDNA3.1(+)flag-apoptin plasmid encoding apoptin fused with a flag-tag at its N-terminus. The phosphorylation-negative flag-apoptin(5Ala)106 mutant was created as described in Danen-van Oorschot et al. (2004). The flag-tagged apoptin(5Ala)106 mutant plasmid was constructed by replacing the five amino acid stretch from position 106-110 by alanines in the pcDNA3.1(+)flag-apoptin construct background.

Transfection method

Cells were transfected with plasmids by using Amaxa nucleofection (Lonza AG, Cologne, Germany) according to the adapted manufacturer's protocol. 10^6 cells were taken up in 120 μ L nucleofector buffer (F44: 140mM Na₂HPO₄/NaH₂PO₄ pH 7.2, 5mM KCl, 10mM MgCl₂ and U2Os: 90mM Na₂HPO₄/NaH₂PO₄ pH 7.2, 5mM KCl, 10mM MgCl₂, 20mM Hepes-KOH pH 7.2), mixed with DNA, transferred to a transfection cuvet (VWR, Amsterdam, The Netherlands) and transfected with program U-20 or X-01 for F44 or U2Os, respectively. After addition of medium, cells were seeded on 6cm dishes.

Inhibitor treatment

The proteasome inhibitors bortezomib and Ada-Ahx₃L₃VS (a kind gift from dr. Bobby Florea, Leiden University, Leiden, the Netherlands) were used to block proteasomal protein degradation (Florea et al., 2010). The proteasome inhibitors were added to cells 16 h after transfection. Increasing concentrations of inhibitors were mixed with medium before addition to cells. After 24 h, unless otherwise indicated, cells were lysed and subjected to Western blot analysis.

Immune fluorescence assay

Cells grown on glass coverslips were first washed with phosphate buffered saline (PBS) at indicated time points after transfection and subsequently fixed at room temperature for 10 min with 1% formaldehyde, 5 min with 100% methanol and 2 min with 80% acetone. After air-drying the slides were used for immunocytochemical staining. For antibody staining, the cells were first incubated with PBS plus 0.05% Tween 20 (PBS-Tween; Sigma Aldrich, Zwijndrecht, The Netherlands) plus 5% normal goat serum (NGS) (Sigma Aldrich) for 1 h.

Next, the cells were incubated with the first antibody (1:150 monoclonal mouse-anti-flag antibody, Sigma Aldrich) in PBS-Tween plus 5% NGS for 2 h, washed with PBS-Tween, and incubated with the second antibody (1:100 rhodamine-conjugated goat-anti-rabbit antibody, Sanbio, Uden, the Netherlands) in PBS-Tween plus 5% NGS for 1 h. After washing with PBS-Tween cells were incubated 15 min with Hoechst 33358 (2 μ g/mL). Stained sections were mounted using PolyMount Mounting Media (Tebu-Bio, Heerhugowaard, The Netherlands) and analyzed with a fluorescence microscope (Olympus, Zoeterwoude, The Netherlands) with the Cell P software version 2.8 (Olympus).

Western blot analysis

Protein samples were separated on 15% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Hybond-P, GE Healthcare, Hoevelaken, the Netherlands). The membranes were blocked with 5% non-fat milk in Tris-HCl-buffered saline containing 0.1% Tween-20 (Sigma Aldrich, Zwijndrecht, the Netherlands) for 1 hour at room temperature. Primary antibodies directed against flag (mouse monoclonal M2, 1:1000, Sigma Aldrich), p53 (mouse monoclonal DO-1, 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) or actin (goat polyclonal I-19, 1:1000, Santa Cruz Biotechnology) were incubated in blocking buffer for 1 hour at room temperature. Peroxidase-coupled secondary antibodies (1:10.000) were obtained from Jackson ImmunoResearch Laboratories, Suffolk, UK, and membranes were incubated for 1 h at room temperature. Detection was achieved with enhanced chemiluminescence. Films were quantified using Quantity One Analysis Software (Biorad, Veenendaal, the Netherlands).

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