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Simultaneous human papilloma virus type 16 E7 and cdk inhibitor p21 expression induces apoptosis and cathepsin B activation

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Abstract

Human papillomavirus type 16 (HPV-16) is the major risk factor for development of cervical cancer. The major oncoprotein E7 enhances cell growth control. However, E7 has in some reports been shown to induce apoptosis suggesting that there is a delicate balance between cell proliferation and induction of cell death. We have used the osteosarcoma cell line U2OS cells provided with E7 and the cdk2 inhibitor p21 (cip1/waf1) under inducible control, as a model system for the analysis of E7-mediated apoptosis. Our data shows that simultaneous expression of E7 and p21 proteins induces cell death, possibly because of conflicting growth control. Interestingly, E7/p21-induced cell death is associated with the activation of a newly identified mediator of apoptosis, namely cathepsin B. Activation of the cellular caspases is undetectable in cells undergoing E7/p21-induced apoptosis. To our knowledge, this is the first time a role for cathepsin B is reported in HPV-induced apoptotic signalling.

Keywords: Human papilloma virus; Apoptosis; Cathepsin B

Introduction

Several studies have indicated a tight coupling between excessive cellular proliferation and apoptosis, as cell cycle regulators such as myc, E1A, E2F-1, cdc25, and ras influence both events (reviewed in (Schmitt and Lowe, 1999)).The transforming human papillomavirus type 16 (HPV-16) E7 oncoprotein binds to and affects the function of several cellular proteins involved in cell cycle regulation and progression, among these pRb, cyclin/cdk2 complexes and may be p21 (Funk et al., 1997; Hickman et al., 1997; Tommasino et al., 1993, for review, see Basile et al., 2001). Interestingly, E7 mediates both pro- and anti-apoptotic effects. The pro-apoptotic functions of E7 are shown by its ability to increase spontaneous cell death (Pan and Griep, 1994; Stoppler et al., 1998) as well as apoptosis induced by the tumor necrosis factor (TNF) ligand family members (Basile et al., 2001; Stoppler et al., 1998), sulfur mustard (Stoppler et al., 1998), actinomycin D (Hickman et al., 1997), γ-radiation (Puthenveettil et al., 1996), and serum deprivation (Jones et al., 1999). Not surprisingly, anti-apoptotic effects of E7 have also been demonstrated (Lee et al., 1998; Thompson et al., 2001). The mechanism by which E7 promotes apoptosis is still dubious. It has been suggested that E7, when inducing spontaneous cell death, mediates its pro-apoptotic effect via a possible p53-independent up-regulation of the inhibitory regulator of the cell cycle p21cip/waf1 (p21) (Basile et al., 2001; Jian et al., 1998, 1999; Jones et al., 1999; Park et al., 2000; Stoppler et al., 1998). This hypothesis is supported by the finding that introduction of p21 cDNA into HPV-16 and HPV-18 positive cancer cells induces apoptosis (Tsao

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et al., 1999). Data also show that inhibitors of the histone deacetylase (HDAC-1) can provoke apoptosis in HPV-infected cells through a mechanism where p21 is up-regulated (Finzer et al., 2001).

In most models of cell demise, including HPV-induced apoptosis, cell death is inevitably associated with the activation of a family of cysteine proteases, the caspases (Chang and Yang, 2000; Liu et al., 2000; Stoppler et al., 1998). Especially, activation of the effector caspase-3 is considered as an essential part of the classical apoptosis pathway (Janicke et al., 1998; Porter and Janicke, 1999). However, human MCF-7 breast carcinoma cells, not expressing caspase-3, undergo apoptosis when exposed to various apoptotic stimuli through other caspases (Eck-Enriquez et al., 2000; Jaattela et al., 1996; Liang et al., 2001), and hepatocytes as well as thymocytes undergo caspase-3 independent apoptosis (Adjei et al., 1996; Doerfler et al., 2000).

Indeed, other proteases than the caspases have been shown to induce apoptotic signalling (Deiss et al., 1996; Guicciardi et al., 2000; Ruggiero et al., 1987; Vanags et al., 1996; Wright et al., 1994). One of them is the lysosomal cathepsin B, a member of the cathepsin family consisting of 12 cysteine proteases with broad exo- and endopeptidase activity (Guicciardi et al., 2000; Polgar and Csoma, 1987). Interestingly, cathepsin B is commonly overexpressed in human primary tumors and induces apoptosis both dependent and independent of caspase activation. The same is true for apoptosis induced in human hepatocytes by either camptothecin (CPT) or bile-salt, where the apoptosis occur independent or dependent of caspase-8, respectively (Adjei et al., 1996; Roberts et al., 1999). Moreover, cathepsin B is reported to act as a dominant execution protease, both dependent and independent of caspases in death receptor triggered tumor cell apoptosis (Foghsgaard et al., 2001). Interestingly, during TNF-α induced apoptosis, cathepsin B is released from the lysosomes to the cytosol where it, possibly via Bid-mediated induction of cytochrome *c* release, engages traditional caspase activation (Foghsgaard et al., 2001; Guicciardi et al., 2000; Mathiasen et al., 2001; Stoka et al., 2001). Thus, active cathepsin B is a mediator of apoptosis and its translocation to the cytosol is essential to cell death.

The present study was initiated by our finding that simultaneous HPV-16 E7 and p21 expression induces cell death. Surprisingly, caspase-like protease activation was undetectable in cells undergoing E7/p21-induced cell death. This finding prompted us to search for non-caspase mediators of apoptosis and resulted in the identification of cathepsin B as a possible mediator of E7/p21-induced apoptosis. Our study is the first reporting the involvement of non-caspase mediators of apoptosis induced by the introduction of a HPV oncogene.

Results

Expression of E7 and p21 transgenes in U2OS cells

To study the influence of HPV-16 E7 and p21 on apoptotic signaling, we developed a cell model system, allowing simultaneous inducible expression of the transforming HPV-16 E7 gene and the cdk inhibitor p21 (cip1/waf1) in U2OS cells. This was done by stably

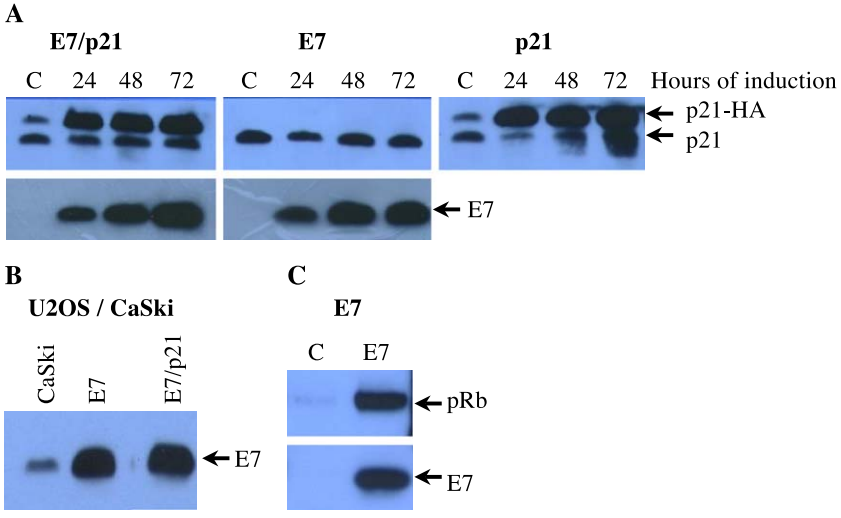

Fig. 1. Western blot analysis showing E7 and p21 protein expression levels and functionality in U2OS cells. (A) Expression of E7, HA-tagged p21, and endogenous p21 in E7/p21, E7, and p21 cell clones using E7 and p21 specific antibodies. Samples include an noninduced control marked C and samples induced for 24, 48, and 72 h. (B) Comparison of expression levels of E7 in E7 and E7/p21 cell clones with levels expressed in CaSki cells. (C) Co-immunoprecipitation, using pRB-specific antibodies, of pRB with E7, verifying E7 functionality in the E7 cell clone.

providing U2OS cells with inducible expression vectors carrying the genes of interest. Single-cell clones, resistant to appropriate selection antibiotics, were analyzed for transgene induction by analysis of E7 and p21 protein expression in Western blot analysis. Massive amounts of E7 and exogenous p21 protein were expressed in E7/p21, E7, and p21 cell clones following protein induction (Fig. 1A). Moreover, the level of E7 expression in our model system was compared to the level of E7 expression in CaSki cells naturally infected with HPV-16. Evidently, the level of E7 expression in the E7/p21 and E7 cells was higher than that present in CaSki cells (Fig. 1B). The endogenous p21 level remained unchanged over time. The intracellular localization of E7 and exogenous p21 was studied by immunofluorescence. Both proteins were expressed exclusively within the nucleus, suggesting functionality of these two proteins when expressed in U2OS cells (data not shown). To further ensure the

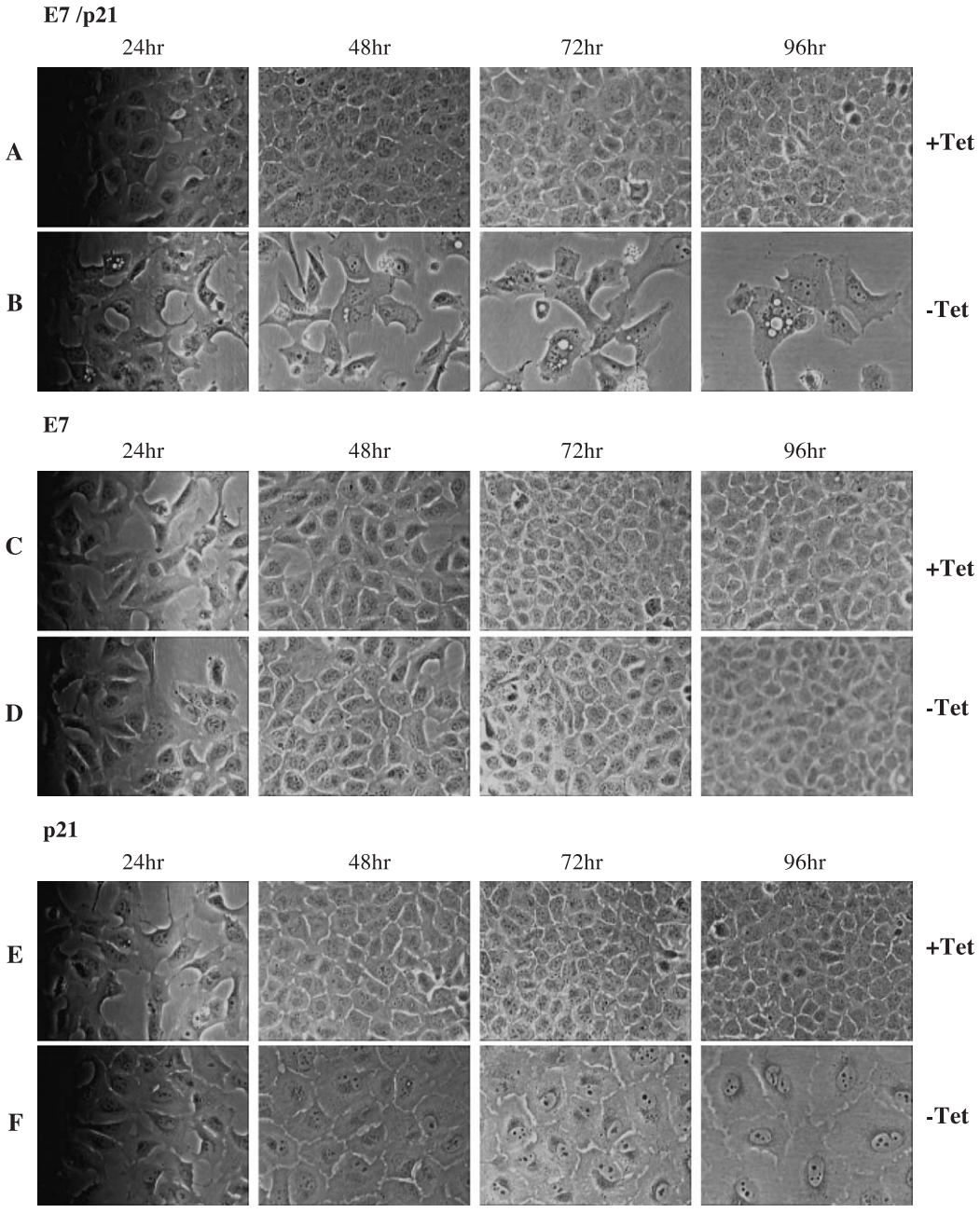

Fig. 2. Light microscopy showing changes in morphology of transgene U2OS cell clones during protein induction. E7/p21 cells show extensive signs of apoptosis following 24–96 h of protein induction (B), E7/p21 noninduced control cells (A). E7 cells retain normal morphology following E7 protein expression (D), E7 noninduced control cells (C). Enlarged p21 cells following protein induction indicating cell cycle arrest (F), p21 noninduced control cells (E).

functionality of E7, we performed co-immunoprecipitation analysis clearly showing coprecipitation of E7 and pRB in the E7 cell line (Fig. 1C).

Simultaneous E7/p21 expression induces apoptosis

Upon protein induction, we first investigated the morphology of the cells. Undoubtedly, E7/p21 expressing cells showed apoptotic features such as membrane blebbing (Fig. 2B). As expected, p21 overexpressing cells showed signs of cell cycle arrest (Fig. 2F), whereas E7 expressing cells retained normal morphology (Fig. 2D). Noninduced cells showed continued growth (Figs. 2A, C, E). Simple protein determinations was used as a measure of cell growth, and both E7/p21 and p21 expressing cells showed reduced cell growth, whereas induced E7 cells exhibited the same growth increase as noninduced controls (Fig. 3). The reduced cell growth of E7/p21 cells as well as stop of the cell cycle progression in the p21 overexpressing cells was verified by the decreased incorporation of bromdeoxyuridine (BrdU) in these cells (Fig. 4A). The viability of E7/p21 expressing cells was further measured using an MTT viability assay. As compared to noninduced cells, the E7/p21 expressing cells grew significantly slower for 72 h whereafter apoptosis was initiated (Fig. 4B). To determine apoptosis in induced E7/p21 cells, TUNEL analysis was performed and a more than fourfold increase in the apoptotic index clearly confirmed the morphological indications of apoptosis in these cells (Figs. 5A, B). TUNEL analysis of E7 and p21 expressing cells, respectively, revealed no apoptosis above control levels in these cells (Figs. 5A, B). TUNEL analysis of apoptosis induced in E7/p21 cells in the presence of the inhibitor of cathepsin B, Ca-074 Me, showed a two- to threefold reduction in the apoptotic index in the presence of the inhibitor (Fig. 5C). Annexin V staining of the noninduced and induced E7/p21 cells 96 h following induction showed an increase in apoptotic cells from 5% to 17%, while no apoptosis was observed in the E7 and the p21 cell lines (data not shown).

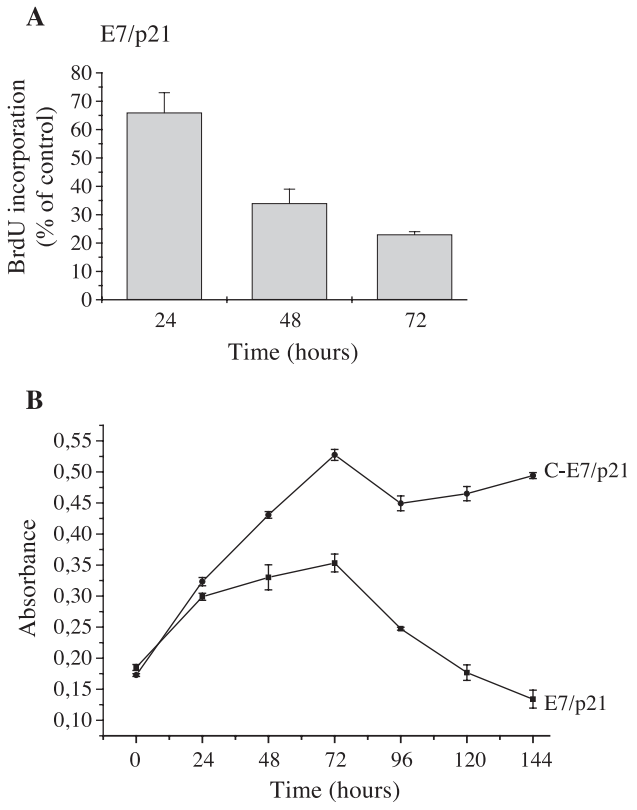

Fig. 4. (A) Incorporation of BrdU in p21 cells following protein induction. Expression of p21 results in decreased incorporation of BrdU showing cell cycle arrest in these cells. Bars indicate standard deviations for each time-point measurement bases on three experiments. (B) Cell viability of E7/p21 cells measured by an MTT assay. The E7/p21 cell clone grew significantly slower following protein induction as compared to noninduced cells. Following 72 h of E7/p21 induction, the MTT measurement shows apoptotic havoc in U2OS cells (solid squares). Noninduced control cells are marked C. Bars indicate standard deviations calculated from readings of six similar wells for each time point.

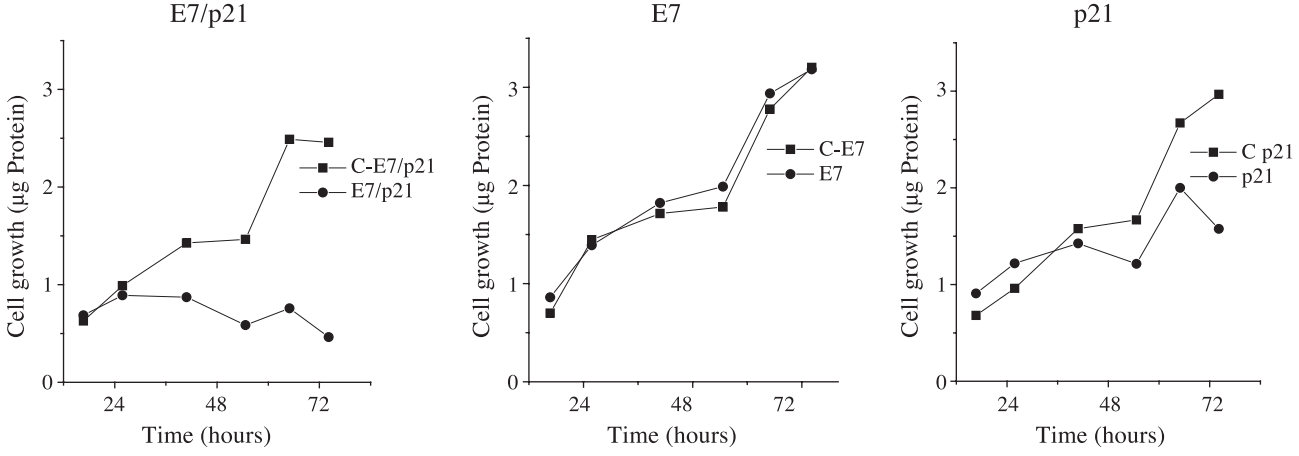

Fig. 3. Protein quantification. A surrogate measure of cell growth. Simultaneous expression of E7 and p21, due to apoptosis, resulted in decreased viability. Expressing E7 individually did not affect growth whereas induction of p21, inducing cell cycle arrest, decreased growth. Noninduced control cells are marked C.

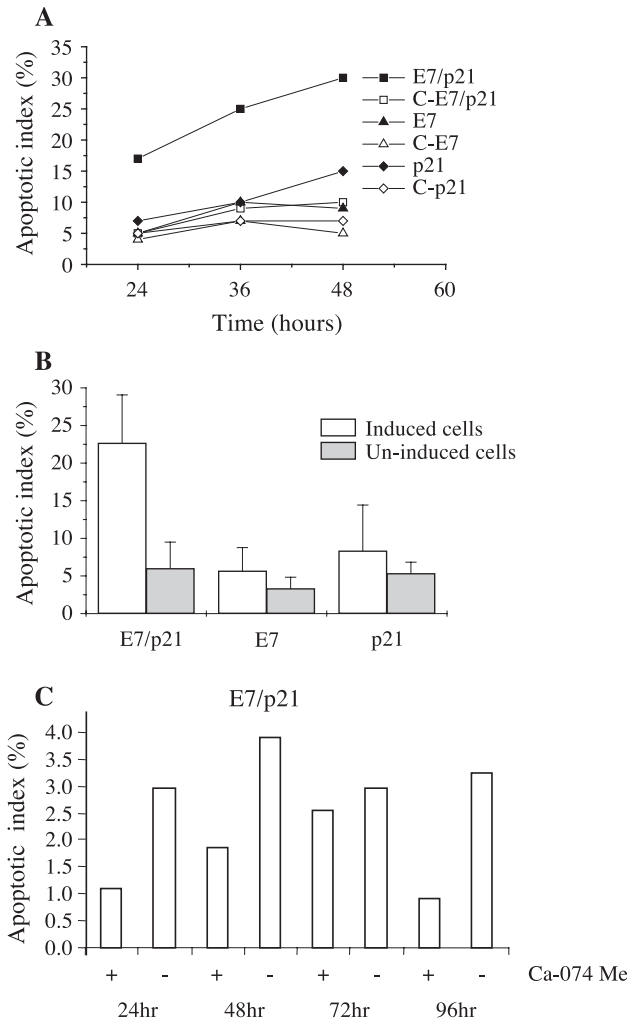

Fig. 5. TUNEL analysis. (A) Kinetics of the induction of apoptosis. The experiment was performed twice with essentially the same results. Noninduced control cells are marked C. (B) Percentage of apoptotic cells following 48 h of induction of protein synthesis. (C) Percentage of apoptotic cells following treatment with the Ca074-Me inhibitor during induction of E7/p21 for up to 96 h. The apoptotic index in (B) and (C) is expressed as a percentage of TUNEL-positive cells. (B) Columns represents mean of three experiments and bars correspond to SD.

E7/p21-induced apoptosis is associated with translocation and activation of cathepsin B

As the release of cathepsin B from lysosomes is essential for its apoptotic capability, we investigated its intracellular localization during E7/p21-induced apoptosis. To determine if cathepsin B is activated during E7/p21-induced apoptosis, cells undergoing apoptosis were examined by both cytochemistry and immunofluorescence (Fig. 6). Cathepsin B exhibits a granular staining equivalent with lysosomal localization in noninduced E7/p21 cells (Figs. 6A, B). Visibly, as shown by immunofluorescence staining, cathepsin B is translocated to the cytoplasm in U2OS cells undergoing E7/p21-induced apoptosis (Fig. 6C). Cathepsin B is synthesized as a catalytic inactive pre-pro-cathepsin B of 39 kDa. Active cathepsin B consists of two alternate forms, one single chain form of 30 kDa and a two chain form consisting of a 5 and 26 kDa fragment (Chan et al., 1986; Erickson, 1989). Western blot analysis of cell extracts shows that E7/p21 expression induces increased levels of cathepsin B in U2OS cells where the endogenous steady state level is rather low. Moreover, a shift from catalytic inactive to active 26 kDa cathepsin B was detected (Fig. 7A). Also, a minor increase of the 30 kDa active form of cathepsin B was detected employing a cathepsin B-specific polyclonal rabbit serum (Fig. 7B). It is recently reported that p21 may regulate the expression of cathepsin B (Chang and Yang, 2000). Thus, to evaluate whether cathepsin B levels in E7/p21 expressing cells is dependent on p21 expression, p21 cells were analyzed for levels of cathepsin B expression. Clearly, p21 expressing cells express constant levels of cathepsin B following induction of p21 and no processing shift was detected either (Fig. 7C). Thus, the rather high level of the 26kD protein relates to the high level of protein loaded on this particular gel. Moreover, no variation of cathepsin B expression in noninduced E7, p21, or E7/p21 cell clones was detected (data not shown).

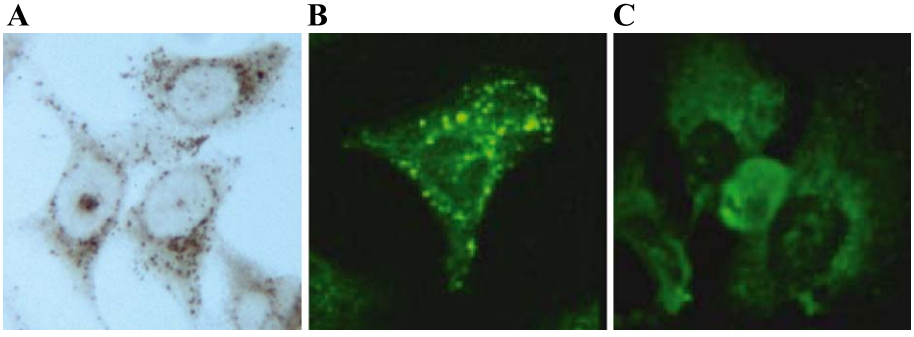

Fig. 6. Microscopy showing intracellular localization of Cathepsin B. (A and B) cathepsin B is localized in lysosomal granular of untreated noninduced E7/p21 cells. In (A), cells were immunostained using antibodies to cathepsin B followed by peroxidase coupled secondary antibodies. In (B), cells were stained using the same primary antibody but followed by FITCH-conjugated secondary antibody. (C) During E7/p21-induced apoptosis, cathepsin B is detected in the cytosol. All immunostainings were made by cathepsin B-specific rabbit serum provided by Dr. E. Weber.

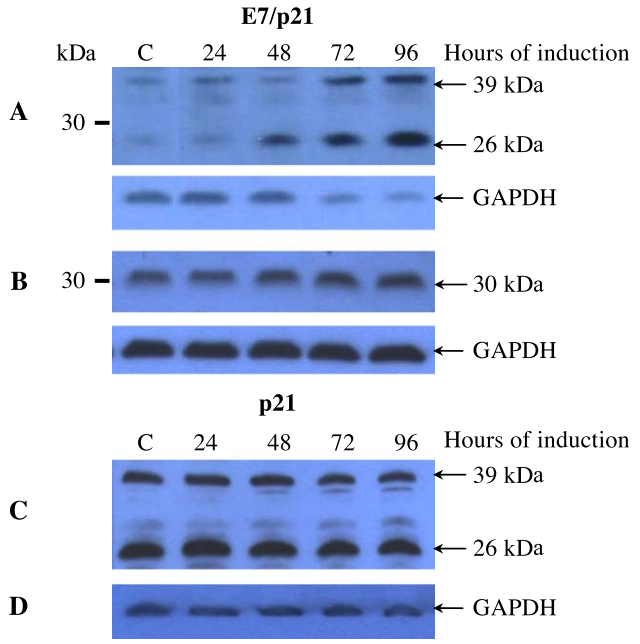

Fig. 7. Western blot showing up-regulation and activation of cathepsin B during E7/p21 expression. (A) E7/p21 expression induces up-regulation of cathepsin B and a shift from in-active 39 kDa to active 26 kDa cathepsin B as detected by using cathepsin B recognizing antibodies (Upstate Biotechnologies Inc.). (B) E7/p21 expression also induces a minor up-regulation of 30 kDa active cathepsin B as detected by using cathepsin B-specific rabbit serum provided by Dr. E. Weber. (C) Induction of individual p21 expression does not affect cathepsin B expression as detected by cathepsin B recognizing antibodies (Upstate Biotechnologies Inc.). (D) GAPDH is used as loading control. The amount of protein loaded per sample was 3 times (40 μg) of that loaded in (A) and (B).

Western blot analysis of extracts from E7/p21 cells treated with the cathepsin B inhibitor Ca-074 Me during induction show delay of cathepsin B activation. Activated cathepsin B protein appeared after 48 and 72 h of treatment compared to activation of cathepsin B already at 24 h in nontreated induced E7/p21 cells (Fig. 8). This corresponds well with the increase in the apoptotic index of the Ca-074 Me treated cells at 48 and 72 h time points (Fig. 5C). Thus, our data show that E7/p21-induced apoptosis is associated with both translocation and increased levels of active cathepsin B in U2OS cells.

E7/p21-induced apoptosis is not associated with caspase-like protease activation

Caspase-3 is considered a pivotal protease in apoptosis, and poly-(ADP-ribose)-polymerase (PARP) is a key target for its activity. Therefore, we investigated both caspase-3 activation and PARP cleavage following E7/p21 induction. Analysis of caspase-3 enzyme activity in E7/p21-induced cells shows no increase in the caspase-3 activity level (Fig. 9A). Camptothecin treated cells served as a positive control showing massive caspase-3 activation. According to Western blot analysis of procaspase-3 and PARP in cell lysates from U2OS cells undergoing E7/p21-induced apoptosis, no signs of caspase-3 like activity was detected following up to 96 h of protein induction (Fig. 9C). To investigate the ability of U2OS cells to induce caspase-3 activation in response to other apoptotic stimuli, noninduced E7/p21 cells were treated for 24 h with various concentrations of etoposide, camptothecin, and actinomycin D. Etoposide treatment induces both PARP cleavage and decreasing procaspase-3 levels as measured in Western blot analysis of cell lysates indicating its processing (Fig. 9B). Similar results were obtained following camptothecin and actinomycin D treatment (data not shown). Western blot analysis of caspases being activated through mitochondrial-, or stress-induced pathways, namely caspase-1, 7, and 8, in E7/p21-induced cells, shows no activation of these caspases (Figs. 9C, D). Unfortunately, caspase-9 was not detectable in U2OS cells. As cas-

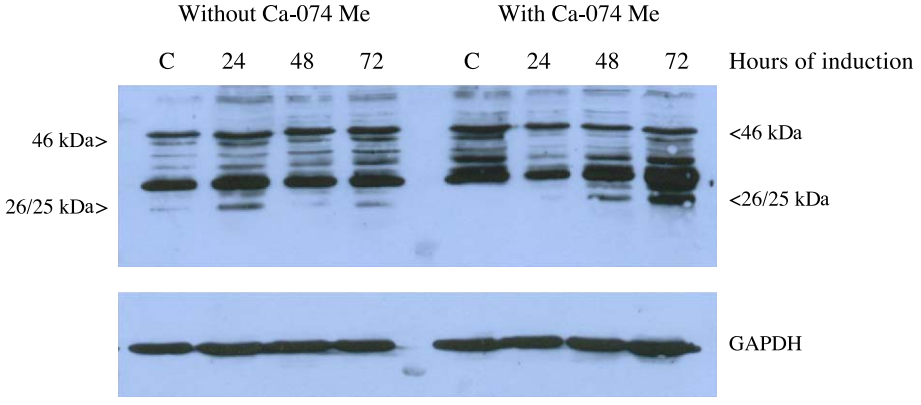

Fig. 8. Western blot analysis of cathepsin B activation during 72 h induction of the E7/p21 proteins. The extracts were prepared also from cells induced in the presence of 5 μM of the cathepsin B-specific inhibitor Ca-074 Me. Extracts from noninduced cells are marked C. GAPDH was used as loading control.

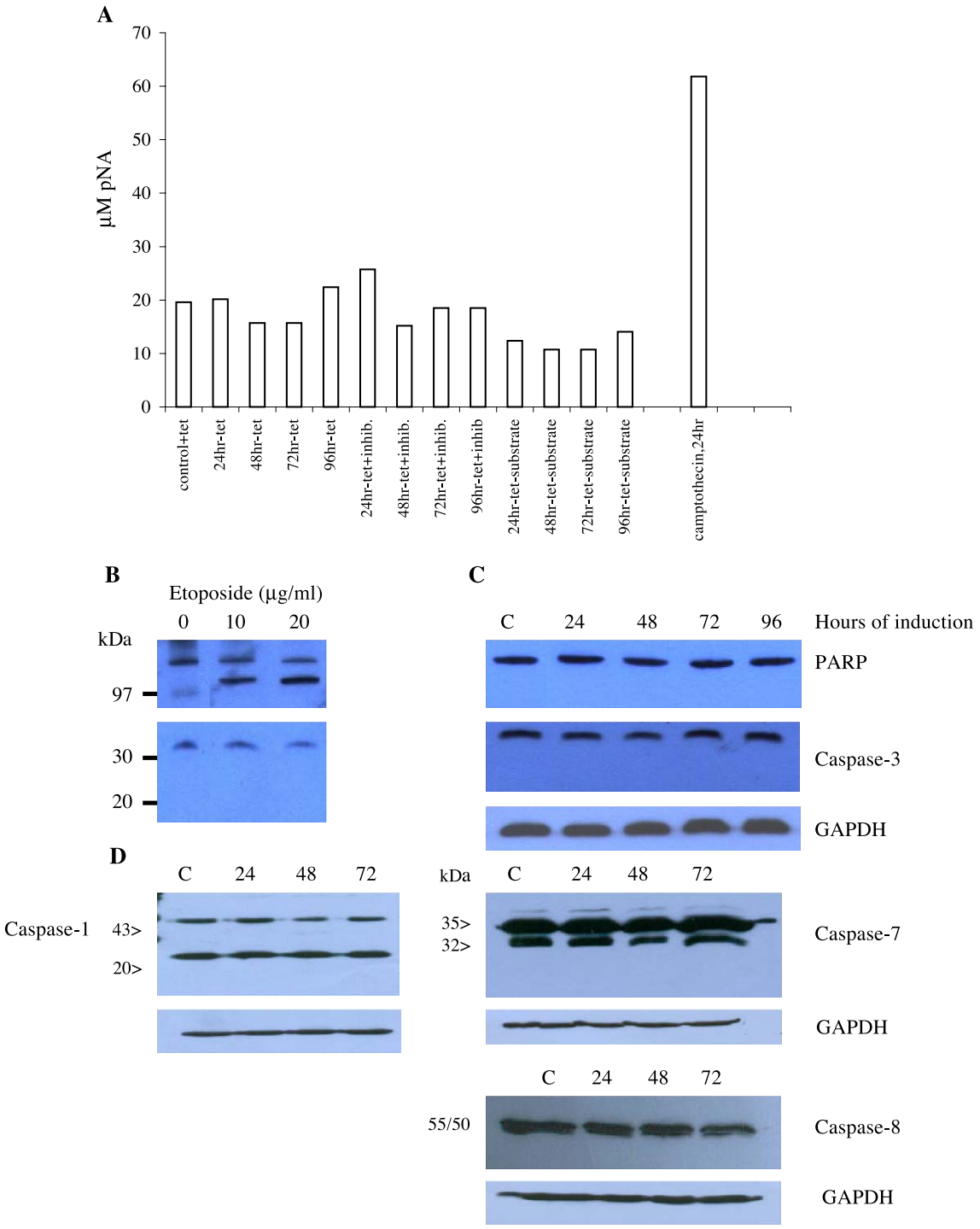

Fig. 9. (A) Caspase-3 enzyme activity measured in E7/p21 noninduced and induced cells. The activity was measured at 24, 48, 72, and 96 h after induction. The caspase-3 inducer camptothecin (10 µM) was used for treatment of control cells for 24 h. The activity was measured in duplicates and the data are presented as the mean value. (B) Western blot showing levels of PARP and pro-caspase processing in E7/p21 cells. Noninduced E7/p21 cells treated with indicated concentrations of etoposide (Sigma) exhibit increased PARP and pro-caspase-3 processing as compared to untreated controls. (C, D) E7/p21 cells undergoing apoptosis show no PARP, pro-caspase-3, pro-caspase-7, pro-caspase-8, or pro-caspase-1 processing. For detection of PARP, caspase-3, caspase-7, caspase-8, and caspase-1 antibodies specific for these proteins were used. GAPDH is used as loading control.

pase-1, 3, 7, or 8 are not activated during E7/p21-induced apoptosis, our data indicate that this particular signalling pathway is mediated by cathepsin B and caspase independent.

Discussion

The data presented above show that simultaneous HPV-16 E7 and p21 expression induces cell death.

Moreover, we are the first to demonstrate that this HPV-related apoptosis is associated with activation of cathepsin B. The initiating apoptotic signal in E7/p21-induced cell death must come from a lethal combination of E7 and p21 expression, as our investigations show that none of these proteins induce apoptosis when expressed individually. The E7 protein has in some studies shown to sensitize cells to apoptosis after treatment with various kinds of chemicals or irradiation (Basile et al., 2001; Finzer et al., 2001; Hickman et al., 1997; Liu et al., 2000; Stoppler et al., 1998).

Here we show that the E7/p21 protein expression by itself induces cell death. In accordance with other models of cell demise (Leist and Jaattela, 2001), we show that cathepsin B is released from the lysosomes to the cytosol during apoptosis. Moreover, as judged from lack of PARP processing as well as no activation of caspase-3 or other caspases in E7/p21-induced apoptosis, this signalling pathway is not associated with caspase activity. We suggest that induction of caspase independent cell demise in our cell model system is E7/p21 specific, as cell death induced by compounds such as etoposide, camptothecin, and actinomycin D is associated with the activation of at least the caspase-3 like proteases. Thus, U2OS cells carry functional caspases, but apparently they remain inactive during E7/p21-induced apoptosis. The criteria and pathway for activating cathepsin B, rather than caspases, in E7/p21-induced apoptosis remain speculative. However, it is tempting to hypothesize that the caspases in some way might be inhibited by E7/p21 expression. One such inhibitory function has been reported for p21, as it by N-terminal binding to pro-caspase-3 in Fas-treated human hepatocytes, hinders caspase-3 maturation, and consequently apoptosis (Suzuki et al., 1998, 1999, 2000). However, such possible caspase inhibitory role of p21 is not the only function of p21 in E7/p21-induced apoptosis, as individual expression of E7 does not induce apoptosis in our model system. Thus, also an apoptosis promoting activity of p21 at least in co-operation with E7 must exist. Adenovirus E1A, which shares many biological functions with HPV-16 E7, is reported to up-regulate caspase-7 and caspase-8 mediated apoptosis through deregulation of E2F (Nahle et al., 2002). HPV-16 E7 does not show the same caspase activation when induced alone or together with p21.

Interestingly, cathepsin B has lately been speculated to function as a backup program for cell death under pathological conditions, where the caspase cascade is suspended (Foghsgaard et al., 2001). Such suspension in, for example, tumor cells may be a result of caspase mutations (Liang et al., 2001; Soengas et al., 2001), overexpression of survival proteins, for example, Bcl-2 (Tsujimoto and Croce, 1984), or, as it may possibly be in the case of our study, elevated p21 expression. Interestingly, cathepsin B is frequently up-regulated in cancerous cells, probably to manage increased protein turnover and invasiveness (MacKenzie et al., 2001). In that sense, it would be an excellent default pathway to a nonfunctional caspase cascade in cancer cells. This theory is supported by the fact that cathepsin B-mediated apoptosis plays a more dominant role in cancerous than in primary cells (Foghsgaard et al., 2001). The mechanism by which cathepsin B function in apoptosis is not known. However, a regulated transfer from the lysosomes to the cytosol and nucleus seems to constitute a key role (Foghsgaard et al., 2001; Roberts et al., 1999). Moreover, cathepsin B-mediated cleavage of Bid, which following its processing promotes caspase-dependent apoptosis by induction of cytochrome *c* release, most likely constitute an amplification step in apoptotic signalling (Mathiasen et al., 2001; Stoka et al., 2001). However, our data, together with data published by others, suggest that cathepsin B can possibly mediate apoptosis even without activation of caspases at all (Adjei et al., 1996; Foghsgaard et al., 2001; Roberts et al., 1999). Therefore, other downstream targets of cathepsin B mediating caspase-independent apoptosis must exist and await further discovery.

Materials and methods

Cell culture

The U2OS-Tet-Off cell line, which is human osteosarcoma cells provided with a tetracycline-dependent expressions vector system (Clontech), were cultured in complete medium (CM) consisting of Dulbecco's modified Eagle's 1885 medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin at 37 °C in humidified air atmosphere with 5% CO_2. Medium used for selection and culture of transfected cells was further supplemented with appropriate antibiotics: 400 μg/ml G418 (Invitrogen), 1 μg/ml puromycin (Sigma), and 75 μg/ml hygromycin B (RocheA/S). Cell lines were regularly tested negative for infection with mycoplasma.

Construction of expression vectors

To create the E7 expression vector (pUHD10-3-E7), an HPV-16 fragment from base pair position 505–875 of the HPV genome containing the E7 open reading frame (base pair 562–858) was inserted into the *Bam*HI/*Eco*RI site in the tetracycline responsive expression vector pUHD 10-3. The expression vector carrying HA-tagged p21 (pUHD10-3-p21HA) was provided by Dr. J. Lukas and Dr. J. Bartek, The Danish Cancer Society, Division for Cancer Biology.

Establishment of U2OS cells with inducible HPV-16 E7 and p21 expression

For the establishment of U2OS cells with inducible expression of either E7 or p21, expression vectors pUHD10-3-E7 and pUHD10-3-p21, respectively, were introduced into U2OS-Tet-Off cells along with pBabepuro (Morgenstern and Land, 1990) conferring pyromycin resis-

tance. To establish U2OS cells inducible of simultaneous E7 and p21 expression, one clone successfully transfected with pUHD10-3-p21 was selected and further provided with pUHD10-3-E7 and pBabehygro conferring hygromycin resistance. All transfections were performed using cells in logarithmic growth phase and LipofectAmine Plus (Invitrogen) according to the manufacturer's instructions. To avoid constitutive expression of the transgenes, the culture media was supplemented with 2 μg/ml tetracycline (Sigma). Confluent drug-resistant single-cell clones were collected and propagated. Where specified in the text cells were grown with 5 μM cathepsin B inhibitor Ca-074 Me (#205531, Calbiochem) added to the medium.

Western blot analysis

For detection of E7 and p21 expression, cells were scraped off the culture dish after incubation in RIPA buffer (1% NP-40, 250 mM NaCl, 50 mM Tris–HCl, 10 mM DTT, 20 mM NaF, 0.5 mM Na_3VO_4) at 4 °C for 10 min. The cell suspension was sonicated and clarified by centrifugation at 15.000 × *g* for 10 min. Samples of 20–40 μg of total protein, as determined by the Coomassie Plus Protein Assay (Pierce), were loaded onto and separated by SDS-PAGE (Biorad) and transferred to PVDF-membranes (Biorad). Membranes were incubated overnight in PBS containing 1% nonfat dry milk (Becton Dickinson) and 0.05% Tween-20 (Sigma). The membranes were probed with antibodies against HPV-16 E7 (monoclonal antibody #ED17, Santa Cruz Biotechnology) and p21 (DCS-61) (Thullberg et al., 2000) followed by two-step secondary antibody detection (DAKO) and ECL (Amersham). For detection of cathepsin B, PARP (poly-(ADP-ribose)-polymerase), and caspases, 2×10^6 cells were seeded in 85-mm dishes and grown for up to 96 h. To collect apoptotic cells, the culture medium was centrifuged at 1500 rpm on ice. Following cell scraping, the adherent cells were collected. Cells were washed in 2 × 5 ml cold PBS. Cells were lysed in 300 μl cold RIPA buffer containing protease inhibitors (complete tablets w/o EDTA, Roche). Samples were sonicated 5 s on ice and centrifuged at 15.000 × *g* for 10 min at 4 °C. Protein levels of the samples were determined by the Coomassie Plus Protein Assay (Pierce). Proteins were separated by SDS-PAGE (Biorad) and transferred to Hybond nitrocellulose membranes (Amersham). Membranes were blocked for 30 min in PBS containing 5% nonfat dry milk. Membranes were probed overnight with antibodies to cathepsin B (# 06–480, Upstate Biotechnology Inc., NY, USA, or cathepsin B-specific rabbit polyclonal antibody kindly provided by Dr. E. Weber, Martin Luther Universität, Halle-Wittenberg, Germany), PARP (#33-3100, Zymed Laboratories Inc., S.F., CA, USA), caspase-1 (#AB1871, Chemicon Int.), caspase-3 (#556425, BD Biosciences), caspase-7 (#551236, BD Biosciences), caspase-8 (#551242, BD Biosciences), caspase-9 (#551246, BD Biosciences), or GAPDH (Biogenesis) followed by two-step secondary antibody detection (DAKO) and ECL (Amersham).

Co-immunoprecipitation analysis of E7 and pRB

Cells were collected and incubated in lysis buffer as described above, but supplemented with 0.1% (v/v) Triton X 100 (Sigma) instead of NP40. The lysates were incubated 10 min on ice and centrifuged at 20.000 × *g* for 10 min. Total protein samples (500 μg) were added 5 μl of E7-specific polyclonal rabbit immunoglobulins, provided by Dr. D. Galloway, University of Washington, Seattle. After 1 h of incubation at 4 °C, 5 μl of swine anti-rabbit IgG (No Z0196, DAKO) was added, and incubation was continued for 30 min. Protein-A Sepharose was added (Amersham-Pharmacia) and the sample was incubated over night at 4 °C. Immunoprecipitates were pelleted and washed twice in PBS and resuspended in 60 μl of loading buffer (5% glycerol, 2% SDS, 0.15 g DTT, 0.12 M TRIS–HCl, pH 6.8, 0.002% bromophenol blue). The co-precipitation was visualized by Western blot analysis after probing of the membrane with antibodies to pRB (G3-245, PharMingen, BD Biosciences) used in the dilution recommended by the manufacturer.

Immunofluorescence analysis

For detection of E7 and p21, E7/p21 cells were seeded in 8-well chambers (Nunc) in medium without tetracycline, which induced protein synthesis. Cells were induced for 48 h and fixed in 4% paraformaldehyde (Fluka Chemie) for 10 min at room temperature. Cells were permeabilized in PBS containing 0.2% NP40 (Sigma) and further incubated for 1 h at room temperature with a mixture of polyclonal rabbit IgG to E7 and mouse monoclonal antibodies to the HA-tag (#F-7, Santa Cruz) diluted in PBS with 1% nonfat dry milk and 0.2% NP40. Finally, the cells were incubated for 20 min with a mixture of FITC-conjugated swine-anti-rabbit IgG (#F205, DAKO) and Cy-3 conjugated sheep-anti-mouse IgG (#C2181, Sigma) diluted in PBS nonfat dry milk and NP40 as above. All antibodies were used in dilutions recommended by the manufacturer. Noninduced cells served as controls. For detection of cathepsin B, cells were seeded on 8-well plastic slides (Nunc) and fixed in ice-cold methanol for 10 min. To permeabilize the cells and block unspecific immunoreactivity, diluting buffer (1% BSA, 0.3% Triton X 100 in PBS) containing 5% swine serum (DAKO) was added for 30 min. Primary antibodies (cathepsin B-specific rabbit serum provided by Dr. E. Weber) or unspecific rabbit serum (DAKO) diluted 1:500 in diluting buffer were added to the cells followed by overnight incubation at 4 °C. The cells were washed 3 × 10 min in washing buffer (PBS containing 0.25% BSA and 0.1% Triton X 100). Secondary antibodies (biotinylated swine anti-rabbit immunoglobulins (DAKO)) diluted 1:500 in diluting buffer were applied for 1 h at room temperature. Cells were washed for 3 × 10 min in washing buffer. Finally, steptavidine Oregon Green 488 (Molecular probes) diluted 1:200 in diluting buffer was applied for 1 h at room

temperature. Cells were washed for 3 × 10 min in washing buffer. In both experiments, the slides were coverslipped with fluoromount (DAKO). Images were recorded with a Nikon Diaphot 200 confocal microscope.

TUNEL assay for apoptosis

Apoptotic cells were identified by the TUNEL method using the ApoAlert DNA Fragmentation Assay Kit (Clontech). Cells were seeded to 70% confluence on coverslips (Nunc). Seeding cells in medium without tetracycline lead to induction of protein expression from the transgenes whereas cells seeded in medium containing tetracycline served as controls. The cathepsin B inhibitor Ca-074 Me (#205531, Calbiochem) was used to verify the specificity of the apoptotic signal. Cells were fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.2% of Triton X 100 (Sigma). Slides were coverslipped with anti-fade mounting media (DAKO). Apoptotic cells were visualized with a Leitz orthomate microscope using a standard fluorescein filter (520 ± 20 nm) (Leitz Wetzlar). The fraction of TUNEL-positive cells was determined out of 150 randomly selected cells. When the effect of cathepsin B inhibitor Ca-074 Me was analyzed, the fraction of TUNEL positive cells was determined out of 200 randomly selected cells.

Detection of apoptosis by flow cytometry

Detection of apoptotic cells was performed at 24, 48, 72, and 96 h after induction of gene expression. Cell cultures were trypsinized and free cells were labeled with annexin V-FITC and propidium iodide (PI) by the use of "Apoptosis Detection Kit" (R&D Systems) according to the manufacturers instructions. Annexin V binds to phosphotidylserine present on the outside of the plasma membrane of apoptotic cells, while PI gain entrance to late apoptotic and necrotic cells. Percentages of apoptotic cells were calculated by registration of annexin V labeled cells. Analysis was performed by the use of a FACS Calibur apparatus and Cell Quest software (Becton Dickinson, BD Biosciences), and 10^4 events were collected for analysis. Debris was excluded from the analysis by electronic gating.

BrdU incorporation assay

The BrdU-ELISA (bromodeoxyuridine) assay (kit #RPN250, Amersham) was performed by plating 5 × 10^3 cells per well in 96-well plates (Nunc). After indicated time points at normal culture conditions, BrdU was added to a final concentration of 10 μM. After another 24 h of incubation, the amount of incorporated BrdU was detected according to the procedure recommended by the manufacturer. The DNA replication was determined as BrdU incorporation per milligram of total protein in all experiments.

Protein determination for growth curves

Cells were grown on 85-mm dishes for indicated time points at normal cell culture conditions. Following the harvest of the cells, they were lysed in RIPA buffer. Protein determination of the samples was done by the Coomassie Plus Protein Assay (Pierce) according to instructions from the manufacturer.

MTT viability assay

The MTT ((3-(4,5-dimethylthizolyl-2)-2,5-diphenyltetrazolium bromide), Sigma) assay was set up by plating 10^4 newly induced cells per well in 96-well plates (Nunc). Cells were grown in 100 μl medium without phenol red (Life Technologies). At each time-point, 25 μl MTT (0.5% MTT in PBS) was added to each well and the cells were incubated for 4 h at 37 °C. After incubation, plates were stored at −80 °C. Before measuring, 100 μl solubilization buffer (20% SDS, 50% *N*,*N*-dimethylformamide) was added to each well and samples were incubated over night at room temperature in darkness. Final measuring was performed by reading samples at 570 nm for 0.1 s per well in a Wallac Victor multi label counter.

Caspase assay

Caspase-3 activity was measured using the ApoAlert^R Caspase-3 kit (Clontech K2027) according to the protocol given by the supplier. In brief, cells were harvested at different time after E7/p21 or p21 induction. Control and induced cells (2×10^6) were solubilized and used in each assay. The supernatant was incubated with the DEVD-pNA (*p*-nitroanilide) substrate, applied to microtiter plates, and the color development was measured at 405 nm in a spectrophotometer after 1 h of incubation at 37 °C. The topoisomerase inhibitors etoposide (Sigma, 20 μg/ml) and camptothecin (Sigma, 10 μM) were used as positive controls. The caspase-3 inhibitor DEVD-fmk was used for control of specificity of the reaction.

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