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Adenoviral-mediated transfer of p53 or retinoblastoma protein blocks cell proliferation and induces apoptosis in culture-activated hepatic stellate cells

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***Background/Aims*: The principal cellular effectors of fibrosis in liver are hepatic stellate cells (HSC). In response to liver injury these quiescent cells undergo a phenotypic change to a myofibroblastic cell type, proliferate and secrete matrix components. Thus, removal of activated HSC should be beneficial for the prognosis of hepatic fibrogenesis and preserve organ function.**

***Methods*: The purpose of this study was to investigate whether administration of adenoviruses constitutively expressing the p53 tumor suppressor or the retinoblastoma protein (pRb) is sufficient to induce cell arrest or apoptosis in culture-activated HSC. The expression of the transgenes was confirmed by Western blot analysis and immunohistochemistry.**

***Results*: Both proteins were expressed mainly in the nucleus and their expression was associated with a marked inhibition of cell proliferation and induction of apoptosis as determined by measurement of phosphatidylserine exposed at the surface, proliferation assay, induction of the p21 cyclin-dependent kinase inhibitor, and an increase of caspase-3 activity. Additionally, electron microscopic analysis confirmed that activation of the p53-mediated pathway in HSC results in chromatin and cytoplasmic condensation, typical features of ongoing apoptosis.**

***Conclusions*: Our results indicate that transduction of p53 or pRb offers a feasible approach to induce apoptosis in activated HSC. Thus, targeted transfer of these proteins into HSC may be potentially useful for the treatment of hepatic fibrosis.**

***Keywords*: Hepatic stellate cell; Myofibroblast; Transdifferentiation; Liver fibrosis; Apoptosis; Induced cell death; Adenoviruses; Gene therapy**

1. Introduction

Apoptosis, or programmed cell death, is a natural process to remove unneeded, senescent, or damaged cells. The morphologic and biochemical alterations during apoptosis have been the subject of much investigation [1,2]. Hepatic stellate cells (HSC) are abundant during fibrogenesis and resolution of liver fibrosis is associated with apoptosis [3]. The development of methods for the isolation and culture of HSC has enabled in vitro studies of these cells and interestingly, culturing quiescent HSC on uncoated plastic surfaces causes activation similar to that seen in liver fibrogenesis [4,5]. This observation has greatly facilitated the study of HSC activation and mechanisms counteracting the execution of the fibrogenic program, e.g. the transdifferentiation into contractile myofibroblasts (MFB). The phenotypic transition includes stimulation of proliferation, loss of cellular retinoid stores, appearance of α-smooth muscle actin, increased synthesis of extracellular matrix proteins, and acquisition of contractility. Given these apparent relevant disease-associated changes, induction of apoptosis in MFB potentially should be beneficial for recovery from established liver fibrosis. Cells undergoing apoptosis exhibit a series of morphological and biochemical changes that allow them to be distinguished from viable cells and from cells dying by

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other cell death mechanisms [6]. These include membrane blebbing, chromatin condensation, DNA fragmentation, exposure of phosphatidylserine to the cell surface, and activation of specific cell death proteases (caspases). In the execution phase of apoptosis, regulators of cell cycle progression as well as growth suppressor genes play important roles [7]. The retinoblastoma protein (pRb) controls entry into the S phase by binding to the E2F transcription factor [8]. In the presence of proliferative signals, pRb is phosphorylated at multiple sites by cyclins and cyclin-dependent kinases (cdks) to generate hyperphosphorylated Rb, which is unable to repress E2F-dependent transcription. In contrast, kinase activity is in turn negatively regulated by a number of cdk-inhibitors that act to prevent phosphorylation of Rb thereby maintaining cells in the G1 phase. p21, one member of the cdk-inhibitor family, is induced by p53 in response to DNA damage [9]. The function of pRb is therefore linked to the activity of p53, that induces several responses in cells, including differentiation, senescence, DNA repair and the ability to regulate cell cycle-checkpoint activity as well as apoptotic responses [10–13]. In glioma cells the experimental transfer of p53 first induces expression of p21 and growth arrest and subsequently induces apoptosis [14,15]. Despite the importance of transcriptional regulation, however, transcriptionally independent activities of p53 also mediate at least some of its downstream effects during apoptosis. The mechanisms underlying these functions remain poorly understood, but include the relocalization of death receptors to the cell surface, a direct role for p53 in mitochondria and an ability to regulate translation by binding to the 5′-untranslated region of certain mRNAs [16–18].

In the last decade remarkable progress has been made in defining how p53 and pRb functions are regulated, determining the pathways that can be activated by these proteins and identifying the downstream mediators of the growth inhibitory and apoptotic cellular response. Ultimately, these insights have initialized the development of novel therapeutic strategies based on activation of the p53 or pRb pathways for the treatment of diverse proliferative disorders in liver [19,20].

We tested whether adenovirus-mediated transfer of p53 or pRb can induce cell death in culture-activated HSC. We demonstrate that both proteins are efficient means of enabling the apoptosis pathway to be initiated and executed in these cells. Thus, local expression of these transgenes in activated HSC may also be appreciable to the treatment of hepatic fibrosis.

2. Materials and methods

2.1. Cell culture

HSC were isolated by liver-perfusion with pronase/collagenase and purified by a single-step density gradient centrifugation as described elsewhere [21–24]. Cells were seeded in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

2.2. SDS-PAGE and immunoblotting

Protein samples were resolved by SDS-PAGE and electroblotted onto Protran membranes. pRb and p53 were detected with monoclonal antibodies directed against human pRb (clone G3-245; BD PharMingen) or p53 (clone PAb421; Oncogene Research Products). For detection of the senescent cell-derived inhibitor-1 a mouse anti-p21 antibody (BD PharMingen) was used. The primary antibodies were visualized using horseradish peroxidase-conjugated anti-mouse IgG and the supersignal chemiluminescent substrate (Pierce).

2.3. Construction of recombinant adenoviruses

\vskip12pt\vskip12ptThe construction of Ad5-CMV-EGFP expressing the enhanced green fluorescent protein was described previously [25]. For construction of the adenoviral shuttle vector pΔE1sp1A-CMV-p53 the filled-in 1471 bp *Eco*RI fragment of clone PbR6 [26] was inserted into pΔE1sp1A-CMV-EGFP, which was filled-in after digestion with *Nhe*I and *Eco*RI. For construction of pΔE1sp1A-CMV-pRb the blunt-ended 2.9 kbp *Eco*RI/*Bsu*36I fragment from the pUC12 derivative pmRb115 [27] harboring murine Rb cDNA was first cloned into pEGFP-C1 which was depleted for EGFP by digestion with *Nhe*I and *Hin*dIII. Thereafter the 3.8 kbp *Ppu*10I/*Mlu*I fragment was released and cloned into pΔE1sp1A [28]. Integration of transgenes into vector pJM17 [29] was performed by in vitro homologous recombination.

2.4. Transfection and luciferase assays

The cirrhotic fat-storing cell line CFSC [30] or adherent 293 cells were transfected with indicated constructs and an internal control (pRL-TK), using the Fugene6™ method (Roche). Firefly luciferase was quantified 48 h later using the Dual Luciferase Assay (Promega) in a Liquid Scintillation & Luminescence counter. Values were normalized to Renilla luciferase activities expressed from pRL-TK. The vectors pp53-TA-Luc, pRb-TA-Luc, and pRL-TK were obtained from CLONTECH.

2.5. Proliferation assay

HSC were seeded in DMEM (10% FCS) and 1 day later serum was reduced to 5% heat-inactivated FCS and cells were infected with indicated adenovirus. Twenty-four hours later, medium was renewed (10% FCS) and cells were exposed to 75 kBq/ml [6-^{3}H]thymidine for another 24 h labeling period. Incorporated radioactivity was measured as described [31].

2.6. Annexin-V-FLUOS assay

For determination of phosphatidylserine we used the FITC-conjugated Annexin-V method (Roche). Briefly, HSC were seeded on glass slides in DMEM (10% FCS) at a density of 2×10^5 cells per six-well chamber. One day later cells were infected at a multiplicity of infection (MOI) of 1000 with indicated viruses in 1 ml DMEM containing 5% heat inactivated FCS. Twenty-four hours later DMEM was renewed (2 ml, 10% FCS) and cells were cultured for another day. For Annexin-V assay 1 ml of the medium was discarded and 20 μl of the Annexin-V-FLUOS solution was added. Subsequently, cells were washed in binding buffer and staining was visualized with an Axiovert-135M fluorescence microscope (Zeiss).

2.7. Cell death detection

HSC were seeded in 12-well chambers at a density of 9×10^4 cells per well and the next day the cells were infected with indicated adenovirus in DMEM containing 5% heat inactivated FCS. The medium was changed (10% FCS) 1 day later and cell death was monitored with the Cell death detection ELISA (Roche) 24 h later. Therefore, cells were harvested in lysis

buffer and intact nuclei were pelleted by centrifugation. Aliquots of the supernatant were transferred to streptavidin-coated wells of a microtiter plate and nucleosomes were bound to monoclonal antibodies against histone (biotin-labeled) and DNA (peroxidase-conjugated), respectively. Antibody-nucleosome complexes were washed three times (PBS) and samples were incubated with the peroxidase substrate 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The amount of immobilized antibody-histone was determined spectrophotometrically at 405 nm and normalized to the content of DNA measured in parallel dishes using Sybr-Green I dye.

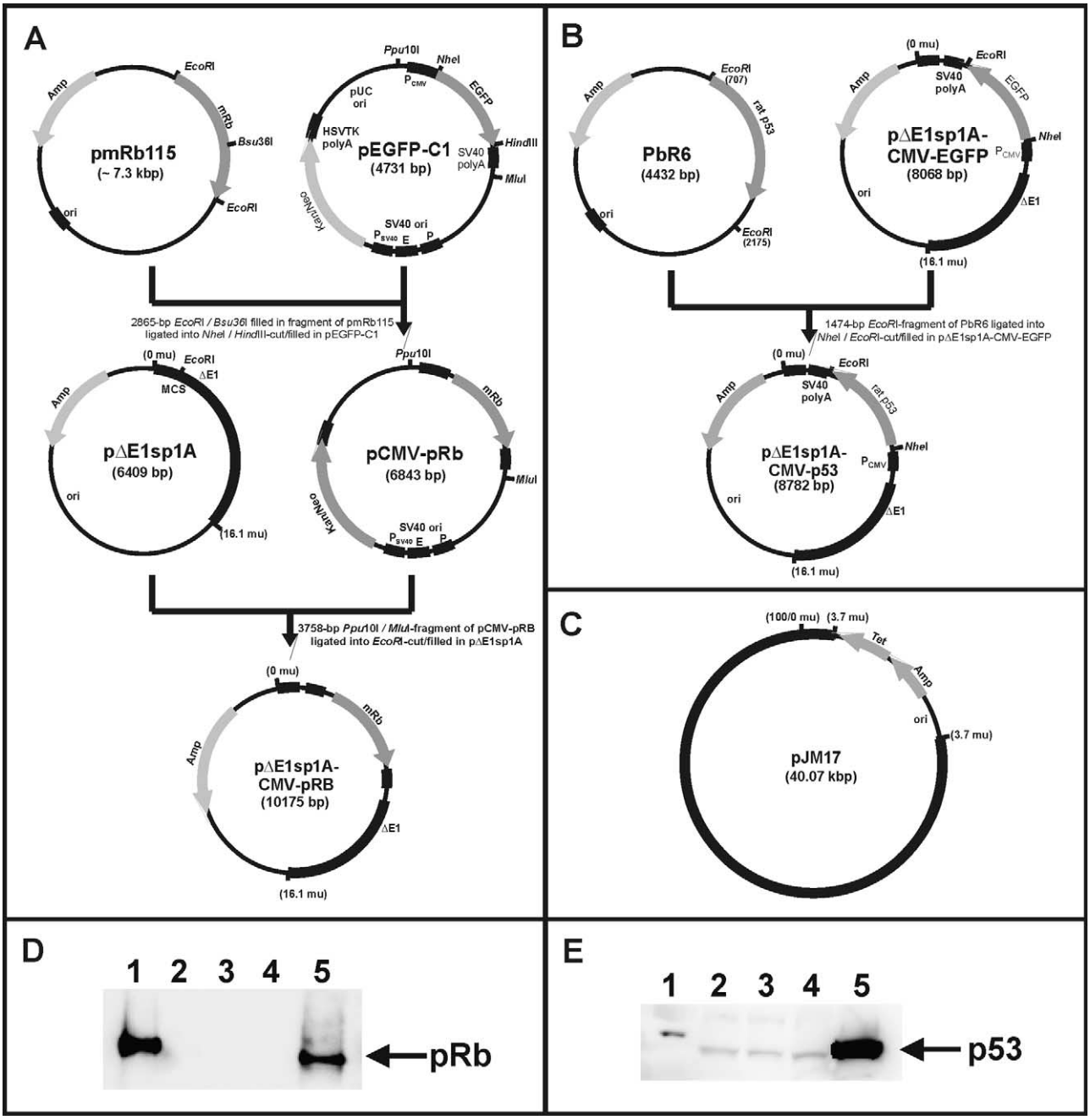

Fig. 1. Adenoviral expression of pRb and p53. (A) Construction of Ad5-CMV-pRb. The clone pCMV-pRb was constructed by fusing the *Eco*RI/*Bsu*36I fragment of pmRb115 into pEGFP-C1 depleted for EGFP. The fragment harboring the CMV promoter, murine Rb, and the SV40-polyadenylation signal was then transferred into adenoviral shuttle vector pΔE1sp1A. (B) Construction of Ad5-CMV-p53. The *Eco*RI fragment of PbR6 was blunt-ended and cloned into EGFP-depleted pΔE1sp1A-CMV-EGFP. (C) pJM17. Integration of transgenes into adenovirus was performed by in vitro homologous recombination into pJM17. In the schematic diagrams (A–C) sequences from Ad5 are shown as thick black lines, bacterial vector sequences as thinner black lines, sequences encoding Rb, p53 or EGFP as thick dark gray lines, and selection markers for ampicillin (Amp), kanamycin (Kan) and tetracycline (Tet) as thick gray lines, respectively. (D) Immunoblot analysis of pRb. HSC infected with wild-type Ad5 (2), mock-infected HSC (3), untreated HSC (4), or Ad5-CMV-pRb-infected HSC (5) were tested for pRb expression. As a positive control lysates from human leukemia cell line MOLT-4 expressing high levels of the 110 kDa pRb with a moderately different M*r* were loaded (1). (E) Immunoblot analysis of p53. Extracts of HSC infected with wild-type Ad5 (2), mock-infected HSC (3), untreated HSC (4), or Ad5-CMV-p53 infected HSC (5) were tested for p53 expression. A human p53 standard with a moderate different apparent M*r* served as control (1).

2.8. Caspase-3 assay

The activity of caspase-3 was analyzed with the ApoAlert caspase-3 fluorescent assay kit (CLONTECH). It measures the shift in fluorescence emission of 7-amino-4-trifluoromethyl coumarin (AFC) after cleavage of the amino acid substrate Asp-Glu-Val-Asp (DEVD)-AFC. AFC emits a yellow-green fluorescence and the level of enzymatic activity in the lysates is proportional to the color reaction, which was quantified at 508 nm.

2.9. Subcellular localization

Cells were seeded on glass coverslips for 24 h in DMEM (10% FCS). One day later, serum content was reduced to 5% FCS and cells were infected (MOI 160) with indicated adenovirus for 24 h. Thereafter, DMEM was changed and 48 h later the cells were washed three times in PBS, fixed for 15 min in 4% paraformaldehyde buffered in PBS (pH 7.4), and permeabilized for 2 min on ice in a solution containing 0.1% sodium citrate and 0.1% Triton X-100. Then the cells were washed three times in PBS, blocked for 30 min at 37 °C in 50% FCS, 0.1% BSA in PBS and then in 0.5% BSA/0.1% fish gelatine in PBS, and washed three times for 5 min at room temperature. Incubations with appropriate antibody solutions were performed at 37 °C for 1 h in 1% BSA following three washes in PBS. Primary antibodies for p53 and pRb were the same as used for immunoblotting and detection was performed using a purified biotin-conjugated anti-mouse antibody and a streptavidin-FITC conjugate.

2.10. Electron microscopic studies

HSC (3×10^6) were infected (MOI 33) in six-well plates 1 day after plating with indicated adenovirus in DMEM (5% FCS). One day later the medium was renewed (10% FCS) and cells were fixed in 3% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) for 15 min after incubation for 24–48 h. Scraped cells were centrifuged for 10 min at low speed and the cell pellet was mixed with 3% agarose at 50 °C by addition of 3% glutaraldehyde. Blocks of embedded cells were washed with phosphate buffer and osmification was performed with 1% osmium-tetraoxide in phosphate buffer for 1 h. Cells were then dehydrated with standard alcohol series, infiltrated in a mixture of epoxy resin/propylene oxide (1:1) for 30 min and flat embedded in pure epoxy resin for 60 min in dried, labeled silicon moulds. The epoxy was allowed to polymerize (8 h at 37 °C, 56 h at 60 °C). Sections (1 μm) were counterstained with Toluidine Blue and ultrathin sections (80–100 nm) were prepared with a diamond knife and mounted on copper grids. The grids were stained with uranyl acetate and lead citrate, air-dried and examined with a Philips transmission electron microscope TEM 400 at 60 kV.

3. Results

3.1. Adenoviral delivery of p53 or pRb

Recombinant adenoviruses are the most widely used vectors to deliver genes to HSC with high transduction efficiencies [25]. This straightforward gene transfer method was chosen to design the adenoviral shuttle vectors pΔE1sp1A-CMV-pRb (Fig. 1A) and pΔE1sp1A-CMV-p53 (Fig. 1B) expressing murine pRb or rat p53 under transcriptional control of the cytomegalovirus (CMV) promoter. The integration of the transgenes into the genome of Ad5 was performed through homologous recombination in 293 cells between common regions of the shuttle vectors and the adenoviral backbone vector pJM17 (Fig. 1C). After two rounds of amplification and purification recombinant Ad5 particles were tested in respect to expression of the foreign gene cassettes. Both viruses, Ad5-CMV-pRb and Ad5-CMV-p53, were able to direct substantial expression of the respective protein products of 110 kDa (pRb) and 53 kDa (p53) in size as indicated by Western blot analysis (Fig. 1D,E). In these experiments total lysates from human leukemia cell line MOLT-4 expressing high levels of the 110 kDa pRb and a commercially available human recombinant p53 standard with moderate higher molecular weights served as controls. In order to follow the cellular localization of expressed proteins we performed immunocytochemistry. As expected, staining of both transgenes occurs predominantly within the nuclei of HSC (Fig. 2). Both transgenes induced nuclear blebbing and shrinking, drastic cellular disintegration and an accompanied degree of mortality. In cells infected with empty control viruses or other heterologous adenoviruses we observed only a weak immunostaining of endogenous p53 or pRb and no features characteristic for cell death (data not shown).

3.2. p53 and pRb induce apoptosis in HSC

To study the effects of recombinant pRb and p53 on HSC proliferation, we determined the cell number following infection by direct counting in a light microscope (Fig. 3). In different experiments, the p53- and pRB-infected cultures showed a significant reduction in cell number (up to 50%). In agreement, the DNA content in these cultures was reduced to half that of control cultures suggesting that p53 and Rb were biologically active. We next tested the functionality in transient co-transfection assays using reporter vectors pp53-TA-Luc or pRb-TA-Luc. The vector pp53-TA-Luc contains a p53 responsive element (p53CE) located upstream of the firefly luciferase reporter gene (Fig. 4A). After binding of p53 to p53CE, transcription is induced and the reporter gene is activated. In contrast, in vector pRb-TA-Luc transcription of reporter is decreased in response to binding of Rb to the Rb-cis-acting element (RCE). When assayed in the fat-storing cell line CFSC [30] or 293 cells the shuttle vector pΔE1sp1A-CMV-p53 confers strong activation of the p53-sensitive reporter (Fig. 4C). In analogy, the functionality of pRb expressed by vector pΔE1sp1A-CMV-pRb was proven in 293 cells (data not shown). We then quantitatively assayed the influence of pRb or p53 on proliferation by [^{3}H]thymidine incorporation in HSC (Fig. 5A). Compared to mock- or virus-controls, the multiplication activity of HSC was significantly reduced by pRb and p53. Concomitant with these findings was the induction of cell death within these cells as monitored by quantitative cell death detection ELISA (Fig. 5B). To investigate whether the observed cell death in HSC was secondary to necrosis or apoptosis, we performed caspase-3 activity assays (Fig. 5C). Increased caspase-3 activity was detected in the Ad5-CMV-pRb and Ad5-CMV-p53 infected cells but not in cells infected with other recombinant adenoviruses, revealing that the observed cell death was due to induction of the apoptotic pathway. To confirm that the type of cell

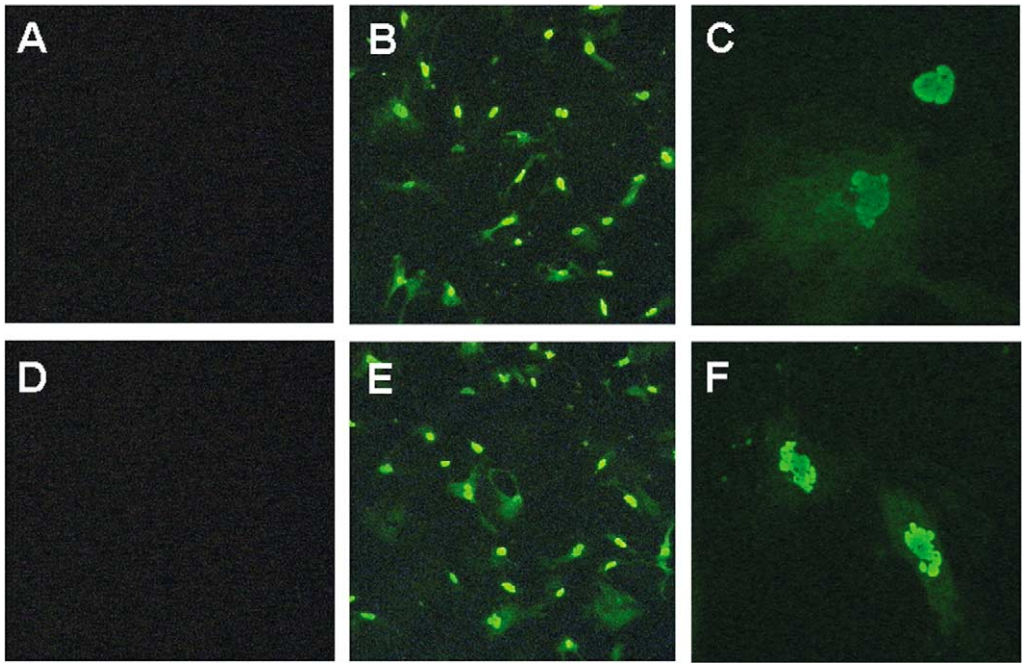

Fig. 2. Apoptosis in HSC. Cells were infected with Ad5-CMV-pRb (A–C) or Ad5-CMV-p53 (D–F) and immunostained with a purified mouse IgG (A,D), an antibody specific for Rb (B,C), or an antibody directed against p53 (E,F). Original magnifications were 100× (B,E) or 400× (A,C,D,F).

death was indeed apoptosis, we also quantified the content of phosphatidylserine on the outer leaflet of the plasma membrane. Again, consistent with the hypothesis that high levels of p53 and pRb induce apoptosis in HSC we found that cells infected with the corresponding Ad5 derivatives were phosphatidylserine-positive as demonstrated by Annexin-V staining, while there was only a weak background staining in untreated or mock-infected cells (Fig. 6).

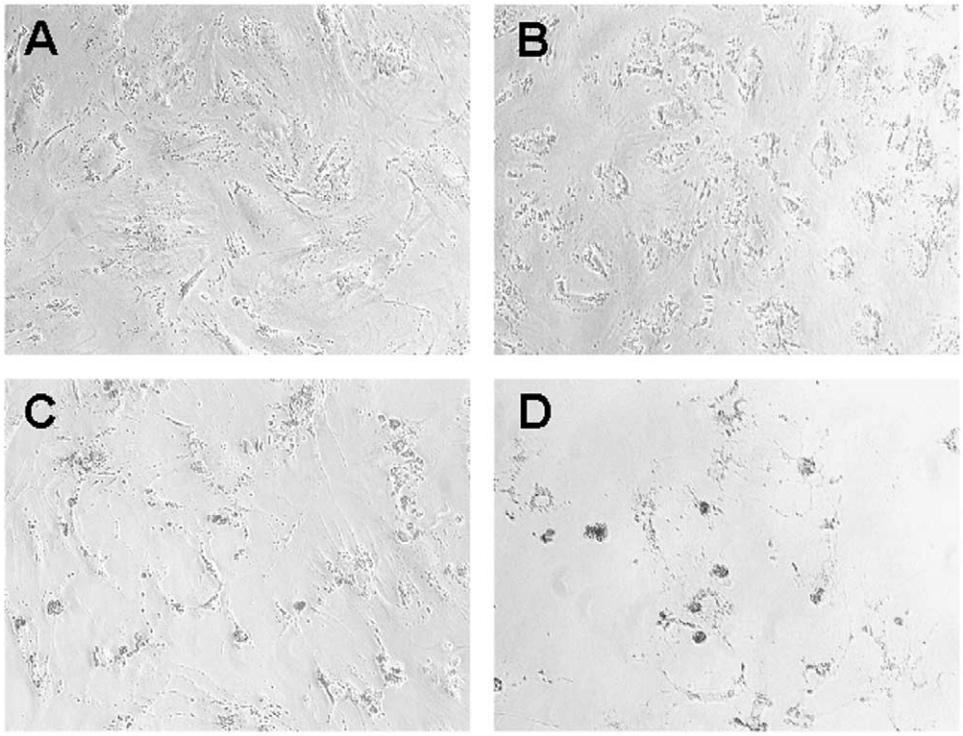

Fig. 3. Microscopic appearance of HSC overexpressing pRb or p53. Photographs were taken from mock-infected HSC (A), control virus-infected HSC (B), Ad5-CMV-pRb infected HSC (C), and Ad5-CMV-p53 infected HSC (D), respectively.

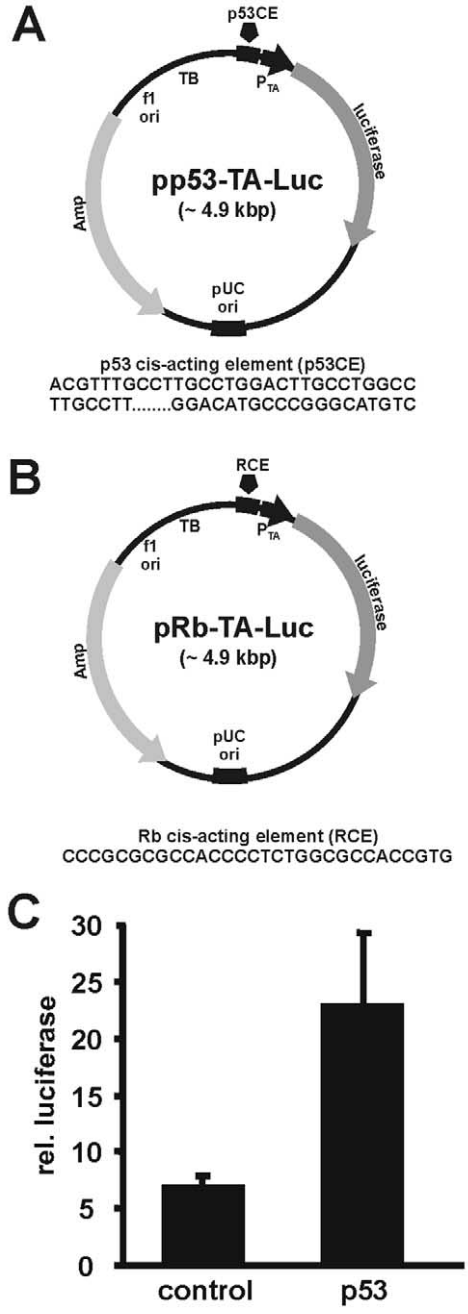

Fig. 4. Functional activity of p53 and pRb. (A) pp53-TA-Luc. The vector pp53-TA-Luc contains a p53 cis-acting element [39], located upstream of the minimal TA promoter, the TATA box from the Herpes simplex virus thymidine kinase promoter (P_{TA}). Located downstream of P_{TA} is the firefly luciferase reporter gene. After transcription factors bind to the p53-response elements, transcription is induced and the receptor gene is activated. (B) pRb-TA-Luc. The vector pRb-TA-Luc contains an Rb cis-acting element (RCE) [40] upstream of the minimal TA promoter. Binding of Rb to the RCE decreases expression of luciferase. (C) Monitoring of p53 activity in pΔE1sp1A-CMV-p53 infected CFSC. CFSC-2G [30] were infected with 2 μg pp53-TA-Luc (control) or co-transfected with 2 μg pp53-TA-Luc and 0.5 μg pΔE1sp1A-CMV-p53 (p53). Two days later the activity of the luciferase reporter was measured and values were normalized to the renilla luciferase activity expressed from pTL-TK co-transfected (0.4 ng) in each experiment.

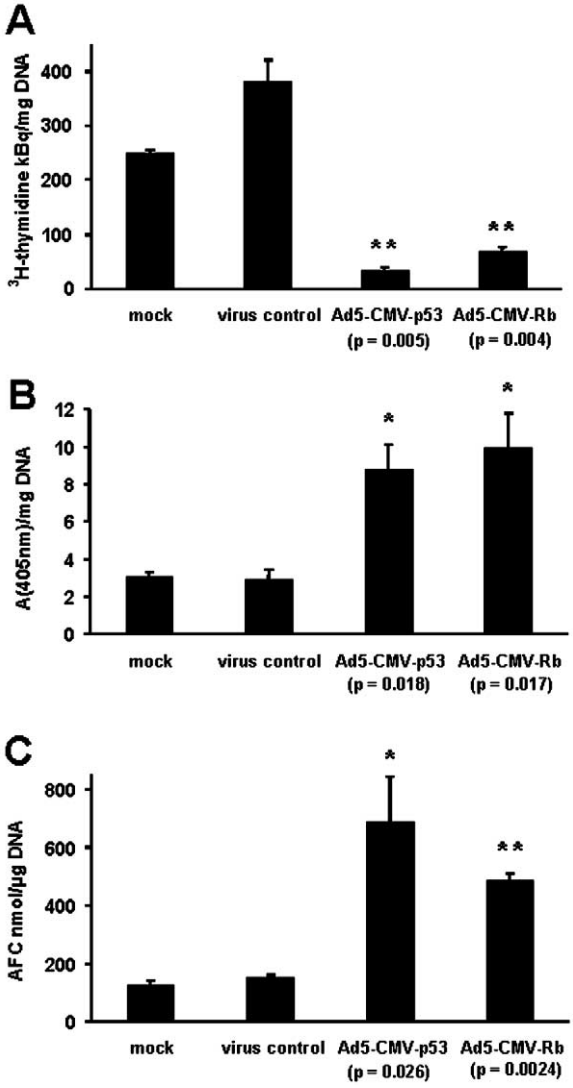

Fig. 5. Determination of cell proliferation, cell death and caspase-3 activity in HSC overexpressing pRb or p53. (A) Proliferation assay. HSC proliferation was analyzed by [^{3}H]thymidine incorporation and the mean values ±SD of triplicate determinations of a representative experiment are shown. (B) Cell death analysis. HSC infected with Ad5-CMV-pRb or Ad5-CMV-p53 were assayed for cell death. Shown is a representative of three independent experiments each performed in triplicate. (C) Caspase-3 activity. Normal, mock-infected or HSC infected with Ad5-CMV-pRb or Ad5-CMV-p53 were assayed for caspase-3 activity. Data are given as average released nmol AFC normalized to the DNA content. In the experiments given (A–C) HSC infected with a virus control served as controls.

3.3. Expression of p21

A key regulator of the p53 pathway is p21/WAF1, which is a transcriptional target of p53 [14,15]. This small regulatory protein is a potent inhibitor of cdks and can also inhibit cell cycle progression by binding to proliferating cell nuclear antigen (PCNA), thereby blocking DNA replication. To address the question of whether the expression of p21 is altered in response to increased levels of p53 we performed Western blot analysis (Fig. 7). In HSC and in control virus-infected

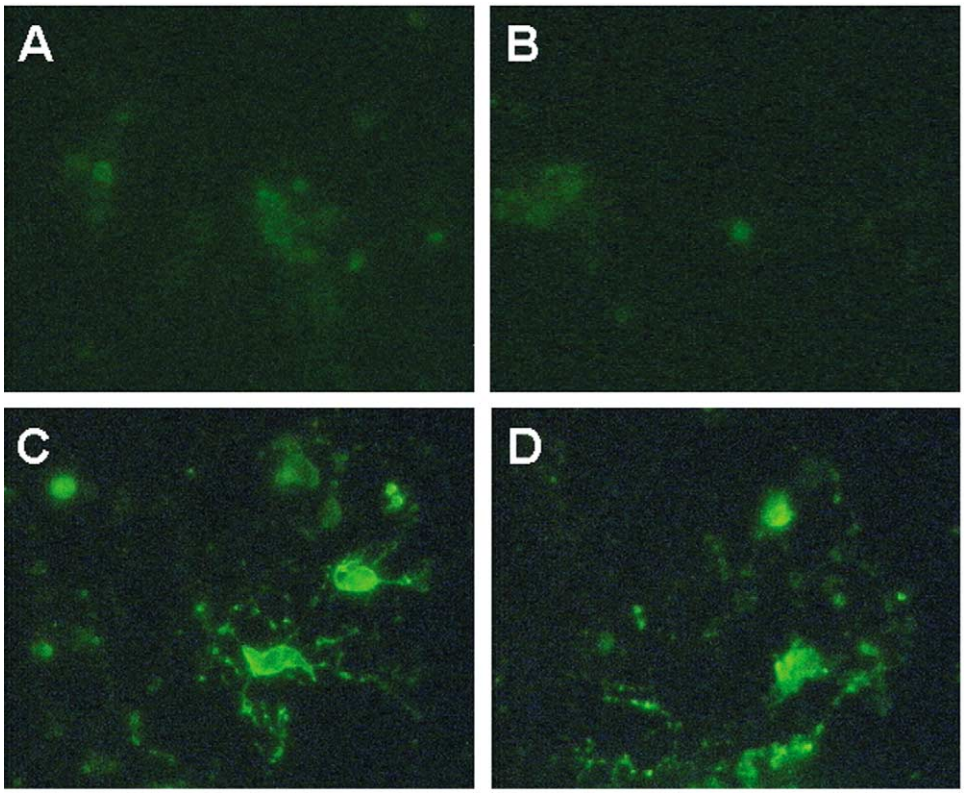

Fig. 6. Staining of HSC with Annexin-V-FLUOS. Untreated HSC (A), HSC infected with a control virus (B), Ad5-CMV-Rb (C) or Ad5-CMV-p53 (D) were stained with the Annexin-V-FLUOS staining kit and analyzed under a fluorescence microscope (400 ×). Shown are representative results of three independent experiments.

cells p21 is only expressed at low levels. However, following infection with Ad5-CMV-p53 we observed upregulation of p21 in a time-dependent manner (Fig. 7A). The experiments also revealed that the level of pRb, the downstream effector of p21, correlates with the expression of p53 (Fig. 7B).

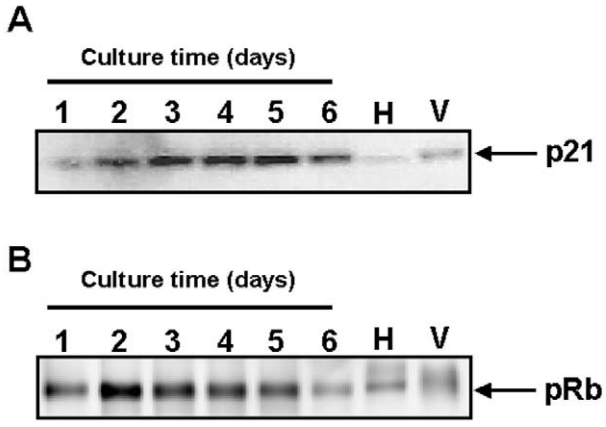

Fig. 7. Western blot analysis. Expression of pRb and p21 in HSC infected for 1–6 days (1–6) with Ad5-CMV-p53. In these experiments, lysates (20 μg) from mock-infected HSC (H) or control virus infected (V) HSC isolated at day 3 after infection served as controls.

3.4. Cell morphology

Two days after infection with Ad5-CMV-p53 HSC showed nuclear blebbing, cytoplasmic vacillation and cellular disintegration, features highly compatible with apoptosis. To further characterize these characteristic changes we performed electron microscopic analysis. HSC or cells infected with control viruses showed the usual ultrastructural morphology and their nucleus was euchromatic and acentric with several prominent nucleoli (Fig. 8A,B). The cytoplasm was well distributed and mitochondria were preferentially located in single organelle-rich cones in close proximity to the nucleus. In contrast, after infection with Ad5-CMV-p53 the chromatin arrangements in the nuclei drastically changed (Fig. 8C,D). Nuclear heterochromatin condensation and fragmentation, typical features of apoptosis, were noted. In later stages of apoptosis nuclear blebbing and shrinking were observed and nuclei were surrounded by typical, highly condensed cytoplasm (Fig. 8E–G). Again, these results show that the introduction of p53 into HSC enables the p53-dependent apoptosis pathway to be initiated and executed.

4. Discussion

Previous studies have demonstrated that apoptosis of HSC may vitally contribute to the spontaneous recovery

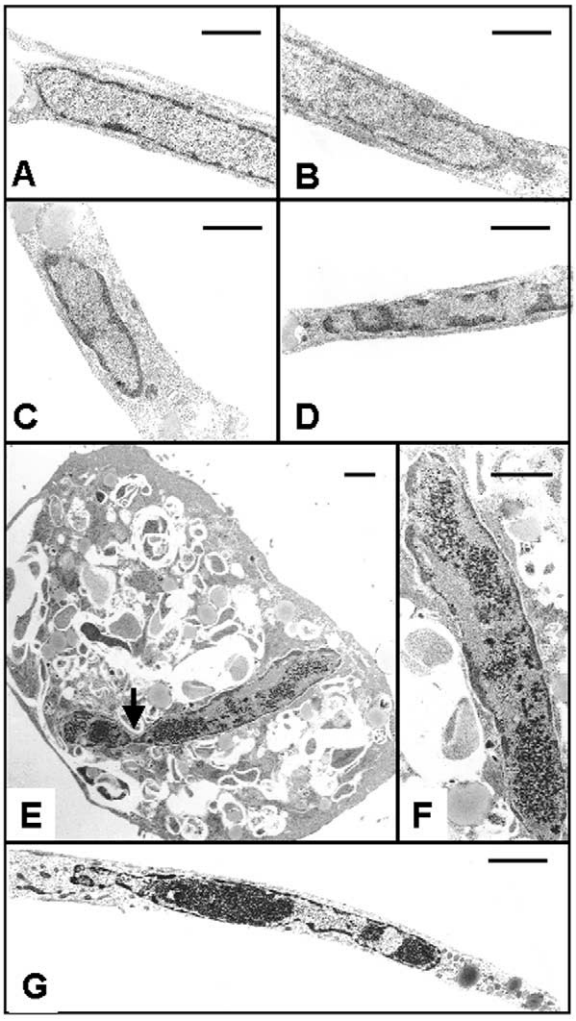

Fig. 8. Electron microscopy. Phenotypic appearance of nuclei from mock-infected HSC (A), or HSC infected with Ad5-CMV-asTGF-β1 [41] (B) or Ad5-CMV-p53 (C–F). Note the chromatin arrangements in nuclei of HSC infected with Ad5-CMV-p53 (C,D). Apoptosis ends in cytoplasmatic disintegration (E) with nuclear blebbing and shrinking (black arrow) and condensed nuclei (F,G). These changes were not seen in mock-infected HSC or in cells infected with other recombinant adenoviruses. Space bars correspond to 1 μm.

from fibrosis [3]. Thus, induction of apoptosis in activated HSC or MFB may have important implications for the development of therapeutic strategies in the liver. Upon activation in culture, HSC undergo spontaneous apoptosis, which can be greatly increased by serum deprivation and fas-ligand [3,32,33]. In other studies it was demonstrated that abrogation of cell adhesion by administration of a soluble integrin antagonist induces apoptosis in primary HSC and ongoing apoptosis was associated with increased expression of p53 [34]. In vitro, TGF-β and TNF-α both inhibit apoptosis in activated HSC and reduced apoptosis may therefore represent the mechanism responsible for prolonged survival of activated HSC in chronic liver failure [35]. As for gene therapy of liver fibrosis, administration of pro-apoptotic genes or transfer of constitutively active negative regulators of the cell cycle are effective means. In light of the broad spectrum of activity, the classical tumor suppressor genes p53 and pRb are strong candidates for use as anti-proliferative agents in a number of disorders that are characterized by inappropriate proliferative responses. With regard to the liver, the cellular activation of HSC in the course of liver fibrogenesis may be a relevant example of such an excessive response, and agents that attenuate the proliferation and activation of HSC could potentially provide a promising strategy for therapy of acute or chronic injury of the liver. In other cellular systems the concept that the activities of p53 and pRb are crucial for the induction of apoptosis and blocking of cell cycle progression is widely accepted [7,36]. In this regard, several studies are tremendously encouraging from the view of therapeutic application, e.g. in hepatocellular carcinoma cells adenoviral-mediated transfer of the p53 gene was shown to initiate cell proliferation arrest and apoptosis [19,20]. However, proof that delivery of p53 or pRb could enable the apoptosis pathway in HSC is uncertain. We therefore constructed replication-defective recombinant adenoviruses expressing wild-type p53 or pRb and examined their effects on HSC proliferation in vitro. Infection with either Ad5-CMV-pRb or Ad5-CMV-p53 resulted in strong expression of the transgenes, which was associated with a drastic suppression of cell proliferation. Both proteins were predominantly localized in the nucleus and were able to induce nuclear blebbing and shrinking. We have used a series of complementary techniques to demonstrate that the observed cell death was mediated by apoptosis and have obtained remarkably consistent results. First, we demonstrate that the activity of caspase-3 was increased. Second, on the basis of staining with an Annexin-V conjugate we were able to show the characteristic externalization of phosphatidylserine, which is usually confirmed to the inner leaflet of the plasma membrane and occurs as a widespread event during apoptosis. Third, the expression of p53 stimulates the activity of the p21 gene. This cdk-inhibitor is known to be a direct p53 target and activation of this downstream mediator confirms that p53-dependent apoptosis in HSC is caused by the classical apoptotic pathway. The prior elevation in the levels of the p21 protein prior to execution of apoptosis via p53 is also known from other cellular systems. Notably, we observed a significant upregulation of pRb expression after transfer of p53 in HSC. Rb is known to signal downstream of p21 and common induction of p21 and pRb again demonstrates that p53 mediates its proper function in the apoptotic program. Furthermore, electron microscopic analysis revealed that HSC infected with Ad5-CMV-p53 generate nuclear heterochromatin condensation and fragmentation with nuclear blebbing and shrinking, typical features of apoptosis.

As for an apoptosis driven gene therapy of liver fibrosis, it is important to induce the transgenes in a HSC/MFB-specific manner. We recently found that in liver the *CSRP2*- and SM22-α genes are exclusively active in

HSC/MFB [37] (unpublished observation). Another potential regulatory element for selective expression in HSC is the promoter of the tissue inhibitor of metalloproteinases-1 [38]. The fusion of p53 or pRb with relevant promoter elements responsible for HSC/MFB-specific expression might offer one way to design specific therapies for liver fibrosis.

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