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Influenza A virus infection of mice induces nuclear accumulation of the tumorsuppressor protein p53 in the lung

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Summary. To investigate whether the tumor suppressor p53 protein, an indicator of DNA damage and cell stress, accumulates in the course of influenza-virus-induced murine pneumonia at the site of inflammation, female BALB/c mice were infected each with 5×10^4 infectious units of influenza virus A, strain Puerto Rico (PR) 8, by instillation into the nose and the pharynx. Two days later the mice became sick. Three and 6 days after infection the lungs of sacrificed infected and uninfected mice were examined. We assessed the presence and localisation of inflammation, the expression of influenza viral and p53 protein, as well as of the WAF1/Cip1/SDI gene product p21. Further, the appearance of nitrotyrosine, as an indicator of the formation of peroxynitrite, and eventually of apoptotic cells was examined. No significant nuclear p53 accumulation was found in influenza virus-infected murine cells in vitro. The results show, that in the course of influenza A virus-mediated murine pneumonia inflammatory bystander cells may cause activation of the tumor suppressor protein p53, due to oxidative stress and DNA damage, with ensuing p53-dependent upregulation of p21. Apoptosis is then mainly due to these indirect processes, with possible involvement of p53.

Introduction

Influenza A virus can induce apoptosis (review in [25]) both in cell culture [6, 10, 28] and in the lung of experimentally infected mice [18]. The virus-induced pneumonia is associated with inflammation-related oxidative stress. Thereby nitric oxide (NO), superoxide ($O_2^{\cdot -}$), with ensuing formation of peroxynitrite ($ONOO^-$), and oxygen-containing and -free radicals are produced [2, 21]. NO and other oxygen-derived radicals are known to induce DNA strand breaks [20, 29]. Hence nuclear accumulation of the tumor suppressor protein p53, an indicator

of chromatin injury [7, 8, 13, 30], eventually leading to apoptosis, may be found also in the murine influenza A virus-infected lung tissue.

We, therefore, investigated as to whether p53 is activated and stabilised in the lung of the influenza virus A-infected (strain Puerto Rico [PR] 8) BALB/c mice. In this case p53-activation-dependent functions should be triggered, such as the transactivation of the WAF1/Cip1/SDI gene [4, 5, 12]. p21, the 21 kD product of this gene, is a universal inhibitor of cyclin-dependent kinases (CDKs) preventing CDK-depending phosphorylation and hence inactivation of the protein product of Rb, which leads to a cell cycle arrest. In addition, p21 inhibits the proliferating cell nuclear-antigen-dependent DNA replication in the S phase of the cell cycle and may also directly inhibit the transcription factor E2F [14].

We assessed the occurrence of pneumonia, influenza A virus proteins, p53, p21, nitrotyrosine (due to peroxynitrite formation), and the presence of cells with signs of apoptosis, using histological, histochemical and immunohistochemical methods. For the investigation whether the virus infection induces nuclear accumulation of p53 directly in cell culture, in the absence of inflammation, murine C3H10T1/2 fibroblasts were used, which are known to give a sensitive p53 response after DNA injury [23, 27].

Materials and methods

Influenza virus A infection of mice and preparation of lung tissue specimens

Female BALB/c mice (6 months old, from Charles-River, Sulzbach, Germany) were infected with influenza virus A, strain Puerto Rico (PR) 8 (kindly donated by Dr. Klenk, Marburg). The virus was produced on embryonated eggs, titre 10^8 infectious units/ml according to titration on dog kidney cells. Fifty µl of the diluted virus (PBS, 5×10^4 infectious units) were instilled into the nose and the pharynx of the mice, which were kept under intraperitoneal xylacin plus ketamin anaesthesia. Control mice were uninfected. Two days later, the infected mice became sick and were sacrificed on day 3 and 6, respectively, after the infection. The lungs were immediately immersed in 4% formalin, embedded in paraffin and then prepared according to standard methods.

Histological analysis

Serial sections were stained with hematoxylin and eosin and analysed by light-microscopy for the presence and localisation of inflammation.

Morphometric analysis and statistical methods

Unstained serial sections were used for immunohistochemical staining. Comparative examination of adjacent sections permitted the assessment of co-localisation of influenza virus antigens, nitrotyrosine-containing proteins and p21-positive cells with nuclear accumulation of p53.

To quantify the distribution of cells with p53 accumulation and p21 expression, adjacent sections from the lungs were evaluated. Using a morphometric software (analySIS, Soft-imaging Software GmbH, Münster, Germany), 5 random microscopic high-power fields at 400-fold magnification from peribronchial areas with severe inflammation were analysed, scoring at least 500 cells as positive or negative for nuclear staining. Moreover, a cell was only scored positive for p53 staining when we found a typical distinct and finely granular

brown staining in the nucleus. The data are reported as mean ± SD. The Wilcoxon 2-Sample Test was applied for comparison of the infected mice with the controls.

Immunohistochemical analysis

First, endogenous tissue peroxidase was quenched with 1% H_2O_2 for 30 minutes, followed by incubation with 0.5% normal bovine serum to reduce non-specific background staining. Thereafter, the sections were incubated with polyclonal antibodies, directed against influenza A virus antigen (dilution 1:20, patients' serum), nitrotyrosine (rabbit, polyclonal, dilution 1:50, cat. no. 06.284 from Upstate Biotechnology, Lace Placid, USA), p53 (CM1, a polyclonal rabbit antibody, dilution 1:200, recognising both wild-type and mutant p53 protein, obtained from Dr. Lane), and p21 (rabbit, polyclonal, dilution 1:500, Oncogene Science), respectively. For immunostaining of nitrotyrosine, p53 and p21, the antigens were unmasked by pressure cooking in 10 mM citric acid/citrate, pH 6, for 5 min. As a positive control for p21 staining we used normal human colon tissue, where p21 staining is restricted to the post-replicative compartment of the crypt and the surface epithelium facing the lumen. The specificity of the rabbit polyclonal anti-nitrotyrosine serum has been validated previously [3]. Negative control stainings were performed by replacing the primary antibodies with preimmune serum of the corresponding species (rabbit, human) or by omitting the primary antibody. All slides were then incubated with biotinylated secondary antibodies at room temperature, followed by incubation with streptavidin and biotinylated horseradish peroxidase complex (ABC-method, Vector Laboratories, Burlingame). In addition, for immunostaining of p53 and p21, the indirect tyramid signal amplification was applied according to the manufacturer's instructions (Dupont). Peroxidase activity was visualized either by 3-amino-9-ethylcarbazole (AEC, from Sigma), yielding a brown reaction product, or with diaminobenzidine, yielding a dark brown reaction product. Cell nuclei were slightly counterstained with hematoxylin.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL)

TUNEL for detecting DNA breaks in tissue sections was performed essentially as described by Gavrieli et al. [9]. In brief, 5 μm sections were incubated with proteinase K (20 mg/ml, Sigma) for 15 min at room temperature. After quenching of endogenous peroxidase, sections were rinsed in TdT buffer (30 mM TRIS, 140 mM sodium cacodylate, 1 mM $CoCl_2$) at pH 7.2 and incubated with 0.3 u./ml TdT and biotinylated-dUTP (1:200, Boehringer, Mannheim, Germany) in TdT buffer for 60 min at 37 °C. Labelled nuclei were detected with Vectastain ABC (Vector Labs, Burlingame) and peroxidase activity was visualised by 3-amino-9-ethylcarbazole (AEC) yielding a brown reaction product. The sections were slightly counterstained with hematoxylin. As a positive control, tissue sections of follicular hyperplasia of the appendix were used and gave the expected positive staining by TUNEL of tingible bodies and some uppermost nuclei of epithelial cells at the edge of the crypt facing the lumen. As a further positive control, lung sections from the uninfected control animals were pretreated with DNAse I (20 mg/ml, Sigma) dissolved in 30 mM TRIS, pH 7.2, 140 mM potassium cacodylate, 4 mM $MgCl_2$, 0.1 mM threo-1,4-dimercapto-2,3-butandiol (DTT, from Sigma) for 20 min at room temperature. After extensive washing, the sections were nick end labelled as described above. As expected, pre-treatment with DNA'se I caused an intensive TUNEL staining of all nuclei.

TUNEL for cytofluorometric detection of apoptotic influenza A virus infected cells in vitro was performed in principle as mentioned previously [23]. In brief, the epitheloid Madin-Darby canine kidney (MDCK) cell line (obtained from Dr. Daniela Kampa, Department of Virology, University of Freiburg), was grown in Dulbecco's modified MEM (DMEM)

with 5% heat-inactivated fetal calf serum (FCS). Cells were infected with trypsin-treated influenza A PR 8 virus, MOI 0.1 and 1, for 24 h or remained uninfected. Upon TUNEL staining, the fraction of apoptotic cells was assayed using a FACSort cytofluorimeter (Becton Dickinson, Heidelberg) and Lysis II software [23].

p53 in influenza A virus-infected murine cells in vitro

Murine C3H10T1/2 cells were cultivated in DMEM with 5% FCS in 6-well plastic dishes, 5×10^5 cells per well, containing glass cover slides. The cells were infected with trypsin-treated influenza A PR 8 virus in serum-free DMEM, MOI 0.2, for 2 h, followed by a medium change with serum-containing DMEM and further incubation for 22 h. Control cells remained untreated or were incubated in the presence of 3 μg mitomycin C per ml for 24 h (until the appearance of cell alterations due to mitomycin cytotoxicity), as a control for DNA-damage-inducible p53 accumulation [8, 27, 31]. p53 and influenza virus antigen expression were detected by immunofluorescence double-staining assay, cell nuclei by simultaneous fluorescence staining with 4′,6′-diamidino-2-phenylindole (DAPI), in principle following previously described fixation and staining protocols [8, 27, 31]. Antisera used were polyclonal rabbit antibodies to p53 (antiserum to p53 expressed in a baculovirus-insect cell system, obtained from Dr. W. Deppert, Hamburg) and human influenza reconvalescent patient's serum. Fluorescent (rhodamine/fluoresceine) secondary antibodies were from Dianova (Hamburg, Germany). Photographs were taken with a Zeiss Axiophot fluorescence microscope.

Results

Pathohistological alterations in influenza A virus-infected lung tissue

In comparison with the tissue of the non-infected mice, the lung tissue from the infected animals exhibited typical features of acute bronchiolitis and peribronchiolar pneumonia, 3 or 6 days after infection. We found widespread plugging of small bronchi and bronchioli by mucous exudation, cell debris, fibrin and inflammatory exudate. Focally, the bronchial epithelium showed extensive destruction and was detached from the basement membrane. The inflammatory infiltrate was composed of cells with typical morphology of macrophages, small lymphocytes and neutrophil granulocytes. In the more severe bronchiolar involvement the inflammatory cells spilled over from the bronchioli into the adjacent lung tissue to produce peribronchiolar pneumonia. In addition, many bronchial epithelial cells exhibited the typical morphologic features of apoptosis, i.e., a homogenous eosinophilic cytoplasm, chromatin condensation and nuclear fragmentation, sometimes in the absence of any inflammation (Fig. 1). No pathological alterations were found in the lung tissue from uninfected mice (Fig. 2). As shown by the investigation of serial sections viral antigen was present in these regions.

Influenza A virus protein in infected mice lung tissue

Immunohistochemical staining demonstrated the appearance of influenza A virus antigen in the lung sections from all infected mice. Viral proteins mainly occurred in the bronchial epithelium, in the desquamated cells in the bronchial lumen and, to a lesser extent, in alveolar lining cells (Fig. 3), whereas the tissue from uninfected animals was negative (not shown).

Nitrotyrosine at the site of inflammation and viral protein expression

Strong nitrotyrosine immunoreactivity was observed extra- and intracellularly in the lung tissue from all the infected mice and was pronounced in the regions with the most severe inflammation, i.e., the bronchiolar compartment of the lung, the peribronchial interstitial tissue and the adjacent alveolar lung tissue. Nitrotyrosine staining occurred in the bronchial epithelium, the inflammatory cells and, to a lesser extent, in alveolar epithelial cells. In addition, intrabronchial cell debris was strongly stained (Fig. 4). Most importantly, investigation of serial sections revealed that nitrotyrosine staining colocalized with p53 staining. In the lung tissue of uninfected animals we found weak and focal nitrotyrosine staining in some bronchi and in the alveolar lung tissue (not shown).

p53 in the lung tissue of infected mice

Immunohistochemical staining disclosed widespread cellular p53 accumulation in all the lungs of the infected mice. p53 staining was finely granular, with varying intensity, and localised preferentially to the nuclei of bronchial and alveolar epithelial cells in areas with acute inflammation (Fig. 5); $32.7 \pm 21.0\%$ of the cells were p53-positive. In the case of uninfected animals, p53 staining was restricted to few bronchial epithelial cells ($2.0 \pm 0.5\%$ p53-positive cells). This difference in the occurrence of p53 between infected and uninfected mice corresponds to $p < 0.001$. In addition, we noted an extracellular p53 staining of the cellular debris in the bronchial lumen, probably resulting from p53 derived from disintegrated cells. By contrast, p53 staining was absent in non-inflamed areas.

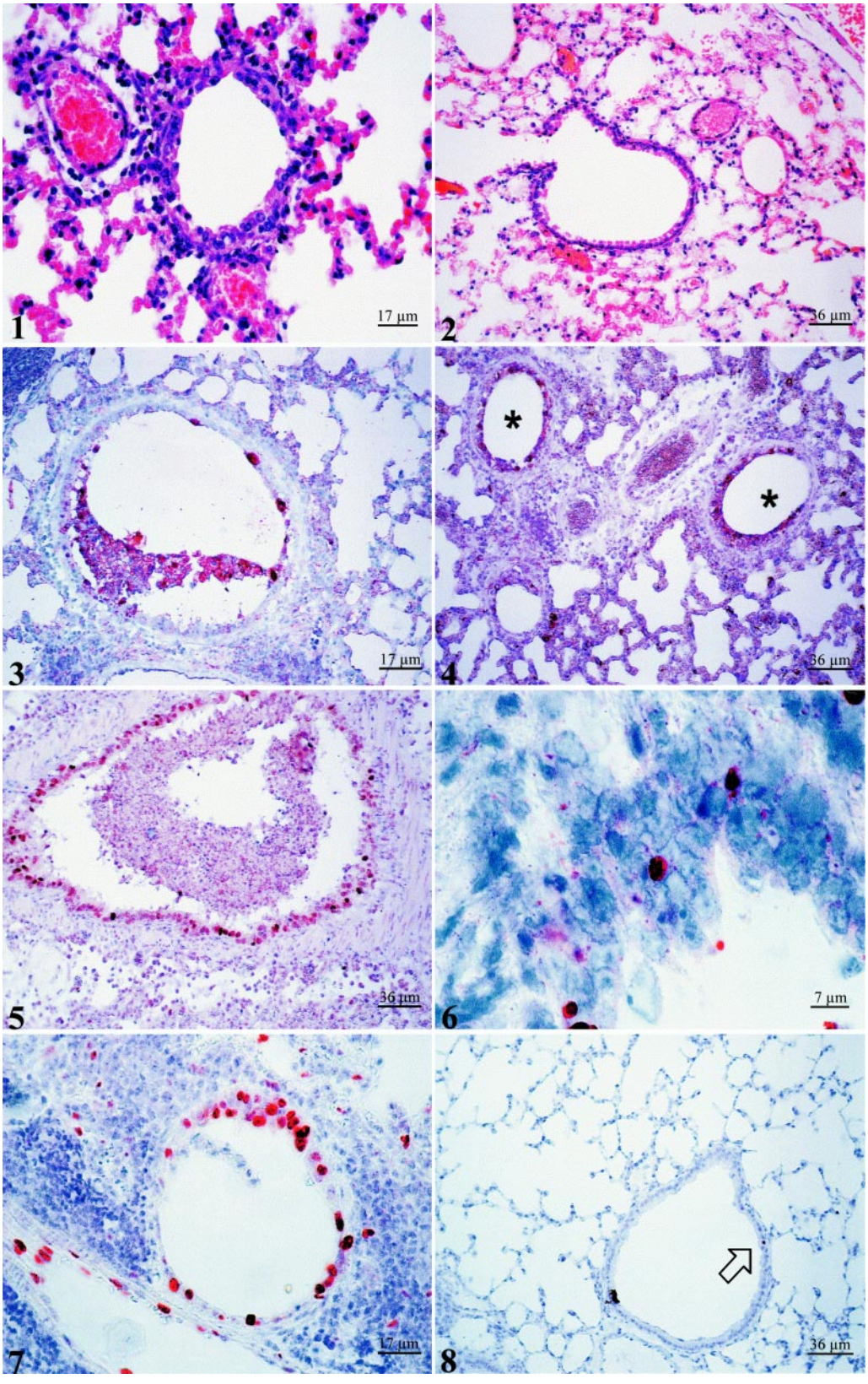
Apoptosis detected by TUNEL assay

Lung tissue specimens from all the infected animals exhibited DNA strand breaks, as shown by TUNEL assay. Most TUNEL-positive nuclear fragments were found in regions with severe inflammation (Fig. 6). In a few microscopic fields TUNEL-positive bronchial epithelial and alveolar cells with the typical nuclear morphology of apoptosis, at the same time exhibiting viral antigen, were found on day 3 after infection, in the absence of any inflammation and p53-staining (not shown).

Lung tissue from uninfected mice exhibited apoptotic cells very rarely (not shown).

p21 expression in influenza A virus-infected lung tissue at the site of p53 occurrence

p21 was found abundantly in the influenza A virus-infected lung tissue. It localised principally to areas with active inflammation and widespread occurrence of p53. p21 was present in the nuclei of bronchial epithelial cells, mesenchymal cells of the peribronchiolar tissue and alveolar lining cells (Fig. 7). Quantitative analysis indicated that $41.9 \pm 1.7\%$ of the cells were positive for p21. By contrast, in the lungs of the uninfected mice we found only few ($4.2 \pm 4.1\%$) bronchial epithelial and alveolar lining cells with nuclear staining for p21 (Fig. 8, arrow shows a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single positive alveolar lining cell). This difference of infected *vs*. non-infected specimens corresponds to $p < 0.001$.

Influenza A virus protein expression at the site of p53 accumulation and TUNEL staining

Importantly, comparative examination of adjacent sections revealed the colocalization of cells with staining for influenza A virus proteins, p53 accumulation, TUNEL-positivity and p21 expression in areas with severe inflammation. However, at some distance from the inflammatory areas we noticed the presence some TUNEL-positive cells exhibiting simultaneously influenza A virus, in the absence of p53 (not shown).

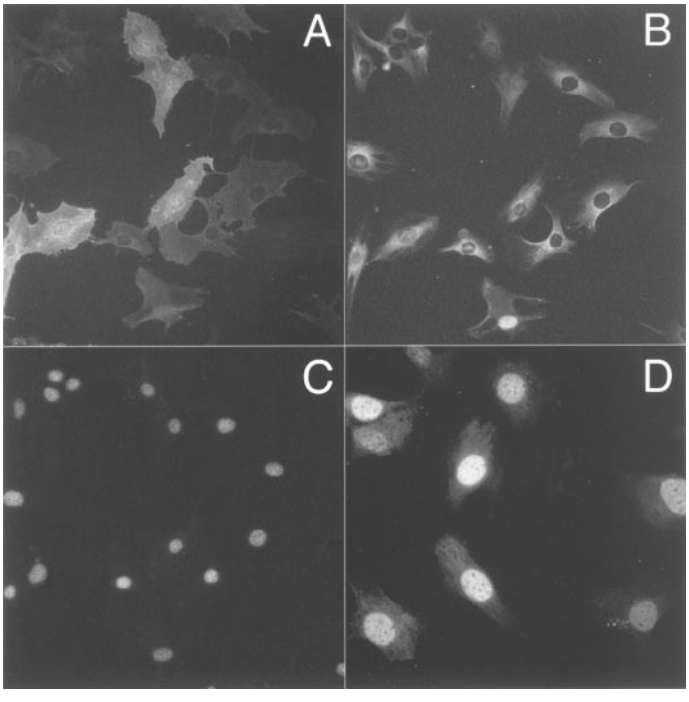

Fig. 9A–D. Influenza-virus-infected C3H10T1/2 fibroblasts, triple staining for p53, virus antigen, and DNA. **A** Influenza virus antigen; **B** same cells as in A, p53; **C** same cells as in **A** and **B**, DNA, stained with DAPI. **D** Control, uninfected C3H10T1/2 fibroblasts, induction of nuclear p53 accumulation by mitomycin C, staining for p53 only

Fig. 1–8. Histochemical and immunohistochemical analysis of lung sections from infected (3 days after the infection) or uninfected (3 days after beginning of the experiment) mice. **1** Infected, stained with hematoxylin and eosin; **2** uninfected, stained with hematoxylin and eosin; **3** Immunoperoxidase staining of influenza virus antigen; **4** infected, nitrotyrosine staining of lining cells of two bronchioli (*); **5** infected, immunoperoxidase staining of p53 of bronchiolar lining cells; **6** infected, nuclear TUNEL staining at a site of inflammation; **7** infected, immunoperoxidase staining of nuclear p21 of alveolar lining cells; **8** uninfected, immunoperoxidase staining of nuclear p21 of one alveolar lining cell (arrow)

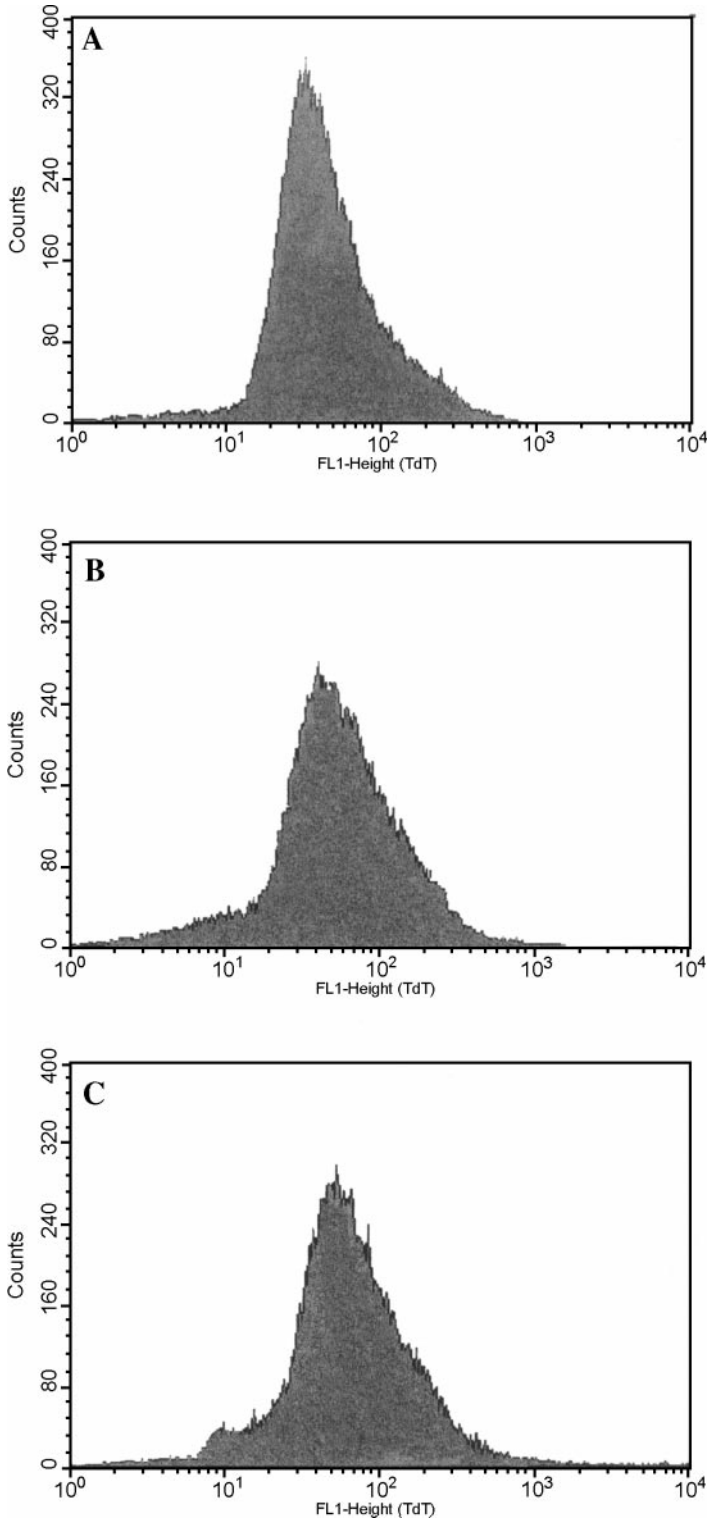

Fig. 10. MDCK cell culture, TUNEL staining and cytofluorimetric evaluation of apoptosis. Abscissa, relative apoptosis-related TdT-incorporation. Ordinate, number of cells exhibiting this signal. **A** Uninfected cells, mean TdT incorporation signal is 60; **B** cells infected with influenza A virus, MOI = 0.1; mean signal is 76; **C** cells infected with influenza A virus, MOI = 1; mean signal is 91

Failure of significant p53 accumulation in murine C3H10T1/2 fibroblasts following influenza virus infection

To investigate whether influenza virus infection per se, in the absence of inflammatory bystander cells, causes p53 accumulation, murine C3H10T1/2 fibroblasts were infected with influenza A virus. Virus protein formation and p53 were assayed by immunofluorescent double staining. Beginning apoptosis was monitored by DAPI staining of the cell nuclei.

Influenza virus infection (MOI 0.2) for 24 h induced virus antigen in about 20% of the fibroblasts. Viral protein was diffusely distributed (*i.e.*, not exclusively in the nuclei), due to the late phase of infection (Fig. 9A; 5 virus-antigen-positive cells), whereas nuclear p53 accumulation was seen rarely, in about one cell per several microscopic fields, and occurred independently of whether the cells were infected or not (Fig. 9B, one p53-positive, virus-negative cell nucleus). This low p53 incidence represents rather spontaneous accumulation, typical for untreated C3H10T 1/2 cells, than infection-related expression.

To control, whether the p53 stabilization of the cells used was intact in response to DNA damage, a separate cell culture was treated with mitomycin C [23, 27]. After 24 h nuclear p53 accumulation was found in the majority of the fibroblasts, as to expect (Fig. 9D).

Infection (MOI 0.1 and 1) of MDCK cells, an epitheloid cell line, resulted in an enhanced rate of apoptosis, 24 h after infection, as shown by TUNEL staining and cytofluorimetric evaluation of the fraction fluorescent apoptotic cells (Fig. 10).

Discussion

The morphology of influenza A virus infection-mediated murine pneumonia has many similarities with that of other acute inflammatory processes. During these inflammatory processes reactive oxygen-derived intermediates (ROI) and compounds [$O_2^{\cdot -}$, H_2O_2, NO, and $ONOO^-$(peroxynitrite)] are formed [3, 16]. These agents are potentially DNA-damaging, which may be harmful for the integrity of the genome. Therefore, nuclear p53 accumulation and activation of the transcription factor p53 may be triggered, especially by the inflammation-generated NO [17, 19, 24]. As a transcription factor, p53 may transactivate "downstream" genes, encompassing the WAF1/Cip1/SDI gene [4, 5, 12]. Since p21, the WAF1/Cip1/SDI gene product, is a universal inhibitor of cell cycle progression, it supports DNA repair, eventually. If the DNA damage is irreversible, activation of p53 contributes to apoptosis [13, 30].

The present study was designed to evaluate the possible involvement of p53 and p21 during influenza-mediated pneumonia in mice. The results show nitrotyrosine staining in areas with ongoing inflammation, co-localising with nuclear p53 accumulation and p21 expression. Moreover, in these areas we found TUNEL-positive cells with the typical nuclear morphology of the execution phase of apoptosis.

Quantitative analysis of serial sections showed, that cells with p53 accumulation and p21 expression were present in a nearly one-to-one ratio, within an area. This co-ordinated upregulation suggests that the increased p21 expression may be caused by transactivation of the WAF1/Cip1/SDI gene by p53. This view is supported by the observation of inflammatory processes, where DNA damage was found in association with both nuclear p53 accumulation and an upregulation of p21 [11].

Nevertheless, p21 expression may also occur independently from p53 accumulation [32], as observed in the lungs of uninfected mice, where p21 occasionally did not co-localise with p53 accumulation (Fig. 8). But the reverse was also found, as small numbers of cells with p53 accumulation were present in uninfected, non-diseased tissue, similar to the occurrence of spontaneous nuclear wild-type p53 in untreated untransformed cell cultures.

Importantly, the failure of significant p53 accumulation in influenza virus-infected murine C3H10T1/2 fibroblast cell culture (Fig. 9) suggests that p53 accumulation is induced indirectly, due to genotoxic stress in the inflamed tissue, not due to the infection itself.

Further, since nitrotyrosine staining and the majority of apoptotic cells occurred in vivo at the site of severe inflammation and were not regularly associated with influenza A virus protein expression, apoptosis is probably caused mainly indirectly, i.e., less due to the influenza virus infection directly. On the other hand, rare virus-antigen-positive alveolar cells were already apoptotic in the pre-inflammatory phase of the infection. In this case apoptosis was indeed triggered by the virus infection directly, as in infected cell culture.

Apoptosis induction by influenza virus seems related to the activation of latent transforming growth factor β (TGF-β) by the viral neuraminidase [25]. Interestingly, in the case of mesangial cells and TGF-β, apoptosis was found induced through NO- and p53-dependent and -independent pathways [15, 22], but it still remains unclear as to whether TGF-β also contributes to NO formation in the mouse lung.

In conclusion, in the course of influenza A virus-mediated murine pneumonia inflammatory bystander cells cause activation of the tumor suppressor protein p53 due to oxidative stress and DNA damage, with an ensuing p53-dependent upregulation of p21. Apoptosis is then mainly due to these indirect processes, with possible involvement of p53.

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