Journal of Cellular Biochemistry 80:441–454 (2001)

An Endoplasmic Reticulum-Specific Stress-Activated Caspase (Caspase-12) is Implicated in the Apoptosis of A549 Epithelial Cells by Respiratory Syncytial Virus

Vira Bitko and Sailen Barik*

Department of Biochemistry and Molecular Biology, MSB 2370, University of South Alabama, College of Medicine, Mobile, AL 36688-0002

Abstract Respiratory syncytial virus (RSV) infection induced programmed cell death or apoptosis in the cultured lung epithelial cell line, A549. The apoptotic cells underwent multiple changes, including fragmentation and degradation of genomic DNA, consistent with the activation of the DNA fragmentation factor or caspase-activated DNase (DFF or CAD). The infection led to activation of FasL; however, a transdominant mutant of FAS-downstream death domain protein, FADD, did not inhibit apoptosis. Similarly, modest activation of cytoplasmic apoptotic caspases, caspase-3 and -8, were observed; however, only a specific inhibitor of caspases-3 inhibited apoptosis, while an inhibitor of caspase-8 had little effect. No activation of caspase-9 and -10, indicators of the mitochondrial apoptotic pathway, was observed. In contrast, RSV infection strongly activated caspase-12, an endoplasmic reticulum (ER) stress response caspase. Activation of the ER stress response was further evidenced by upregulation of ER chaperones BiP and calnexin. Antisense-mediated inhibition of caspase-12 inhibited apoptosis. Inhibitors of NF-kappa B had no effect on apoptosis. Thus, RSV-induced apoptosis appears to occur through an ER stress response that activates caspase-12, and is uncoupled from NF-kappa B activation. J. Cell. Biochem. 80:441–454, 2001.

Key words: apoptosis; caspase; endoplasmic reticulum; fas; NF-κB; respiratory syncytial virus

Lung cells, by virtue of their unique location, are constantly traumatized by pollutants and infectious agents present in the inhaled air. Perhaps the single most important lung pathogen is the respiratory syncytial virus (RSV), a negative-stranded RNA virus of the *Paramyxoviridae* family, that causes bronchiolitis and pneumonia in infants [Anderson and Heilman, 1995]. RSV infects the respiratory epithelium and causes substantial immunopathological alterations of the infected bronchi and alveoli. Pathological examination of fatal RSV-induced respiratory diseases has revealed extensive cellular damage including peribronchial infiltration, plugging of lumen, hyperinflation, atelectasis, and destruction of the epithelium—symptoms that are also observed in patients who die in *status asthmaticus* [Hayes, 1976]. However, the actual mechanism by which RSV may kill specific host cells remains unknown.

Two major models of cell death have been described: apoptosis and necrosis. Apoptosis, also known as programmed cell death, is characterized by specific morphological as well as molecular events [recently reviewed by Song and Steller, 1999] including changes in the plasma membrane, a condensation of the cytoplasm and nucleus, and cleavage of the chromatin into nucleosomal oligomers. A large variety of cellular traumas have been shown to cause cell death through the induction of apoptosis. These include exposure to genotoxic agents, treatment with enzyme inhibitors, and recently, infection by many pathogens. There is now mounting evidence that induction of apoptosis contributes directly to the pathogenesis of viruses [reviewed by Everett and McFadden, 1999] such as influenza A and B [Hinshaw et al., 1994; Takizawa et al., 1993], measles virus [Esolen et al., 1995], and human immunodeficiency virus type 1 (HIV-1), the major causative agent of AIDS [Bartz and Emerman, 1999]. Despite enormous progress in our know-

*Correspondence to: Sailen Barik, Department of Biochemistry and Molecular Biology, University of South Alabama, College of Medicine, 307 University Blvd., Mobile, AL 36688-0002. E-mail: sbarik@jaguar1.usouthal.edu

Received 1 April 2000; Accepted 13 July 2000

ledge of the cellular mechanisms of apoptosis, the apoptotic pathway(s) triggered by viruses are only beginning to be unraveled.

As part of our interest in cellular signaling pathways activated by RNA viruses, we have recently initiated studies of cellular apoptosis by RSV. While our work was in progress, apparently conflicting reports regarding the ability of RSV to cause apoptosis appeared. Takeuchi et al. [1998] showed that RSV infection of lung epithelial cells (A549) activated IL-1 converting enzyme (ICE); however, it was stated that no apoptosis could be detected. In contrast, O'Donnell et al. [1999] demonstrated apoptosis in about 15% of RSV-infected cells using a TUNEL assay. Cell-sorting assays revealed that such cells expressed an approximately 2–3-fold higher level of CD95 (Fas). The Fas pathway and the ICE subfamily of caspases are established mediators of apoptosis [Vincenz and Dixit, 1997; Song and Steller, 1999]. A similar increase of CD95 accompanied by increased apoptosis was also observed in RSV-infected neutrophils [Wang et al., 1998], suggesting a general importance of the activation. Interestingly, however, cross linking of Fas by anti-Fas antibody increased RSV-mediated apoptosis by two fold (from 15% apoptotic cells to 30%), whereas cross-linking alone (in the absence of RSV infection) did not cause apoptosis. Thus, the relative importance of the Fas pathway in RSV-induced apoptosis needs to be addressed.

In addition, studies in several laboratories including ours have demonstrated a rapid and persistent activation of the ubiquitous cellular transcription factor NF-kappa B (NF-κB) upon RSV infection [Fiedler et al., 1996; Garofalo et al., 1996; Mastronarde et al., 1996; Bitko et al., 1997]. NF-κB appeared to play a dual role in apoptosis; while it offered protection against apoptosis in some cases [Baichwal and Baeurle, 1997; Beg and Baltimore, 1996; Marusawa et al., 1999; Van Antwerp et al., 1996; Wang et al., 1996], it was required for apoptosis in others [e.g., Abbadie et al., 1993; Dumont et al., 1999; Grimm et al., 1996; Lin et al., 1995; Marianneau et al., 1997; Ouaaz et al., 1999]. Activation of NF-κB has been shown to be essential for the transcriptional induction of a number of cytokines and ICAM-1 by RSV [Bitko et al., 1997; Fiedler et al., 1996; Garofalo et al., 1996; Mastronarde et al., 1996; Chini et al., 1998]. Whether it also plays a role in RSV-mediated cell death, however, remains an important question that needs to be answered.

In the present study, we have, therefore, examined the phenomenon and the mechanism of RSV-induced apoptosis. Our results convincingly demonstrate apoptosis in RSV-infected A549 cells by a variety of available criteria, and thus, agree with those of O'Donnell et al. [1999]. Additionally, we have attempted to identify the cellular apoptotic pathway activated by RSV.

MATERIALS AND METHODS

Growth of Cells and RSV

A549 cells were maintained in MEM supplemented with 5% of fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The Long strain of RSV, used in this studies, was grown on monolayers of HEp-2 cells in MEM containing glutamine and fetal bovine serum, and purified by centrifugation as described [Bitko et al., 1997]. The virus-free supernatant for mock-infection was prepared by centrifugation of RSV stock virus at 120,000*g* for 2 h. The monolayer, at a confluency of 70–80%, was infected at an m.o.i. of 5 (or the same volume of virus-free supernatant in "mock infection"). In the experiments using the caspase inhibitors and Na-salicylate, the cells were exposed to the inhibitors 2 h prior to the addition of the virus. Two hours after the addition of the virus, the media were removed and replaced with prewarmed fresh media (supplemented with inhibitors, where used).

Transfection with Plasmids and Antisense ODN

For introduction of plasmids and antisense oligodeoxynucleotides (ODNs) into cells, we used the SuperFect transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Infection of the transfected cells with RSV was carried out as described [Bitko et al., 1997]. AU-1 epitope-tagged recombinant full-length FADD protein and its dominant negative deletion mutant, containing amino acids 80–208 of FADD (called NFD4, or simply, FADD-DN), were expressed by transient transfection of the corresponding pcDNA-3 clones [Chinnaiyan et al., 1995]. Transient transfection with the IκB-α mutant expression clone [Traenckner et al, 1995; Van Antwerp et al., 1996] was performed in the same manner. The transfected cells were allowed to

grow another 8 h before they were infected with RSV. In the ODN experiments, fresh ODN (200 μM) was added to the culture medium at 10 h post-infection. The ODN sequences have been described earlier [Nakagawa et al., 2000]; antisense (5′-TGTCCTCCTGGCCGCCATGG-CTGT-3′), and scrambled (5′-GTCGCTCTGT-GACGCCTGTGCCTG-3′). Both ODNs had 5′-thiol modifications as described [Nakagawa et al., 2000].

Multiparametric Staining of Cells

Triple staining of cells was performed essentially as described [Negri et al., 1997]. A549 cell monolayers that had been grown on coverslips were subjected to various treatments and infected with RSV as described above. At 20 h p.i. (unless otherwise indicated), the monolayer was rinsed in PBS and fixed with ice-cold 10% trichloracetic acid for 15 min, followed by washes in cold 70 and 90%, and absolute ethanol for 3 min each. Apoptotic DNA fragments were end-labeled using the TUNEL-based Apoptosis Detection System (Promega). Briefly, after fixation, the samples were again rinsed in PBS and incubated for 10 min with equilibration buffer (200 mM potassium cacodylate and 25 mM Tris–HCl, both pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA, and 2.5 mM $CoCl_2$). The enzymatic-labeling reaction was performed for 60 min at 37°C in the dark in the equilibration buffer supplemented with following reagents: 10 μM dATP, 1 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 5 μM fluorescein-12-dUTP and 25 units of TdT (final concentrations). To remove unincorporated dUTP, the slides were washed three times for 15 min each with 2 × SSC and three times with PBS. Since this TUNEL assay gave us virtually no background (e.g., the dark panel of uninfected cells in Fig. 3), all cells with green nuclei were counted as "apoptotic" for quantitative estimates (e.g., Table I).

RSV infection of A549 cells was evaluated by immunofluorescence. After washing the TUNEL-processed cells with PBS, they were incubated for 30 min at room temperature with monoclonal antibodies against RSV (Chemicon International, Temecula, CA) as described by Burke et al. [1998]. Thereafter, the cells were washed three times for 5 min each with PBS, incubated for 30 min with rhodamine-conjugated anti-mouse antibody diluted in PBS, and washed again with PBS. Nuclei were stained by 4′,6′-diamidino-2-phenylindole (DAPI). After washing, the coverslips were mounted on glass slides and observed by fluorescence microscopy using an Olympus IX70 microscope equipped with a digital image capture device.

TABLE I. Effect of Prospective Inhibitors on RSV-Induced Apoptosis

Treatment	Percent apoptotic cells
None (i.e., uninfected)	< 0.5
RSV	95 ± 5
RSV+Ac-IETD-CHO (40 μM)	80 ± 5
RSV+Ac-DEVD-CHO (40 μM)	8 ± 2
RSV+zVAD-fmk (40 μM)	5 ± 2
RSV+FADD (wild type), 2 μg	94 ± 5
RSV+FADD-DN, 0.5 μg	82 ± 6
RSV+FADD-DN, 1 μg	80 ± 5
RSV+FADD-DN, 2 μg	81 ± 4
RSV+Na-salicylate, 20 mM	90 ± 5
RSV+IκBα-DN, 1 μg	85 ± 5
RSV+IκBα-DN, 2 μg	80 ± 5
RSV+Antisense ODN, 0.5 μM	24 ± 6
RSV+Scrambled ODN, 0.5 μM	88 ± 4

Note: A549 monolayers at 60–70% confluency were pretreated with inhibitors as and where indicated, then infected with RSV at an m.o.i. of 5, and the number of apoptotic cells determined by TUNEL assay as described in detail under Materials and Methods. IκBα-DN represents the Ser32,36Ala double mutant IκBα, detailed under Results. In different transfection experiments, roughly 70–80% cells expressed the recombinant proteins (IκB or FADD); however, co-staining for TUNEL did not reveal any difference of apoptosis between the cells that expressed the recombinants and the few that did not. Each treatment was carried out in triplicate, and 200–250 cells were observed each time. Percent apoptotic cells = (Number of apoptotic cells/total number of cells counted) × 100. Each number represents the mean of at least three experiments with the variation as indicated. For each inhibitor, a range of concentrations was tested, and only a representative concentration showing maximal inhibition of apoptosis is presented. At higher concentrations, many inhibitors started to inhibit RSV growth for unknown reasons (data not shown).

DNA Fragmentation Assay

Fragmentation of DNA into the characteristic apoptotic ladder was assessed as described [Park and Patek, 1998]. Infected and uninfected cells were collected in 1.5 ml microcentrifuge tubes and washed once with 1 ml of 0.02% EDTA in PBS without Ca^{2+} or Mg^{2+}. The cells were pelleted by centrifugation for 5 min at 700*g*, then 12 μl of lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM sodium-EDTA, 0.25% NP-

40) was added along with 4 μl of an RNase A solution (10 mg/ml). The mixture was suspended and incubated for 20 min at 37°C. To the samples, 4 μl of proteinase K solution (10 mg/ml) was added and incubation continued for an additional 20 min. The samples were then analyzed by electrophoresis on a 1.8% agarose gel in TE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8,0) for 3 h at 36 V. The ethidium bromide-stained DNA was visualized and photographed by transillumination with UV light.

Caspase Assay

To determine the increased enzymatic activity of caspases-3, -8, and -9 in apoptotic cells colorimetric assay kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions. In brief, cells are first lysed to collect their intracellular contents, and the lysate was tested for protease activity by addition of caspase-specific peptide conjugated to the color reporter molecule, *p*-nitroanilide. The level of caspase activities was quantitated by spectrophotometric measurement at a wavelength of 405 nm.

Where used, cell-permeable caspase inhibitors were continuously present at a final concentration of 40 μM starting at 2 h prior to infection. The following inhibitors were used: caspase-3 inhibitor Ac-DEVD-CHO (BD Transduction Laboratories, San Diego, CA); caspase-8 inhibitor, Ac-IETD-CHO, and the broad-spectrum caspase inhibitor, zVAD-fmk. The last two inhibitors were from Calbiochem (La Jolla, CA).

Immunoblot (Western Blot)

The preparation of cell lysates, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the lysates, and immunoblot analyses were carried out as described [Bitko and Barik, 1998]. Anti-caspase-10 antibody used in these experiments was purchased from Calbiochem (La Jolla, CA). Polyclonal anti-DFF antibody was raised against the N-terminus of DFF45 as described by Liu et al. [1997]. Monoclonal mouse antibody against caspase-12 has been described [Nakagawa et al., 2000] and was kindly provided by Dr. Junying Yuan (Harvard Medical School, Boston, MA). The last two antibodies recognize the full-length unprocessed form as well as the processed fragments of their respective antigens (DFF45 and caspase-12, respectively). Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce, Rockford, IL).

RESULTS

Activation of Cellular DNA Fragmentation in RSV Infection

Chromosomal DNA fragmentation of uninfected, mock-infected and RSV-infected cells was examined at different times postinfection by agarose gel electrophoresis and visualized by staining with ethidium bromide. As shown in Figure 1a, fragmentation of nuclear DNA of the infected cells was obvious by 4 h p.i., whereas in uninfected A549 cells and in cells that were mock-infected with virus-free media supernatant, the nuclear DNA remained unaffected. The amount of fragmentation increased up to about 18 h p.i., indicating maximal apoptotic outcome at this point in infection.

Processing of DNA Fragmentation Factor in the RSV-Infected Cell

In the last few years, the DNA fragmentation factor (DFF; also called caspase-activated DNase or CAD) has been shown to be a major mediator of DNA fragmentation characteristic of apoptotic cells [Liu et al., 1997; Tang and Kidd, 1998, and references cited therein]. DFF is a heterodimer composed of the 40 kDa nuclease subunit (DFF40) which is normally inhibited by its association with the 45 kDa inhibitory subunit (DFF45 or ICAD). Apoptosis-activated caspases may proteolytically degrade DFF45, which leads to the release, and hence activation, of DFF40 that in turn fragments nuclear DNA. The posttranslation mechanism allows for rapid activation of DFF in response to activated caspases.

As DNA fragmentation was observed in RSV-induced apoptosis (Fig. 1a), we wanted to investigate if this effect correlates with the activation of DFF. To test this, we looked for the diagnostic cleavage products of the inhibitory subunit, DFF45. Indeed, within about 1 h of RSV infection, substantial quantities of the 28 kDa cleavage intermediate began to appear (Fig. 1b), with increasing amounts of the shorter end-product of ~17 kDa that reached maximal levels at about 4 h. Recent evidence has shown that it is the final cleavage event, signified by the appearance of the 17 kDa fragment, which actually correlates with the

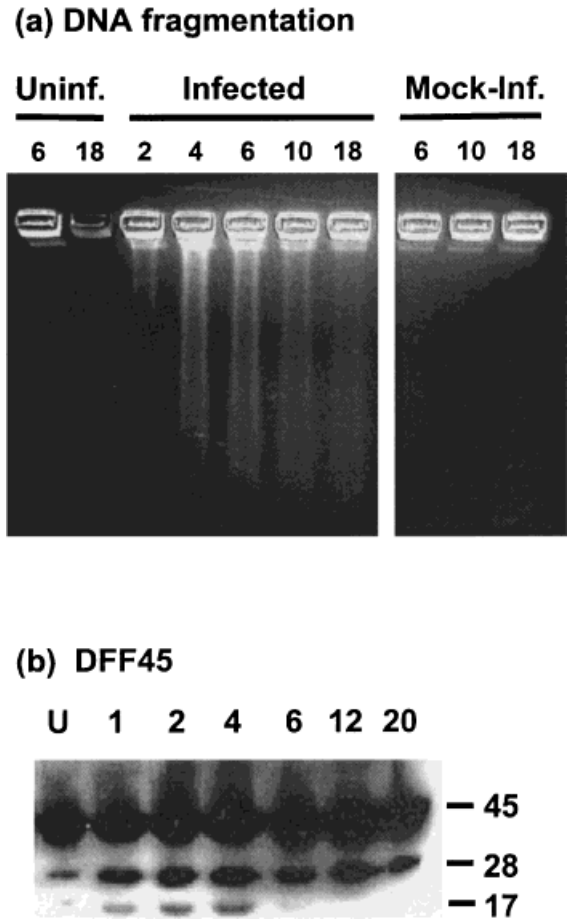


Fig. 1. Apoptotic (**a**) DNA fragmentation and (**b**) DFF45 degradation in RSV-infected cells. Analyses were carried out as described in Materials and Methods. The numbers are hours postinfection at which cells were harvested for analysis. Western blotting of total extracts of uninfected (U) and RSV-infected A549 cells (at various hours p.i. indicated above or below the lanes) were carried out using anti-DFF45 antibody as described in Materials and Methods. Mock-infected cells were ''infected'' with virus-free 100,000*g* supernatants of the RSV preparation. The 43 kDa unprocessed DFF45 polypeptide and its 28 and 17 kDa processed fragments are so marked.

functional inactivation of DFF45 and activation of DFF40 [Liu et al., 1997; Sakihara et al., 1998, and references therein]. The disappearance of the 17 kDa fragment at later times is likely due to its degradation by general proteolysis. In conclusion, these results suggest a relatively rapid and early activation of DFF in the RSV-infected cell. The fact that the onset of visible DNA degradation (about 2 h) closely follows that of activation of DFF (1 h) is consistent with a causal relationship between the two.

Relationship Between Apoptosis and RSV Infection

To confirm the RSV infection of the apoptotic A549 cells, we have carried out a multi-parametric fluorescence analysis of the infected cells. In this experiment, cells were infected with a multiplicity of about five RSV particles per cell, which resulted in infection of essentially every cell in the field, as monitored by immunostaining with antibody against RSV (Fig. 2). At the same time, nuclear DNA nicks of the same population of cells were monitored by TUNEL assay. Results revealed that about 90% of the infected cells had the strong green fluorescence in their nuclei characteristic of chromatin breaks (Fig. 2). Co-localization of the blue fluorescing DNA-binding dye, DAPI, with the green TUNEL signal ascertained that the TUNEL stain was indeed at the nuclei. These results provided independent evidence of apoptosis and demonstrated that under optimal conditions virtually all RSV-infected cells undergo gross chromosomal degradation, confirming irreversible apoptosis.

In order to further correlate the TUNEL signal with RSV-mediated apoptosis, we have determined its kinetics. As shown in Figure 3, apoptotic nuclei started to appear as early as 4 h p.i. and reached a maximal number at around 18 h p.i., in agreement with the DNA ladder analysis presented in Figure 1.

Apoptosis is a Direct Result of RSV Infection

The results presented so far proved that infection was essential for apoptosis; however, they did not exclude the possibility that a diffusible product of infection may induce apoptosis. HIV-infected T-cells are known to elaborate viral Tat protein that causes apoptosis of "bystander" T-cells in the vicinity [McCloskey et al., 1997]. In fact, it is possible that such killing of the uninfected cells gave the appearance of a violation of Koch's postulates for infectious disease and was used as one of the arguments for the non-HIV theory of AIDS [Duesberg and Rasnick, 1998]. To investigate whether a diffusible entity liberated from RSV-infected cells may have apoptosis-promoting activity, we performed a co-culture experiment in which A549 cells grown in 6-well dishes were first infected with RSV. At about 2 h p.i., the unadsorbed virus and spent media were removed and replaced with fresh media.

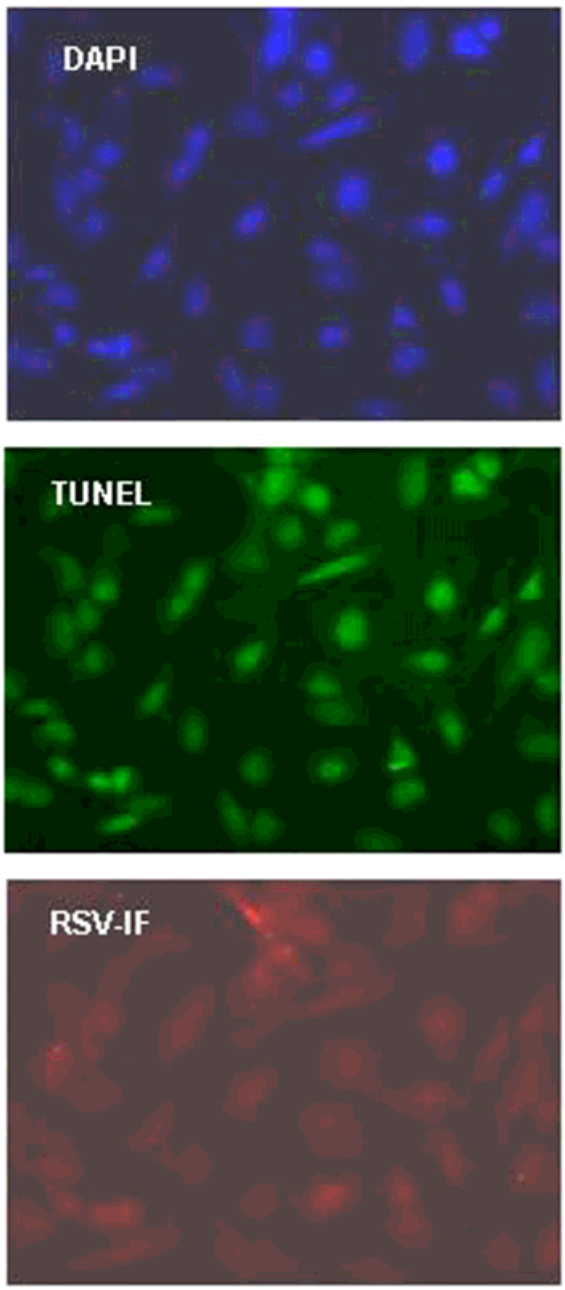


Fig. 2. Apoptosis of individual RSV-infected cells. A549 monolayer was infected with RSV, and at 18 h p.i. cells were subjected to multiple staining for nuclei, DNA break, and RSV growth using DAPI, TUNEL assay, and anti-RSV antibody (immunofluorescence, IF), respectively as shown.

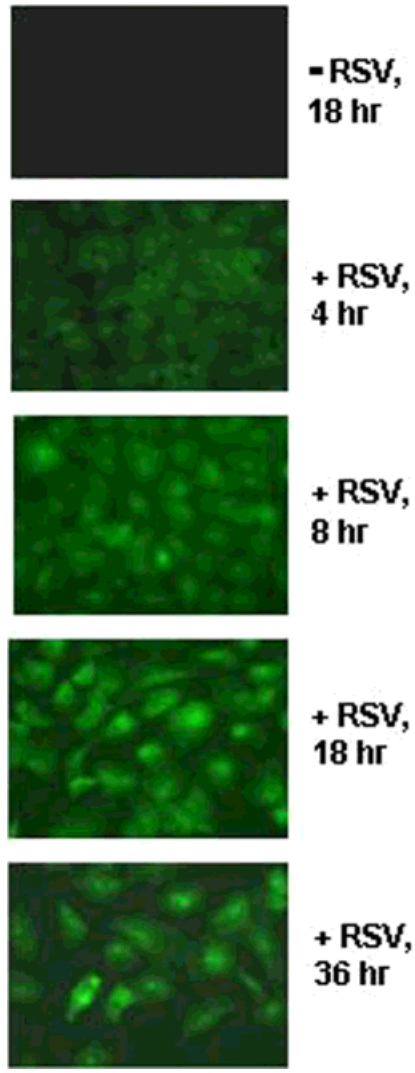


Fig. 3. Kinetics of the TUNEL assay. At indicated times after infection with RSV, A549 monolayers were probed for nuclear DNA breaks by TUNEL assay as described in Materials and Methods. Uninfected control cells, otherwise treated identically, are shown on top.

Uninfected A549 monolayers grown on semipermeable membrane inserts (Nunc polycarbonate tissue-culture inserts, from Fisher Scientific) were then placed inside the wells containing the infected monolayer. The infected and uninfected cells were thus separated only by the membranes that had pores of 0.2 μM diameter. These pores allow passage of small molecules, cytokines, and proteins, but retain cells. Cells of both the monolayers were harvested at various times and analyzed for DNA fragmentation. While the DNA of the infected cells showed the usual fragmentation pattern (Fig. 4), DNA of the uninfected cells remained intact. Together, these results suggested that apoptosis is most likely due to a direct effect of infection and not triggered by a diffusible product liberated from a few initially infected cells.

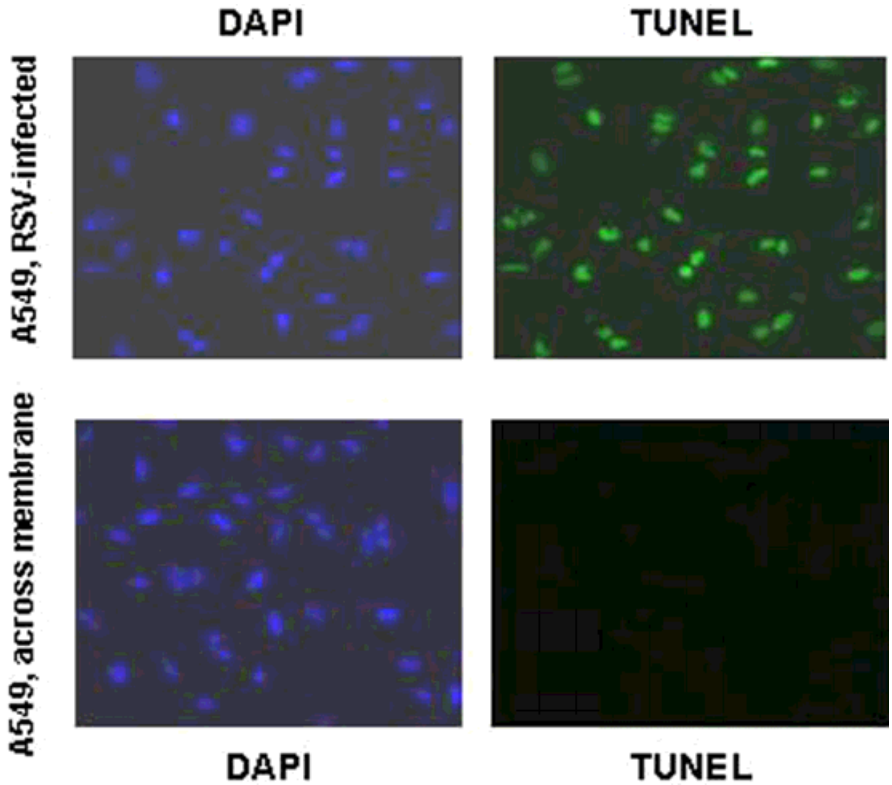


Fig. 4. Apoptosis is a direct effect of RSV infection. Two monolayers of A549 cells were grown seaprated by a semipermeable membrane as described under Results. One monolayer was infected with RSV, and at 18 h p.i. both were analyzed for apoptosis by TUNEL assay as shown. Nuclei were stained with DAPI to indicate their locations. Note that only the infected cell nuclei stained TUNEL-positive.

Limited Role of the Fas-FasL Pathway in RSV-Mediated Apoptosis

Since RSV infection was found to upregulate Fas receptors on A549 cell membrane [O'Donnell et al., 1999], the first logical question was what serves as the ligand for these receptors. Thus, we investigated whether FasL, a natural activator of the Fas receptor, is also activated upon RSV infection of these cells. A time course of the FasL levels determined by immunoblot analysis clearly revealed a rapid induction of FasL protein upon RSV infection (Fig. 5). Within 2 h of infection, a nearly three-fold higher amount of FasL was detected in the infected cells compared to the uninfected ones, which rose to about five-fold by 4 h. Combining these results with the previous ones [O'Donnell et al., 1999], we conclude that RSV infection upregulates both the Fas receptor (CD95) and the Fas ligand (FasL) in A549 cells.

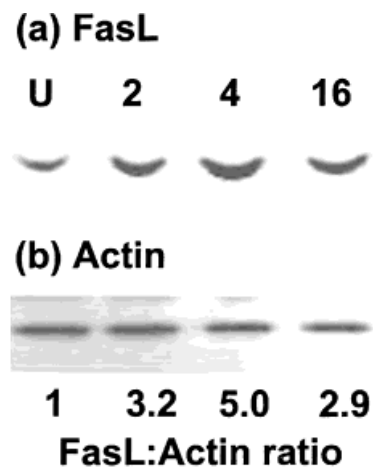


Fig. 5. Induction of FasL in RSV-infected cell. Western blot analyses of (**a**) FasL and (**b**) actin (control) were performed on extracts of uninfected (U) and RSV-infected A549 cells harvested at the hours post-infection (2, 4, and 16 h) indicated on the top. The relative intensities of the bands were determined by densitometry and the fold induction of FasL over actin is presented at the bottom.

In an attempt to determine the relative importance of the Fas–FasL pathway in RSV-induced apoptosis, we decided to inhibit this pathway using the transdominant negative FADD mutant. FADD is a death-domain protein that serves as a conduit for death signals from Fas-family receptors that include the TNF receptor [Nagata, 1999]. A well-characterized deletion mutant of FADD, NFD4 (hereon referred to as FADD-DN), lacks 80 N-terminal amino acids, but contains the death domain responsible for association with the related death domain of Fas/CD95 [Chinnaiyan et al., 1995]. Thus, FADD-DN is able to interact with Fas, but failed to initiate apoptosis, thereby acting as a transdominant negative inhibitor of apoptotic signaling through Fas. As shown in Table I, optimal expression of FADD-DN produced only a modest inhibition of apoptosis, resulting in the killing of about 80% cells as opposed to 94% in wild-type FADD-expressing cells. A robust expression of FADD-DN protein in the transfected cells was ascertained by immunobloting with anti-AU antibody [Chinnaiyan et al., 1995; data not shown]. These results suggested a limited role of the Fas death system, if any, in RSV-induced apoptosis of A549 cells.

RSV Infection Weakly Activates Cellular Caspases-3, -8, but not -9, -10

In an attempt to further understand the mechanism of RSV-induced apoptosis, we next measured the enzymatic activity of the caspases-3, -8 and -9 in RSV-infected A549 cells by using a colorimetric reaction as detailed under Materials and Methods. Activated caspase-10 was estimated by the available immunoblot assay (Western blot) using antibodies that recognize the processed, activated caspase-10 (59 kDa), as described under Materials and Methods. A small but reproducible increase in the levels of caspase-3 and -8, but not of caspase-9 or -10, was observed after 18 h of infection. The increases for caspases-8 and -3 were about 1.8 fold and 1.5 fold, respectively, over the uninfected controls (data not shown).

To determine whether the small amounts of activated caspase-8 and caspase-3 have any role in RSV-induced caspases, we tested the effect of caspase inhibitors on RSV-mediated apoptosis by analyzing DNA laddering as well as nuclear TUNEL assays. In the absence of any inhibitor, about 95% cells were TUNEL-positive (apoptotic), whereas the pan-caspase inhibitor z-VAD-fmk lowered this number to 5%, which is near background (4%, for uninfected cells). The inhibitor of caspase-3 (Ac-DEVD-CHO) also had a strong effect and reduced apoptosis to about 8% cells (Table I). In contrast, the inhibitor of caspase-8 (Ac-IETD-CHO) had a very modest effect even at optimal concentration, and allowed apoptosis in about 80% cells (Table I). Higher concentrations of all three caspase inhibitors did not result in any further inhibition of apoptosis, but instead started to inhibit RSV replication, as evidenced by a lower amount of viral proteins detected by immunoblot (data not shown). Together, these results and those of the FADD-DN studies suggest that the Fas-FADD-triggered caspase-8 does not play a major role in RSV-mediated apoptosis. However, other caspases, including caspase-3 may play a significant role.

RSV Infection Strongly Activates Caspase-12, a Stress-Induced ER Caspase

Caspase-12, originally discovered in mice, is a member of the ICE (interleukin 1-β converting enzyme) subfamily of caspases that includes caspases-1, -4, -5, and -9. Pioneering recent studies by Yuan and colleagues have revealed some of the unique features of caspase-12 [Nakagawa et al., 2000; reviewed by Mehmet, 2000]. It is a resident of the endoplasmic reticulum (ER) and is proteolytically activated by ER stress, including disruption of ER calcium homeostasis and accumulation of excess proteins in ER, but not by membrane- or mitochondrial-targeted apoptotic signals [Nakagawa et al., 2000]. In the same study, activated caspase-12 was also shown to cause amyloid-β neurotoxicity, which is characteristic of Alzheimer's disease. The status of expression of caspase-12 in human lung or in epithelial cells (such as A549) remained undetermined.

We have recently found that RSV infection causes ER stress (manuscript in preparation). A common indicator of ER response to stress is the upregulation of ER resident chaperones such as BiP (grp78) [Nakagawa et al., 2000; Pahl and Baeuerle, 1997]. Representative results in Figure 6a and b show that the expression of two ER chaperones, viz., BiP and calnexin, was strongly stimulated in RSV-infected A549 cells. This result prompted us to

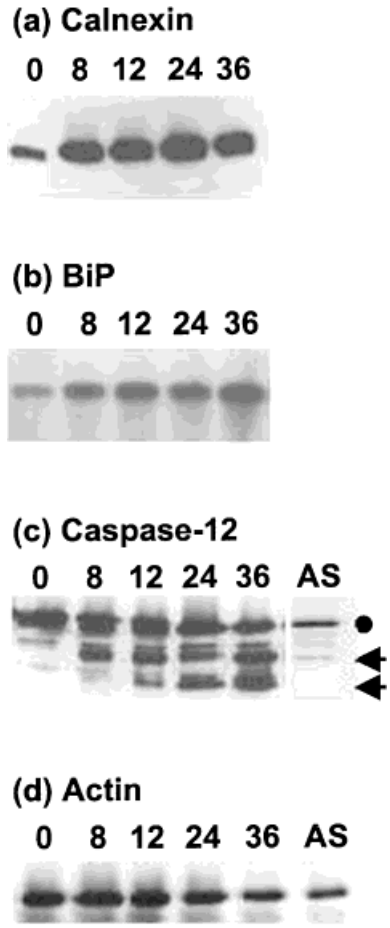


Fig. 6. ER stress and caspase-12 activation by RSV infection. Western blots of (**a**) calnexin, (**b**) BiP (grp78), (**c**) caspase-12 and (**d**) actin (as control) were performed on extracts of RSV-infected cells at the indicated hours post-infection. The procaspase-12 band (Mr 60 kDa) is indicated by the dot, and the processed bands (Mr ~40 and 25 kDa), by arrowheads. AS means antisense ODN-treated cells at 36 h p.i.

investigate whether RSV infection leads to activation of caspase-12. Immunoblot analyses (Fig. 6c) shows that the 60 kDa procaspase-12 was detected in uninfected as well as RSV-infected A549 cells, whereas the cleaved caspase-12 (~40 kDa) was detected in the RSV-infected cell only. These results demonstrated that caspase-12 is indeed expressed in lung epithelial cells and that it is strongly activated by RSV infection.

We then tested whether caspase-12 may play a role in RSV-induced apoptosis. Since no specific inhibitor of caspase-12 has yet been reported, we took advantage of an antisense technology that has been used successfully [Nakagawa et al., 2000]. In brief, a specific oligodeoxynucleotide (ODN) antisense to the translation start region of the caspase-12 mRNA abrogated caspase-12 function and resultant apoptosis in cortical neurons [Nakagawa et al., 2000]. A control "scrambled" ODN had no effect. We, therefore, employed the same ODNs in our studies, and a summary of the results is presented in Table I (bottom). Pre-treatment of A549 cells with the antisense ODN had a pronounced effect on RSV-mediated apoptosis and lowered the number of apoptotic cells by roughly four-fold (75% reduction of apoptosis). The scrambled ODN, as expected, had no significant effect. These results suggest that caspase-12 may play a major role in RSV-induced apoptosis.

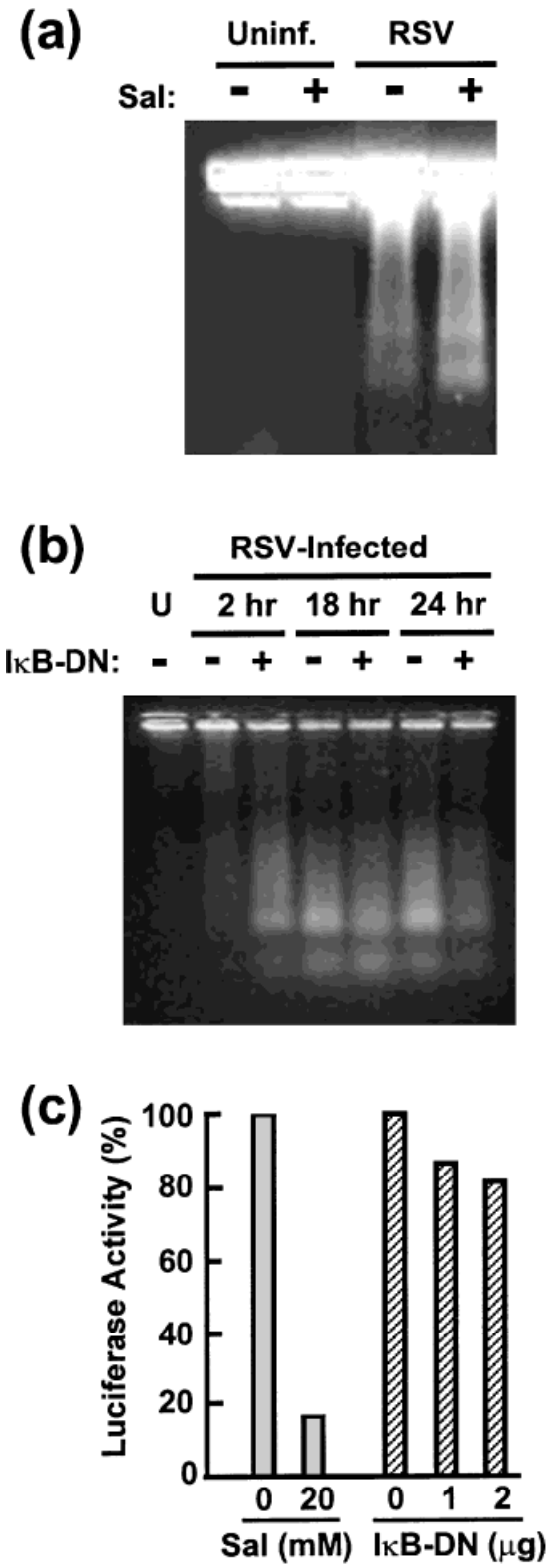
RSV-Activated NF-κB Does Not Modulate RSV-Induced Apoptosis

Our studies at this point indicated that RSV induces apoptosis in A549 cells. As we mentioned earlier, RSV also activates NF-κB in these cells. It was, therefore, reasonable to ask if these two interactions of RSV with the host are related; in other words, if the NF-κB is involved in apoptosis.

To test this, we have used two different inhibitors of NF-κB: salicylate [Kopp and Ghosh, 1994] and a dominant IκBα mutant [Traenckner et al., 1995]. The exact mechanism of action of the salicylate is not known although it prevents degradation of the IκBα [Kopp and Ghosh, 1994]. In the IκBα mutant, mutation of both Ser32 and Ser36 to Ala abrogated the ability of the IκBα to be phosphorylated and degraded from NF-κB/IκB complex [Traenckner et al., 1995; Van Antwerp, 1996]. RSV-mediated activation of NF-κB was shown to be strongly inhibited by salicylate [Bitko et al., 1997]; however, inhibition by the Ser32,36Ala mutant IκBα appeared to be modest and variable [Fiedler and Wernke-Dollries, 1999; Thomas et al., 1998].

We confirmed the expression of the mutant IκBα by Western blotting as well as immunostaining using an antibody against IκBα [Bitko and Barik, 1998; data not shown]. The inhibitory activities of salicylate and the mutant IκBα were directly tested under our experimental conditions by monitoring the intracellular NF-κB activity using the luciferase reporter assay [Bitko et al., 1997]. Results (Fig. 7c) showed that while the mutant IκBα only marginally

inhibited NF-κB, salicylate caused a strong inhibition, confirming earlier reports [Bitko et al., 1997; Fiedler and Wernke-Dollries, 1999]. As shown in Table I and Figure 7, neither the mutant IκBα nor salicylate had any effect on the induction of cell death by RSV. Together, results presented in this section suggest that RSV-activated NF-κB is not required for the RSV-induced apoptotic response.



DISCUSSION

Apoptotic cell death participates in the pathogenesis of several metabolic and infectious diseases [Song and Steller, 1999]. Studies of O'Donnell et al. [1999] and those reported here confirm apoptosis of lung epithelial cells (A549) by RSV. Although we did not present the results for space constraints, we have also observed extensive apoptosis in primary cells of normal human bronchial epithelial origin (NHBE cells from Clonetics, San Diego, CA). Our major conclusions regarding RSV-induced apoptosis of A549 cells are: (a) Fas receptor [O'Donnell et al., 1999] and Fas ligand (Fig. 5) are upregulated; however, the Fas pathway does not play a significant role in RSV-induced apoptosis (Table I). (b) This is further supported by the finding that cytoplasmic caspase-8, a cytoplasmic effector of the Fas pathway, has little role in apoptosis (Table I). (c) Caspase-3, which is further downstream, is required for apoptosis. (d) NF-κB activated by RSV is not required for apoptosis (Fig. 7 and Table I). Finally, (e) Caspase-12 is activated to a greater extent (Fig. 6), most likely as a result of viral stress on the ER, and plays an important role in apoptosis (Table I).

The contribution of RSV-induced apoptosis to the injury and pathology of RSV-infected lung cannot be over-emphasized. It is not clear to us why Takeuchi et al. [1998] did not observe RSV-mediated apoptosis; however, O'Donnell et al. [1999] have already offered a few possible explanations. In brief, the observed discrepancy could result from a subtle but critical change in any or all of the following contributing factors: (i) Exact composition of the virus preparation: RSV is known to package specific

Fig. 7. Lack of requirement of NF-κB in RSV-induced apoptosis. The effect of Na-salicylate (**a**) and the dominant negative (DN) IκBα mutant (**b**) on RSV-induced apoptosis of A549 cells was tested as detailed under Results. Representative DNA laddering results are shown (**c**): represents NF-κB activity assays in inhibitor-treated cells using the pBIIxluc reporter plasmid [Bitko et al., 1997]. Note the strong inhibition by salicylate and very little inhibition by the IκBα mutant.

cell components [Burke et al., 1998, 2000]. It is possible that some of them are potent apoptotic molecules such as cytokines, and that their amounts may vary depending on the exact procedure of virus growth, cell lysis, and virion purification. (ii) Factors in the particular formulation of the cell culture medium may influence RSV-induced apoptosis. Lastly, (iii) the cell line itself (A549) may have some variability from one laboratory to another. It is interesting to note here that HEp-2 cells have long been considered as nasopharyngeal carcinoma cells of epithelial nature and traditionally used to grow RSV ex vivo. However, at least certain stocks of HEp-2 cells are in fact HeLa cells, possibly as a result of an inadvertent contamination, and are, therefore, essentially fibroblasts (ATCC cell number CCL-23). It will be interesting to see if some RSV-susceptible cell lines are apoptosis-resistant, which may eventually lead to the identification of a novel cellular apoptotic factor.

In the last few years, a family of cysteine proteases named caspases, highly related to the interleukin-1-converting enzyme (ICE) and the proapoptotic CED-3 gene of *Caenorhabditis elegans*, have emerged as important mediators of the apoptotic process [Humke et al., 1998, and references therein]. The vast majority of caspases exist in the form of inactive proenzymes that are processed to form the active enzyme. Different apoptotic signals are believed to activate "initiator" caspases such as the cytosolic caspase-8 (also known as FLICE) or the mitochondrial caspase-9 [Loeffler and Kroemer, 2000]. The activated initiator caspases in turn activate downstream "effector" or "executioner" caspases [Stennicke and Salvesen, 2000] such as caspase-3 through proteolytic cleavage. An effector caspase then activates specific DNases such as DFF [Liu et al., 1997, Sakahira et al., 1998], and the activated DFF breaks down the chromosomal DNA. Some stages of apoptosis are known to produce morphological changes in the nuclei without major DNA damage [Sakahari et al., 1999]. The combination of the TUNEL assay and the DNA ladder, as described here, confirm internucleosomal fragmentation of DNA in RSV-infection.

The major mechanism of activation of these two caspases utilizes the following signaling pathway: FasL→Fas (CD95)→FADD→caspase-8→caspase-3 [Nagata, 1999]. Although RSV infection activated all of these signaling molecules to one extent or another, the inhibitor experiments documented that only the caspase-3 molecule of this pathway plays an important role in apoptosis. The corollary is that caspase-3 may be activated through a caspase other than caspase-8. Since caspase-12 is activated and required for apoptosis, it is quite possible that it is responsible for the processing and activation of caspase-3 in RSV-infected cells. As mentioned earlier, caspase-12 is a relatively new discovery [Nakagawa et al., 2000], and its relationship with other caspases is yet to be determined [Mehmet, 2000]. We propose that in the RSV-infected cells, caspase-12 may be an "initiator", and caspase-3, an "effector" caspase that in turn activates DFF. This is testable.

The lack of the Fas pathway in RSV-induced apoptosis was also surmised in previous studies. As mentioned, addition of anti-CD95 antibody led to an increase (two-fold) in the number of apoptotic cells in RSV-infected cell culture, leading to the suggestion that RSV may use a Fas-independent pathway [O'Donnell et al., 1999]. Furthermore, carcinoma cells (such as A549) in general are known to be resistant to FasL-mediated killing. A549 cells, in particular, do not undergo appreciable apoptosis when exposed to TNF-α or anti-CD95 [Nambu et al., 1998]. The mitochondrial pathway of apoptosis is also ruled out because of a lack of activation of caspase-9.

In unstimulated lung epithelial cells, NF-κB is present in the cytoplasm in complex with a family of inhibitory proteins (IκB), of which the two major members are IκBα and IκBβ [recently reviewed by Foo and Nolan, 1999]. Following stimulation, IκBα is phosphorylated and then degraded by as yet uncharacterized protease(s), resulting in the translocation of NF-κB to the nucleus. The activated NF-κB participates in a variety of cellular processes, such as activation of cytokine genes, stress response, and apoptosis. NF-κB may either stimulate or suppress apoptosis, perhaps depending on the cell and the signaling pathway. Pioneering studies showed that NF-κB suppressed apoptosis by the tumor necrosis factor, TNF [Baichwal and Baeuerle, 1997]; thus, inhibition of NF-κB, as mentioned earlier, leads to TNF-mediated apoptosis. The anti-apoptotic function of NF-κB has in fact been exploited by other mechanisms; in a recent example, the

hepatitis C virus core protein inhibited Fas- and TNFα-mediated apoptosis via NF-κB activation [Marusawa et al., 1999]. In apparent contrast, NF-κB played a pro-apoptotic role in many systems, such as in apoptosis of bone marrow [Abbadie et al., 1993, Grimm et al., 1996] and neurons [Qin et al., 1999], hydrogen peroxide-induced apoptosis of T cells [Dumont et al., 1999], and dengue and Sindbis virus-infected cells [Lin et al., 1995, Marianneau et al., 1997]. Interestingly, RSV provides a unique situation whereby it activates NF-κB as well as apoptosis; however, the two events appear to be uncoupled. In other words, although NF-κB is activated by RSV, it plays no detectable role in RSV-induced apoptosis. This is reminiscent of a fibroblast cell line, L929, in which TNFα-induced cell killing was not affected by regulating the activity of NF-κB [Hehner et al., 1998]. RSV-mediated activation of NF-κB has recently been shown to be incompletely resistant to the dominant negative IκBα mutant that we have used here [Fiedler and Wernke-Dollries, 1999; Thomas et al., 1998], and our results confirm it (Fig. 7c). One can argue that this is the reason why the mutant failed to inhibit RSV-induced apoptosis (Fig. 7b and Table I). However, salicylate, which was able to inhibit RSV-mediated activation of NF-κB (Fig. 7c) [Bitko et al., 1997], also failed to inhibit apoptosis (Fig. 7a and Table I). Clearly, RSV-induced apoptotic pathway may include novel signaling steps that do not share molecule(s) normally regulated by NF-κB, and it is tempting to speculate that an overlapping or similar pathway may also be shared in TNF-mediated killing of L929 cells. A detailed elucidation of both pathways is clearly needed.

Our finding that RSV infection activates ER caspase-12 and that the inhibition of caspase-12 substantially abrogates RSV-mediated apoptosis, opens up new avenues of research in this area. As mentioned earlier, caspase-12 is a newly discovered enzyme and its downstream substrates have not yet been characterized. By the same token, it is not known whether caspase-12 functions as an initiator or an effector caspase, or both. The other non-mitochondrial members of the ICE subfamily, namely caspases-1, -4, and -5, have the potential to act as either initiator or effector [Esolen et al., 1995; Song and Steller, 1999; Stennicke and Salvesen, 2000]. It will also be important to determine how the RSV infection generates stress in the ER. RSV codes for three major glycoproteins, F, G, and SH, which are structural components of the virion. F and G, in particular, play essential roles in infection, are synthesized in relatively large amounts in the RSV-infected cell, and are processed through the ER [Anderson et al., 1992; Ding et al., 1987]. We hypothesize that the large flux of these viral glycoproteins through the ER is a major cause of ER stress [Pahl and Baeuerle, 1997]. This is currently under investigation. Moreover, knockout mice mutants deficient in caspase-8 (*Lpr* mutant), caspase-3 (*Gld* mutant), or caspase 12 are available [Nagata and Suda, 1995; Nakagawa et al., 2000]. These mutants can be used to further confirm the role (or lack thereof) of these caspases in viral apoptosis.

Lastly, we conclude that RSV-induced cell death may be a major cause of destruction of the lung epithelia in infected individuals. A substantial remodeling of the scarred epithelia may explain the symptoms of RSV infection that are not much unlike the emphysema characteristic of tobacco smokers, such as an impaired respiratory exchange and a predisposition to shortness of breath and asthma. We propose that a comparative study of emphysema and RSV pathogenesis may lead to revealing insights into both disorders. Such studies are in progress.

ACKNOWLEDGMENTS

Generous help of a number of investigators made this study possible. We are particularly indebted to: Dr. Junying Yuan (Harvard Medical School) for the anti-caspase-12 monoclonal antibody; Dr. Xiaodong Wang (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center) for the anti-DFF45 antibody; Dr. Vishva Dixit (Genentech) for the FADD clones that were provided to us by Dr. Claudius Vincenz (University of Michigan, Ann Arbor); Dr. Sankar Ghosh (HHMI, Yale University) for the mutant IκBα expression clone; and Dr. Anthony Gard (Department of Cell Biology and Neurosciences) for the use of his microscope.

REFERENCES

Abbadie C, Kabrun N, Bouali F, Smardova J, Stehelin D, Vandenbunder B, Enrietto PJ. 1993. High levels of c-rel

expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells in vitro. Cell 75:899–912.

Anderson K, Stott EJ, Wertz GW. 1992. Intracellular processing of the human respiratory syncytial virus fusion glycoprotein: amino acid substitutions affecting folding, transport and cleavage. J Gen Virol 73:1177–1188.

Anderson LJ, Heilman CA. 1995. Protective and disease-enhancing immune responses to respiratory syncytial virus. J Infect Dis 171:1–7.

Baichwal VR, Baeuerle PA. 1997. Activate NF-kappa B or die? Curr Biol 7:R94–96.

Bartz SR, Emerman M. 1999. Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. J Virol 73:1956–1963.

Beg AA, Baltimore D. 1996. An essential role for NF-kappa B in preventing TNF-alpha-induced cell death. Science 274:782–784.

Bitko V, Barik S. 1998. Persistent activation of RelA by respiratory syncytial virus involves protein kinase C, underphosphorylated IkappaBβ, and sequestration of protein phosphatase 2A by the viral phosphoprotein. J Virol. 72:5610–5618.

Bitko V, Velazquez A, Yang L, Yang YC, Barik S. 1997. Transcriptional induction of multiple cytokines by human respiratory syncytial virus requires activation of NF-kappa B and is inhibited by sodium salicylate and aspirin. Virology 232:369–378.

Burke E, Dupuy L, Wall C, Barik S. 1998. Role of cellular actin in the gene expression and morphogenesis of human respiratory syncytial virus. Virology 252:137–148.

Burke E, Mahoney NM, Almo SC, Barik S. 2000. Profilin is required for optimal actin-dependent transcription of respiratory syncytial virus genome RNA. J Virol 74:669–675.

Chini BA, Fiedler MA, Milligan L, Hopkins T, Stark JM. 1998. Essential roles of NF-kappa B and C/EBP in the regulation of intercellular adhesion molecule-1 after respiratory syncytial virus infection of human respiratory epithelial cell cultures. J Virol 72:1623–1626.

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81:505–512.

Ding MX, Wen DZ, Schlesinger MJ, Wertz GW, Ball LA. 1987. Expression and glycosylation of the respiratory syncytial virus G protein in *Saccharomyces cerevisiae*. Virology 159:450–453.

Duesberg P, Rasnick D. 1998. The AIDS dilemma: drug diseases blamed on a passenger virus. Genetica 104:85–132.

Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML. 1999. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappa B. Oncogene 18:747–757.

Esolen LM, Park SW, Hardwick JM, Griffin DE. 1995. Death by design: mechanism and control of apoptosis. J Virol 69:3955–3958.

Everett H, McFadden G. 1999. Apoptosis: an innate immune response to virus infection. Trends Microbiol 7:160–165.

Fiedler MA, Wernke-Dollries K. 1999. Incomplete regulation of NF-kappa B by IkappaBα during respiratory syncytial virus infection in A549 cells. J Virol 73:4502–4507.

Fiedler MA, Wernke-Dollries K, Stark JM. 1996. Inhibition of viral replication reverses respiratory syncytial virus-induced NF-kappa B activation and interleukin-8 gene expression in A549 cells. J Virol 70:9079–9082.

Foo SY, Nolan GP. 1999. NF-kappa B to the rescue: RELs, apoptosis and cellular transformation. Trends Genet 15:229–235.

Garofalo R, Sabry M, Jamaluddin M, Yu RK, Casola A, Ogra PL, Brasier AR. 1996. Transcriptional activation of the interleukin-8 gene by respiratory syncytial virus infection in alveolar epithelial cells: nuclear translocation of the RelA transcription factor as a mechanism producing airway mucosal inflammation. J Virol 70:8773–8781.

Grimm S, Bauer MK, Baeuerle PA, Schulze-Osthoff K. 1996. Bcl-2 down-regulates the activity of transcription factor NF-kappa B induced upon apoptosis. J Cell Biol 134:13–23.

Hayes JA. 1976 In: E. Weiss, and M.S. Segal, editors, Bronchial Ashtma, Little Brown: Boston.

Hehner SP, Hofmann TG, Ratter F, Dumont A, Droge W, Schmitz ML. 1998. Tumor necrosis factor-alpha-induced cell killing and activation of transcription factor NF-kappa B are uncoupled in L929 cells. J Biol Chem 273:18117–18121.

Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D. 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J Virol 68:3667–3673.

Humke EW, Ni J, Dixit VM. 1998. ERICE, a novel FLICE-activatable caspase. J Biol Chem 273:15702–15707.

Kopp E, Ghosh S. 1994. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 265:956–959.

Lin KI, Lee SH, Narayanan R, Baraban JM, Hardwick JM, Ratan RR. 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. J Cell Biol 131:1149–1161.

Liu X, Zou H, Slaughter C, Wang X. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184.

Loeffler M, Kroemer G. 2000. The mitochondrion in cell death control: certainties and incognita. Exp Cell Res 256:19–26.

Marianneau P, Cardona A, Edelman L, Deubel V, Despres P. 1997. Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. J Virol 71:3244–3249.

Marusawa H, Hijikata M, Chiba T, Shimotohno K. 1999. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappa B activation. J Virol 73:4713–4720.

Mastronarde JG, He B, Monick MM, Mukaida N, Matsushima K, Hunninghake GW. 1996. Induction of interleukin (IL)-8 gene expression by respiratory syncytial virus involves activation of nuclear factor (NF)-kappa B and NF-IL-6. J Infect Dis 174:262–267.

McCloskey TW, Ott M, Tribble E, Khan SA, Teichberg S, Paul MO, Pahwa S, Verdin E, Chirmule N. 1997. Dual

role of HIV Tat in regulation of apoptosis in T cells. J Immunol 158:1014–1019.
Mehmet H. 2000. Caspases find a new place to hide. Nature 403:29–30.
Nagata S. 1999. Fas ligand-induced apoptosis. Annu Rev Genet 33:29–55.
Nagata S, Suda T. 1995. Fas and Fas ligand: *lpr* and *gld* mutations. Immunol Today 16:39–43.
Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403:98–103.
Nambu Y, Hughes SJ, Rehemtulla A, Hamstra D, Orringer MB, Beer DG. 1998. Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinomas. J Clin Invest 101:1102–1110.
Negri C, Donzelli M, Bernardi R, Rossi L, Burkle A, Scovassi AI. 1997. Multiparametric staining to identify apoptotic human cells. Exp Cell Res 234:174–177.
O'Donnell DR, Milligan L, Stark JM. 1999. Induction of CD95 (Fas) and apoptosis in respiratory epithelial cell cultures following respiratory syncytial virus infection. Virology 257:198–207.
Ouaaz F, Li M, Beg AA. 1999. A critical role for the RelA subunit of nuclear factor kappaB in regulation of multiple immune-response genes and in Fas-induced cell death. J Exp Med 189:999–1004.
Pahl HL, Baeuerle PA. 1997. The ER-overload response: activation of NF-kappa B. Trends Biochem Sci 22: 63–67.
Park DJ, Patek PQ. 1998. Detergent and enzyme treatment of apoptotic cells for the observation of DNA fragmentation. Biotechniques 24:558–560.
Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, Chase TN. 1999. Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J Neurosci 19:4023–4033.
Sakahira H, Enari M, Nagata S. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99.
Sakahira H, Enari M, Ohsawa Y, Uchiyama Y, Nagata S. 1999. Apoptotic nuclear morphological change without DNA fragmentation. Curr Biol 9:543–546.
Song Z, Steller H. 1999. Death by design: mechanism and control of apoptosis. Trends Biochem Sci 24:M49–52.
Stennicke HR, Salvesen GS. 2000. Caspases—Controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477:299–306.
Takeuchi R, Tsutsumi H, Osaki M, Haseyama K, Mizue N, Chiba S. 1998. Respiratory syncytial virus infection of human alveolar epithelial cells enhances interferon regulatory factor 1 and interleukin-1β-converting enzyme gene expression but does not cause apoptosis. J Virol 72:4498–4502.
Takizawa T, Matsukawa S, Higuchi Y, Nakamura S, Nakanishi Y, Fukuda R. 1993. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. J Gen Virol 74:2347–2355.
Tang D, Kidd VJ. 1998. Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. J Biol Chem 273:28549–28552.
Thomas LH, Friedland JS, Sharland M, Becker S. 1998. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on nuclear factor-kappa B nuclear binding and is inhibited by adenovirus-mediated expression of inhibitor of kappa B α. J Immunol 161:1007–1016.
Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle P. 1995. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls Ikappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J 14:2876–2883.
Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274:787–789.
Vincenz C, Dixit VM. 1997. Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. J Biol Chem 272:6578–6583.
Wang CY, Mayo MW, Baldwin AS Jr. 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 274:784–787.
Wang SZ, Smith PK, Lovejoy M, Bowden JJ, Alpers JH, Forsyth KD. 1998. The apoptosis of neutrophils is accelerated in respiratory syncytial virus (RSV)-induced bronchiolitis. Clin Exp Immunol 114:49–54.