International Reviews of Immunology, 22: 425–449, 2003

ISSN: 0883-0185 print/1563-5244 online.
DOI: 10.1080 / 08830180390211303

Taylor & Francis
healthsciences

INFLUENZA VIRUS INDUCTION OF APOPTOSIS BY INTRINSIC AND EXTRINSIC MECHANISMS

R. JOEL LOWY
Armed Forces Radiobiology Research Institute, Bethesda, Maryland, USA

It is now firmly established that apoptosis is an important mechanism of influenza virus-induced cell death both in vivo and in vitro. Data are predominately from experiments with influenza A virus and in vitro experimental systems. Multiple influenza virus factors have been identified that can activate intrinsic or extrinsic apoptotic induction pathways. Currently there is no evidence for influenza virus directly accessing the apoptosis execution factors. The best-studied influenza virus inducers of apoptosis are dsRNA, NS1, NA, and a newly described gene product PB1-F2. PB1-F2 is the only influenza virus factor to date identified to act intrinsically by localization and interaction with the mitochondrial-dependent apoptotic pathway. Both dsRNA and NA have been shown to act via an extrinsic mechanism involving proapoptotic host-defense molecules: PKR by induction of Fas-Fas ligand and NA by activation of TGF-β. PKR is capable of controlling several important cell-signaling pathways and therefore may have multiple effects; a predominant one is increased interferon (IFN) production and activity. NS1 has been shown to be both proapoptotic and antiapoptotic. Use of influenza virus NS1 deletion mutants has provided evidence for NS1 interference with apoptosis, IFN induction, and related cell-signaling pathways. Influenza virus also has important exocrine paracrine effects, which are likely mediated via TNF family ligands and oxygen, free radicals capable of inducing apoptosis. Little is known about activation of inhibitors of apoptosis such as inhibitory apoptotic proteins. Whether all these factors always have a role in influenza virus-induced apoptosis is unknown. The kinetics of synthesis of influenza virus factors affecting apoptosis during the replication cycle may be an important aspect of apoptosis induction.

Keywords: influenza virus, apoptosis, mechanism, induction

Study of the induction of apoptosis by influenza virus [1,2], as for other viruses, has become a central issue in developing our understanding of

Support for R. J. Lowy was from the Armed Forces Radiobiology Research Institute.
Address correspondence to R. Joel Lowy, Armed Forces Radiobiology Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5603, USA. E-mail: lowy@afrri.usuhs.mil

the mechanisms of pathogenesis in vivo and in vitro [3–9]. Several of these reviews contain thorough descriptions and diagrams of the general apoptotic pathways and should be consulted to clarify the following discussion. The apoptosis induction/activation pathways are divided into the intrinsic mitochondrial-dependent and the extrinsic death receptor–dependent pathways that are thought to converge to specific cysteine proteases, caspase-9 and -8, respectively [3,4,10]. These in turn activate caspase-3, and possibly others, which begins the effector phase of the pathway. Activation of the effector phase, to date, is considered irreversible [11]. Complicating matters in understanding influenza virus-induced apoptosis is that infection induces a large list of host cell responses that in other systems, viral and nonviral, are implicated in activation of one or both of these general induction mechanisms. The complexity of cellular factors putatively involved is an important problem in understanding apoptosis in general. Furthermore, because these factors regulate the two primary, but possibly linked, apoptosis pathways, there is ample potential for multiple interacting excitatory and inhibitory control points [3,10]. Part of this complexity is reflected in observations that suggest that viral and other inducing agents' impact on apoptotic processes can be context sensitive [12–16]. Therefore, increasingly, like other complex metabolic pathways, virus interactions with cellular processes resulting in apoptosis are probably best viewed as a network rather than a simple set of linearly linked enzymes. Molecular network techniques may prove to be an important development in further understanding influenza virus-host interactions. To simplify this discussion, influenza virus factors will be, insofar as possible, associated with particular apoptotic mechanisms. However, cross-talk between apoptotic pathways and the possibility of influenza virus factors having as-yet-undiscovered multiple actions should be kept in mind.

The framework used for this article divides influenza virus-induced apoptosis into (1) intrinsic mechanisms, which can be either direct or indirect, and (2) extrinsic mechanisms, which can be either autocrine or paracrine [10]. Direct intrinsic mechanisms will be those in which, in influenza virus, macromolecules interact directly with unique apoptotic pathways, either the direct effectors of apoptosis (execution factors) or upstream control points of induction/activation pathways unique to apoptosis. Indirect intrinsic mechanism will be considered to be those in which influenza virus macromolecules interact intracellularly with more general cell-signaling pathways and gene regulation process that, in turn, interact with apoptotic-specific pathways. Extrinsic mechanisms are those that occur when influenza virus infection induces the host cell to produce and secrete host defense

molecules through more general cell-signaling pathways. Once produced, these host defense molecules, in turn, can act on the infected cell in an autocrine manner, or on bystander cells in a paracrine fashion, or both. Extrinsic mechanisms are often, but not necessarily, mediated through receptor activation, especially the TRAIL (TNF-related apoptosis induction ligands) family of "death receptors" [3,17], with the action of oxygen free radicals being an example. Evidence exists primarily for activation via extrinsic autocrine pathways, but the host-cell signaling pathways already identified suggest the possibility that these other mechanisms exist. The purpose of this article is to describe what is currently known for each mechanism.

Not unsurprisingly, considering the relative recent interest in apoptosis as a mechanism for influenza virus pathogenesis, the distribution of information is not uniform. One additional complication is that not all studies, particularly early ones, provide unequivocal information allowing for a putative pathway to be assigned. In many instances only terminal markers were used, such as DNA fragmentation or nuclear condensation and/or fragmentation. These end-state markers are easily assayed and in earlier studies were virtually the only ones available. Therefore, in interpreting specific studies, it should be kept in mind that understanding of apoptosis pathways is continuing to evolve and for a given set of experiments markers may not have been known at the time. Even for identified markers, placement within the extrinsic or intrinsic activation or execution pathways may not have been well defined. Examples of these additional biochemical markers include intracellular events, such as mitochondrial depolarization; cytochrome c release; specific caspase activation, notably -8, -9 and -3; Apaf-1; and activation of members of the death-inducting signaling complex (DISC factors) associated with TRAIL receptors. Zeiosis, one of the most characteristic and defining features of apoptosis, as originally defined, is unfortunately difficult to observe [18,19]. It is a highly specialized form of cytokinesis, fragmenting the cytoplasm by release of small membrane-bound bodies. Most important apoptotic markers have now been demonstrated in vivo and in vitro for influenza virus-induced cell death. Zeiosis and nuclear and cytoplasmic condensation, combined with the characteristic maintenance of membrane integrity, were shown to occur during influenza A virus-induced cell death in some systems [20–22], as was sensitivity to caspase inhibitors [23–25] and PARP cleavage [25]. Nuclear condensation and nick-end DNA labeling (TUNEL assay) studies demonstrated that apoptosis occurs in vivo in the lungs, spleen, and thymus of infected mice [26] and in the vascular endothelium, liver, kidney, and brain of chickens [27]. The majority of information is from

human influenza A virus strains, with much more limited information on equine, avian, and B strains. Importantly, it is not yet clear where there is comparatively less information on a particular mechanism whether this reflects the mechanism's relative importance for influenza virus-induced apoptosis or the newness of this area of investigation and a need for further exploration.

EARLY STUDIES

The initial reports of influenza virus-induced apoptosis occurred in three experimental systems: leukocytes, MDCK cells, and HeLa cells. The conclusion that the mechanism of cell death was apoptosis and not necrosis was primarily based on the DNA fragmentation, resulting in the characteristic small nucleotide laddering pattern observed on electrophoretic gels, combined with limited observations of nuclear condensation in some cells from the infected cultures. In addition to describing the occurrence of the mechanism, these early studies made a number of important observations. Evidence indicated apoptosis was likely to be a general phenomenon for both influenza A virus and other orthomyoxvirus types, notably B, and not restricted to a limited number of sera types [28]. However, efficacy of different influenza virus sera types in inducing apoptosis varied. Strain variation in apoptotic induction was confirmed and used to address the issue of mechanism, using both natural strains and genetically engineered variants to a great advantage (see below). Apoptosis was dependent on replicating virus, as chemically or UV-inactivated virus failed to induce apoptosis [28–31]. As for other apoptotic systems, new protein synthesis was necessary, as cycloheximide treatment inhibited virus-induced DNA fragmentation. A critical time window of 2–4 h post-infection (pi) was identified for induction of apoptosis, but dependence on the virus, host factor protein synthesis, or both, was not distinguished [29]. Virus-induced apoptosis was blocked in an MDCK cell line stably transfected to express bcl-2. An importance of this observation was that it demonstrated, at least in MDCK cells, that influenza A virus induction acted via one of the general apoptotic control pathways being defined at the time, those involving the bcl-bax family of proteins. These molecules have subsequently been most strongly associated with the apoptotic pathway involving mitochondrial depolarization, release of cytochrome c, and formation of the Apaf-1 complex, which activates caspase-9. Furthermore, bcl-2 expression reduced the virus titer, spread in culture, and permitted eventual virus clearance, leading to the suggestion that apoptosis might facilitate influenza A virus production [32]. Infection of human

monocytes with A/PR8 was virtually nonproductive [30]. However, infection induced microvilli and pseudopodia loss, chromatin condensation, the characteristic apoptotic DNA fragmentation pattern, and high levels of internal vesicle formation. This latter phenomenon had been observed in macrophages undergoing apoptosis in response to other stimuli but has not been clearly reported for other cells and may be unique to monocytic cells [20,33]. As with other cell types, UV-inactivated virus failed to induce apoptosis. While the sum of these experiments demonstrated that influenza virus was inducing apoptotic cell death in cells that do and do not support influenza virus replication, it was not established whether intrinsic or extrinsic pathways were involved or which virus macromolecules had a major role.

CASPASE ACTIVATION

Studies of influenza virus apoptosis induction demonstrate that caspase activation occurs, and they also suggest that these host enzymes are important in both apoptosis and virus protein processing. Western blot analysis of Udorn/72-infected HeLa cells demonstrated a time-dependent increase in caspase-3 cleavage activity from 12 to 36 h pi [24]. In this same study the use of a broad caspase inhibitor (Z-VAD-fmk), or caspase-8 (z-IETD-fmk) or caspase-3 (Ac-DEVD-CHO) inhibitors, increased cell survival and decreased DNA fragmentation in a concentration-dependent fashion. Hydrolysable substrates demonstrated caspase-3 activation by A/Aichi/68 and B/HK/72 in MDCK cells [23] and WSN activation of caspase-8, but not -9, in fibroblast 3T3 L1 cells [34]. Activation of caspase-3 is evidence that influenza A virus, at least in part, uses the prototypical pathway for the execution phase of apoptosis. However, caspase-3 inhibition did not completely block apoptosis, nor was it as effective as the less specific broad caspase inhibitors. It remains to be determined whether this is evidence that influenza virus activates other effector caspases. Potentially, M1 protein may be an activator of caspase-8 but not -9, as M1–caspase-8 interaction has been demonstrated in protein binding studies [35]. Inhibition of apoptosis in MDCK cells by caspase inhibition differed from the effects of bcl-2 [32], as viral replication was not decreased; however, viral spread was not evaluated [24]. It was suggested that caspases may a have a role in the influenza virus replication of mammalian but not avian viruses. Caspases may be involved in influenza virus replication, based on evidence they are responsible for NP protein processing during synthesis, with caspase-3 inhibitors preventing the normal A and B virus NP intracellular cleavage [23]. If caspase-dependent cleavage is important or necessary for virus

replication, this may be a partial explanation for bcl-2 allowing nonimmune cells in vitro to clear themselves of infection [32]. Experiments with A/Beijing and human monocytes/macrophages in vitro demonstrate that production of IL-1β and IL-18 is dependent on influenza A virus-induced caspase [36]. This observation is very interesting in the context of the macrophage/monocyte apoptosis, where there is the potential for antiapoptotic caspases activity. Caspase-1 is not now included as a direct inducer or effector of apoptosis; however, it is important in its originally defined role of converting prointerleukines to active forms [10,37]. Withdrawal of both TNF and IL-1β was shown to be proapoptotic in monocytes, but the presence of either cytokine supported cell survival [38,39]. Taken together with the observation that caspases may have importance in influenza virus protein processing suggests that expanded roles for both host and viral factors need to be considered. In addition, modulating the activation and execution phases of apoptosis caspases may be involved in influenza virus replication.

INTRINSIC APOPTOSIS ACTIVATION

PB-F2

PB1-F2 is a newly described influenza virus gene product produced by an alternate reading frame coding of the influenza virus viral RNA polymerase subunit PB1 [2,40]. The peptide was discovered during screening for antigenic peptides recognized by $CD8^+$ T lymphocytes and had high affinity for the MHC $H\text{-}2D^b$ binding site. The peptide is derived from a +1 frame of an 87-residue open reading frame of the PB2 gene consisting of residues 62–70. Originally identified for influenza strain A/Puerto Rico/8/34 (H1N1), e.g., PR8, the 87 ORF was identified in 64 of 84 influenza A variants but not in influenza B viruses. MDBK cells infected with influenza A virus WSN7 + 1 were reported to first produce protein at 2 h pi, with maximal translation at 3 h pi, just before cell death was reported to begin at 3 h pi. Inhibiting proteosome degradation pathways extended the $t_{1/2}$ of 30 min and increased PB2-F2 detection. PB2-F2 also induced cell death in MDBK cells when added extracellularly at low concentration. Immunofluorescence assays of virus-infected MDBK cells, HeLa cells transfected with PB2-F2 cDNA, and MDBK cells microinjected with synthetic full-length peptide showed a strong mitochondrial localization, with low levels in the nucleus and cytoplasm. Electron microscopic immunocytochemistry showed both inner and outer leaflet localization. Changes in mitochondrial morphology were evident in many, but not all, PB2-F2 expressing cells, with elongated structures

changing to fragmented and more rounded ones. Loss of mitochondria membrane potential was shown by a membrane dye distribution assay. Use of HeLa cells constitutively expressing GFP-cytochrome c demonstrated PB2-F2–induced release of cytochrome c, which could be inhibited by coexpression of bcl-2. Influenza virus A/PR8 also induced changes in the apoptotic markers annexin V binding and mitochondrial depolarization in human primary monocytes and in the human monocyte cell line U937. Infection of these cells with A/PR8 having a mutated PB1-F2 resulted in a 50% reduction in cells having both markers. Decreases in PB2-F2 expression did not decrease apoptotic markers in MDCK, MDBC, or A549 cells, which support productive influenza A virus replication, or in HeLa cells, which do not. Taken together, these data well support the idea that PB1-F2 can activate the apoptotic pathway at the mitochondria regulator step. Modulation of mitochondrial potential, cytochrome c release, and bcl-2 antagonism of apoptosis are established indicators of interaction with this important apoptotic regulator pathway. Unfortunately, the molecular mechanisms of these intracellular physiological changes and their relationships to one another and the apoptotic pathway are still being defined. The ability of PB2-F2 to associate with cell membranes is important, as this is a characteristic shared with the bcl-bax family of apoptotic regulatory proteins. It leaves open the possibility that PB2-F2 may associate with integral mitochondria factors, the bcl-bax family members, or both.

EXTRINSIC APOPTOSIS ACTIVATION

Currently much of the information on influenza virus-induced apoptosis involves studies in which an extrinsic mechanism is postulated or implied. Influenza virus infection elicits the production of a typical spectrum of inflammatory cytokines [41–43], a list that increases with further investigation [44–48]. Notable are TNF, interferons type I (alpha/beta) and type II (gamma), and TGF-β, all generally considered proapoptotic. The production of free radical chemical species, known as reactive oxygen intermediates (ROI) or reactive oxygen species (ROS), is associated with influenza infection and tissue damage, especially in the lung [49]. Another indication of the complexity of influenza virus pathogenesis was the demonstration that influenza virus infection activated important transactivation factors, NF-κB and AP-1 [50]. NF-κB and AP-1 are established mediators of oxygen free radicals, stress kinases pathways, inflammatory cytokine response, and IFN, which in turn modulate apoptosis. Recently, progress in demonstrating more direct roles for important cell signaling pathways

in influenza virus pathogenesis has been reported. These are the stress-activated protein kinases, such as JNK/SAPK [51,52], MEK/MAP [53], and the dsRNA-dependent kinase PKR [5,44,46,54,55]. For some, but not all, of these host-cell transduction pathways a link to influenza A virus-induced apoptosis has been made. In viral-induced and other apoptotic induction systems it is suggested that pro- and antiapoptotic factor, such as TNF or NF-κB, activation may be context and/or kinetically dependent [12,13,16]. To add to this complexity, NF-κB is also important in that its activation in other experimental systems results in the induction of inhibitor apoptotic proteins (IAPs) [8,10]. Currently, there does not appear to be any published evidence for influenza virus induction of known IAP factors. Therefore, it is worthwhile to recall from the subsequent discussion that influenza virus factors have multiple entry points into cell activation pathways that putatively modulate apoptosis both up and down.

Fas and Fas Ligand

Part of the earliest work on influenza virus apoptosis induction implicated extrinsic autocrine mechanisms. Experiments with HeLa cells supported the idea that a pathway involving the Fas antigen (APO-1/CD 95) was sufficient for influenza A virus-induced apoptosis in these cells [56]. Infected HeLa cells expressed a large transient increase in Fas antigen as assayed by surface marker immunofluorescent FACS analysis. *De novo* synthesis was the likely mechanism as surface expression was preceded by virus-induced transient expression in Fas mRNA [57]. Furthermore, this work supported the idea that dsRNA was the virus factor that induced the host cell response, as polynucleotides, polyI-C, was also capable of causing cell surface Fas expression. However, unlike influenza A virus, antibody cross-linking of Fas elicited by polyI-C was required to induce cell death. Both virus- and polyI-C–induced death were partly blocked by Fas-neutralizing antibodies. This partial block was suggested to be evidence for the possibility of alternate influenza A virus apoptosis induction pathways not involving Fas. Cloning and expression of the Fas promoter in HeLa cells using a luciferase (luc) reporter system demonstrated the ability of influenza A virus to interact with and activate this important host-cell gene regulatory element [58]. Like Fas surface expression, activation was transient, occurring 1–6 h pi.

Because the cloned Fas promoter contained six NF-IL6 binding sites, activation of NF-IL6 was examined, and shown to occur and to be capable of stimulating the Fas promoter. Both influenza A virus and polyI-C activated NF-IL6 [58] but with very different kinetics.

The virus induction occurred earlier and was less prolonged. An important missing piece necessary for a completely functional in vitro pathway was subsequently demonstrated [21]. Virus also induces Fas ligand (FasL) in HeLa cells and its coexpression with Fas has similar kinetics. In contrast, polyI-C failed to induce FasL, providing an explanation of the need for Fas cross-linking to induce apoptosis in HeLa cells in the earlier studies. The presence of additional apoptotic markers, outer-leaflet phosphoserine exposure, chromatin condensation, and maintenance of membrane integrity were also demonstrated. These studies in HeLa cells also suggested linkages between Fas-mediated influenza A virus pathogenesis and the IFN pathway, with evidence for involvement of viral dsRNA and PKR [57–59]. Influenza A virus infection and polyI-C increased levels of IFN-β production, with polyI-C increasing PKR autophosphorylation. The addition of IFN-β to infected cells increased cell mortality, Fas expression, and PKR phosphorylation, whereas anti–IFN-β partially blocked these effects. It was also reported that the PKR inhibitor 2-AP blocked polyI-C induction of Fas mRNA. Therefore, in HeLa cells at least, a complete extrinsic autocrine influenza A virus-induced pathway is present and functional, but influenza A virus factors, in addition to dsRNA, may be necessary for its full induction.

Evidence for the role of the Fas-FasL apoptotic mechanism in influenza A virus-induced human leukopenia comes from in vitro experiments with human monocytic cells and lymphocytes [60]. Influenza viruses A/AA/Marton/43, A/Bethesda/85, and A/Ann Arbor/6/60 all induced DNA fragmentation, as evaluated by TUNEL staining in $CD3^+$ lymphocytes. Heat-inactivated A/Marton failed to induce TUNEL staining, consistent with the requirement for actively replicating virus as observed with other cell types. A/Marton induced a time-dependent increase, from 24 to 72 h pi in TUNEL staining, and at 24 h pi A/Marton induced DNA electrophoretic laddering in $CD4^+$, $CD8^+$, and $CD19^+$, as well as $CD3^+$, lymphocyte subsets. The broad caspase inhibitor Z-VAD-fmk and caspase-3 inhibitor Ac-DEVD-CHO significantly reduced TUNEL staining of virus-infected $CD3^+$ cells. Virus increased total Fas (CD95) expression in $CD8^+$ cells and increased Fas surface density on $CD8^+$, $CD4^+$, and $CD3^+$ cells. Expression of Fas ligand, evaluated in pooled mononuclear leukocytes, increased. These data are consistent with a Fas-FasL death receptor–mediated induction of apoptosis. Additional evidence is that Fas antibody, increased TUNEL staining in $CD8^+$ cells, and anti-FasL decreased this apoptotic marker in $CD3^+$, $CD4^+$, and $CD8^+$ cells. However, anti-Fas did not alter TUNEL staining in $CD4^+$ cells. Depletion of monocytic cells from pooled mononuclear leukocyte

preparations prior to infection resulted in large decreases in virus-induced TUNEL staining. Interestingly, anti-TNF did not alter the response. This observation bears further investigation due to the importance of this usually proapoptotic cytokine present in influenza A virus infections in vivo. This result suggests that the source of either Fas or FasL activating the lymphocytes might be dependent on parcrine immune cell-cell interactions.

dsRNA and PKR

dsRNA production is an inevitable consequence of viral infection resulting in activation of dsRNA-dependent protein kinase R and L (PKR; PKL). Due to their importance in single-strand RNA viral pathogenesis, there is considerable effort to understand both viral and host factors involved in regulation and downstream consequences of their activity [61,62]. Presently, most studies are on PKR, and to date there appear to be no reports directly investigating the role of PKL relative to influenza virus-induced apoptosis. There are two broad mechanisms where PKR could be upstream of the apoptotic pathway: suppression of host and viral protein synthesis and activation of signal transduction pathways. Relative to apoptosis, work on the latter mechanism currently predominates and emphasizes the role of PKR as a pleuripotent activator of extrinsic autocrine or parcrine pathways. Much of the work on influenza virus PKR activation pathways has not been primarily directed toward understanding apoptosis per se, but toward the regulation of pathways important in production of host defense molecules, with particular emphasis on IFN. This is not surprising, considering the well-established importance of IFN in host antiviral defense. Recent papers and excellent reviews on cell signaling pathways, cytokine induction, and IFN host defense should be consulted for details beyond the scope chosen for this article [5,45,46,63,64].

The PKR suppression of translation is via phosphorylation of eukaryotic initiation factor 2 alpha (eFI2α), decreasing translation of both host and viral proteins [61]. Therefore, in principal, an intrinsic indirect mechanism for influenza virus-induced apoptosis could exist based on this pathway. The loss of negative regulators, e.g., a loss or reduction in IAP activity, could result in de novo induction of apoptosis or increased apoptosis induced by other initiators. Considerable work has been done to understand the mechanism of host translation control in the context of influenza A virus replication [62,65,66]. However, interestingly, there are no data directly linking influenza virus suppression of host-cell transcription and apoptosis induction. Per-

haps it will be difficult to observe the contribution of such a mechanism due to the dominance of extrinsic apoptosis activation factors. Also there is evidence that influenza virus-induced eIF2α phosphorylation is relatively weak, compared with other viruses, possibly in part due to influenza A virus interference with this PKR-mediated mechanism [62]. Because influenza A virus requires host mRNA for replication, it cannot completely suppress host translation processes [65–67].

The studies described above for Fas-mediated influenza A virus apoptosis of HeLa cells are evidence for PKR involvement in influenza virus extrinsic induction of apoptosis [58,59]. Additional evidence for importance of a PKR-mediated pathway comes from experiments using 3T3 L1 fibroblasts overexpressing wild-type PKR (PKR-wt) or a dominant negative inhibitor (PKRΔ6) [34]. Infection with influenza virus A/WSN increased cell blebbing, cell shrinkage, annexin staining, trypan blue staining, and TUNEL labeling in PKR-wt but not in PKRΔ6 cells. IFN alpha/beta pretreatment increased all of these markers in PKR-wt but not PKRΔ6 cells, despite IFN treatment decreasing virus titers. In this same system activity of caspase-8 and caspase-9 were evaluated using fluorogenic substrates. WSN elevated caspase-8, but not -9. Overexpression of PKR (PKR-wt) slightly enhanced caspase-8 activity, whereas the negative dominant construct PKRΔ6 suppressed its activation by WSN. These data suggested WSN activated PKR, resulting in activation of TRAIL receptor pathways mediated by FADD/caspase-8 control of apoptosis effectors, but not the mitochondrial/Apaf-1/caspase-9 pathway.

Dependence of Fas and FADD activity on PKR was further supported by Western blotting studies showing that the activity was suppressed in the PKRΔ6 cells relative even to control cells and that activity levels were not increased by WSN infection. Consistent with this idea, results from FADD and Apaf-1 nul mutant cells showed WSN-induced apoptosis was dependent on FADD but not Apaf-1. IFN increased cell death in all cells having active FADD, regardless of Apaf-1 status, although the fibroblast cells' poorly supported virus replication suppression of cell death was not necessarily correlated with changes in titer. Both PKRΔ6 and FADD-nul cells had enhanced survival, but only the latter showed large increases in titer over control cells. PKRΔ6 cells suppressed influenza virus replication, reminiscent of the decrease seen with bcl-2 [32]. In vitro and in vitro observations using animals and cells from two different mouse lines with genetically disrupted PKR that results in nul phenotypes had different outcomes [54]. In vivo PKR$^{-/-}$ mice infected with A/WSN/33 had greater and more rapid mortality. Consistent with this increased viral virulence, lung caspase activity, apoptotic markers, and virus

titers were elevated in the PKR$^{-/-}$ l mice. In contrast to the in vivo observations, both caspase-8 and -9 were activated. It was suggested that the mitochondrial/Apaf-1/capspase-9–dependent pathway might be activated in vivo by caspase-8 activation of Bid, a bcl-bax family member. The increased virus virulence was attributed to loss of IFN-mediated defense mechanisms not being activated due to lack of PKR activity [54]. Different results were obtained in vivo with the mouse-adapted strain A/W29 influenza A virus derived from the human A/FN/1/47 strain, as very little change in influenza A virus virulence or tissue virus titers was observed between wild-type and PKR$^{-/-}$ mice [55]. In vitro, induction of apoptosis, as monitored by annexin V staining, in mouse embryonic fibroblasts (MEF) infected with A/HK/1/68 was not different between cells from the two strains. IFN α/β pretreatment partly rescued both wt and PKR$^{-/-}$ cells at low multiplicity of infection. The MEF cells from the two strains also were equally responsive to TNF induction of apoptosis. Activation of eIF2α differed as well; remaining at normal levels in contrast to being greatly reduced compared with the wild type in the studies with WSN influenza A virus.

NS1, M, and NP

Understanding the role of NS1 may be particularly important for a complete view of influenza A virus-induced apoptosis. NS1 was one of the first influenza virus proteins suggested to have a role in apoptosis and was proposed to be proapoptotic. Expression of the full-length NS gene from A/Ty/Ont/66 and two molecular clones, NS D1 and NS D5, in MDCK cells resulted in DNA fragmentation [68]. Similar results were reported to occur in chick embryo fibroblast (CEF) and mink epithelial cells. Of the two proteins encoded by the NS gene, only expression of NS1, but not NEP (NS2) [65], was successful. NS1 expression resulted in MDCK cell apoptosis at 24 h, which increased at 48 h as judged by DNA laddering. Expression of A/Udorn/72 NS1 mutants with deletions in the RNA-binding/dimerization domain or the effector domain demonstrated the RNA-binding domain only was necessary for inducing DNA laddering. A/WSN/33 viruses that have reassortment mutations within the NS1 RNA-binding/dimerization region were reported to be growth attenuated in MDCK cells but not in CEF cells or mink lung epithelia, but were still capable of inducing DNA fragmentation in all cell types. These experiments are consistent with NS1, which has intact RNA-binding regions, being sufficient for inducing apoptosis, but not required, in these cells. That NS or NS1 expression in the absence of other influenza A virus factors could

induce DNA fragmentation implies that NS1 interacts with apoptotic pathways. Whether this is an intrinsic or extrinsic mechanism needs to be defined. Extrinsic mechanisms were suggested as the explanation for NS1 mutant viruses retaining the ability to induce apoptosis. It was postulated that these influenza A virus variants might be inducing apoptosis by a loss of antiapoptotic activity via failure to suppress dsRNA PRK activation, leading to increased IFN production and cell death.

Other studies suggest that NS1 can be antiapoptotic. Discovery of viral factors interfering with apoptosis has been important for understanding viral pathogenesis and for providing important molecular tools for studying apoptotic pathways [3,6,69]. Presently NS1 is the only influenza A virus macromolecule for which evidence of antiapoptotic activity has been reported. NS1 was known to be an important component of influenza virus-host interactions for modulating translational control (see above), and NS1 complexes with PKR inhibiting its activity [70]. Influenza virus also interferes with PKR activity by an intrinsic indirect mechanism in that influenza virus infection activated the $P58^{IPK}$ pathway capable of inhibiting PKR activity [67]. Additional evidence for NS1's importance comes from the use of an influenza A virus NS1 deletion mutant (NS1-del). The deletion mutant is impaired in its ability to replicate in the presence of functional IFN activation [71] due to the inability to prevent activation of IFN-related pathways in vivo and in vitro [72,73]. Deletion mutant experiments provide extensive evidence that NS1 is antagonistic to IFN effects. NS1 also impacts pathways having more general regulatory roles, including NF-κB [74] and c-Jun and AP-1 activity [75]. A more direct test of NS1 antagonism to apoptosis was performed in MDCK and Vero cells. MDCK cells infected with the NS1-del virus showed earlier cell morphological changes, cell rounding and detachment, and DNA fragmentation compared with cells infected with A/PR8 wild-type virus [76]. These kinetic differences were not observed in Vero cells, and onset of the apoptotic makers was delayed for both the wild-type and mutant virus in these cells. The differences were attributed to IFN pathways present in MDCK cells but not in Vero cells. It was suggested that influenza A virus apoptosis is at least in part dependent on IFN-mediated factors, with PKR-mediated events a likely mechanism.

The role of NS1, M, and NP were examined in HeLa cells using an expression system based on Herpes simplex virus (HSV) VP22 chimeras [77] from two influenza A virus strains, A/Fiji and clone7a. These influenza virus strains produce very different pathogenicities in vivo and in vitro, with A/Fiji less virulent and a poorer inducer of

apoptosis in both MDCK and U937 cells [78]. The HSV tegument protein VP22 was used to amplify the effect of the proteins because it allows translocation and internalization of the protein chimeras to additional cells beyond those transfected. Apoptosis was evaluated by nuclear fragmentation. A dual pro- and antiapoptotic role for NS1 was observed. Individually the VP22-NS1 proteins induced apoptosis, as did polyI-C, whereas exposure to both factors resulted in an intermediate level of cell death. Modest increases in apoptosis occurred with M1, M2 chimeras from both strains, whereas cell survival was not altered by NP from either strain [77].

NA and TGF-β

The important influenza virus envelope protein NA, having neuraminidase activity, has been shown to have a proapoptotic role. Experiments with Ty/Ont-infected MDCK cells demonstrated that influenza A virus neuraminidase could activate latent TGF-β to its biologically active form [79], a broad inducer of apoptosis, including in MDCK cells. Secretion of TGF-β was elicited in both a time- and titer-dependent fashion. The activation was dependent on neuraminidase activity, as judged by blocking antibody treatment of infected cell-conditioned media. TGF-β antibodies, however, only partially blocked apoptosis. Similar results were observed for equine influenza virus (EIV) [52]. EIV induced apoptosis, TGF-β production, and activation of the JKK/SAPK pathway kinase c-jun. Blocking TGF-β or c-jun activation also reduced EIV cytopathic effects [52]. Additional studies of NA and apoptosis were with the strains A/Fiji and clone7a, with low and high pathogenicity and low and high neuraminidase activity, respectively [78]. Inhibitors of neuraminidase decreased cell death induced by both viruses by approximately two-thirds [80]. However, UV inactivation of clone 7a and A/Fiji, as with other strains, greatly reduced apoptosis induction despite both clones retaining most of their neuraminidase activity. NH_4 treatment, which prevents influenza A virus entry from endosomes, acted as expected and reduced cell death. Surprisingly, treatment with a neuraminidase inhibitor further reduced levels of apoptosis for both virus strains. Both groups suggested their data supported the idea of neuraminidase-dependent and -independent mechanisms for influenza A virus-induced apoptosis [79,80]. Use of HSV VP22 protein chimeras with NA from A/Fiji and 7a supports this idea. The 7a chimeras induced apoptosis more strongly than the A/Fiji ones [77]. Anti–TGF-β and the neuraminidase inhibitor GG167 (zanamivir) inhibited the 7a-NA chimera induction of apoptosis but not the modest induction by A/Fiji. Differ-

ent results were found with A/Unidorn in HeLa cells, where zanamivir increased apoptotic markers [81]. Whether this was due to differences in influenza virus or host cells, as suggested, remains unclear. However, it is interesting to note that in this study annexin V marker for apoptosis increased as WGA binding decreased, indicating a concurrent increased surface expression of neuraminidase [81].

Oxygen Free Radicals

Oxidative stress is an important mediator of apoptosis [82,83], with oxygen free radicals potentially having multiple roles in influenza virus apoptosis induction. To date the evidence is that influenza virus-induced ROI interaction with apoptotic pathways is mediated by host-cell transduction factors. Therefore, ROIs produced during influenza virus infection could act as both intrinsic indirect and extrinsic factors. Interestingly, there are no reports suggesting that ROIs are capable of directly activating apoptosis for any biological system either in the induction or execution phases. Caspases, bcl-2 family members, or control of mitochondrial cytochrome c release might be expected to directly interact with ROIs due to their high chemical reactivity. Because influenza virus-induced ROI activity is mediated by host-cell transduction processes, there is potential for both pro- and anti-apoptotic effects, although only proapoptotic activity has been suggested or reported.

Immune system cells that infiltrate tissues during respiratory infections [84–86], notably neutrophils and macrophages, are important sources of ROIs and host defense molecules, e.g., cytokines, known to increase ROI production. These primary innate immune system effectors respond to influenza virus by release of nitric oxide (NO), superoxide, other ROI species, or those formed by subsequent ROI interactions, including hydroxyl radical, hydrogen peroxide, and various adducts, such as peroxynitrils. All of these species are good candidates for in vivo autocrine and paracrine tissue damage by direct free radical attack, as well as inducers of apoptosis. The importance of host oxidative stress in influenza A virus was re-emphasized by gene array studies showing that antioxidant enzymes are upregulated [87]. ROIs shown to be present in lungs of influenza A virus-infected mice include superoxide anions [49,88], nitric oxide radicals [88,89], and hydrogen peroxide [90]. Increases in iNOS, responsible for NO production [88,89,91,92], and xanthine oxidase, responsible for superoxide production [49,93], were observed in murine lung tissues and blood. Host responses indicate defense against influenza A virus-induced ROIs is important, as virus infection results in an upregulation

of superoxide dismutase (Mn-SOD), indoleamine-2,3-dioxygenase (DEO), and hemeoxygenase-1 [85]. Glutathione peroxidase increased in C57Bl/6 infected with A/PR8 [85] but decreased in ICR-ZH mice infected with A/PR8 [90,94].

The decrease or lack of change in oxidative capacity in these latter studies led to the suggestion that the lungs retain sufficient oxidative capacity to defend against influenza A virus damage. Contrary to this idea is the extensive evidence that administration of antioxidants—including superoxide dismutase and the iNOS inhibitor L-NMMA—and erythromycin [95] increased in vivo survival [88,89] as well as survival in an SOD gene therapy murine model [92]. Recently it was suggested that influenza A virus induction of NO in human airway epithelial via iNOS is PKR mediated [91], providing a putative link between ROIs and this important enzyme. As in other reports, A/Japan/305/57, polyI-C, and IFN-gamma induced NOS2. PolyI-C induced PKR and iNOS activation in these cells. Consistent with this mechanism, NOS2 induction was diminished in PKR-nul mouse embryonic fibroblast cells, but influenza virus was not tested directly. Transgenic mice expressing enhanced levels of SOD had less lung inflammation and injury [92]. In mice genetically deficient in NOS2 ($NOS2^{-/-}$) there was little pneumonitis and complete survival after infection with titers of A/PR8, which caused complete mortality in wild-type mice. Interestingly the experiments involving $NOS^{-/-}$ mice suggested that viral clearance was not due to NO but another IFN-sensitive mechanism [89].

One of the most important connections between influenza A virus, ROI, and apoptotic pathways appears to be mediated by the activity of transactivation factor NF-κB and, possibly, AP-1. Evidence for this interaction comes from upregulation of these transactivation factors by influenza A virus and the inhibition of both their activity and apoptosis by antioxidants, notably PDTC and NAC. Although mechanisms of PDTC and other thiol-containing antioxidants, such as NAC, are not entirely clear, they have been repeatedly shown to have antiapoptotic activity for a wide spectrum of virus–host cell combinations. What is not clear is how these observations can be reconciled with the pro-IAP role for NF-κB activation. Both NF-κB and AP-1 activity are increased in vivo by A/PR/8/34 [85] and in vitro by A/Port Chambers in A549, a human lung epithelial cell line [96]. NF-κB was activated by A/Beijing/353/89 in human monocytes [47]. AP-1 was activated by EIV in MDCK cells [52] and by the avian (FPV) A/Bratislava/79 influenza virus in MDCK and U937, human promonocytic, cells [51]. However, FPV did not activate AP-1 in A3.01, human T lymphoma, or HeLa cells, which are not permissive for

influenza A virus replication [51]. In vitro, infected cell oxidative stress, as monitored by changes in dichlorodihydrofluorescein intensity, increased in both A549 [96] and MDCK cells [52,97]. Early reports found that NAC and PDTC did not block A/Ty/Ont apoptosis in MDCK cells [98]. More recently NAC, PDTC, and trolox, a vitamin E analog, in vitro reduced influenza A virus-induced oxidative stress [25,96,97]. In these studies, as well as those conducted with the murine macrophage J774.1 cell line, NAC and PDTC also inhibited apoptotic cell death [20]. Trolox, however, failed to inhibit apoptosis in chorion cells [97] or J774.1 macrophages [20; Lowy, unpublished data].

Specific influenza A virus proteins interacting with NF-κB and AP-1 have been identified. Use of an expression vector demonstrated that HA alone was capable of upregulating NF-κB activity [50] and that the antioxidants dithiothreitol and PDTC blocked this upregulation. This suggested that the putative action of antioxidants was on upstream production of ROIs by the HA expression. Recent work extended these observations, demonstrating that HA, NP, or M proteins were all capable of driving the expression of luciferase under the control of HIV-1 LTR or κB but not AP-1 [99]. Activation was IKK dependent and sensitive to the antioxidant PDTC. IκB kinase (IKK) activation is a well-established regulator of NF-κB, via phosphorylation of IκB, resulting in release of the active form of NF-κB. These data support the idea that influenza virus proteins induce ROI formation that is upstream of IKK, resulting in the subsequent NF-κB activation. In this context it is interesting to note that, although NS1 inhibits dsRNA activation of NF-κB, it was reported that NF-κB activation by TNF was not inhibited by NS1 [74]. It was suggested that NS1 might not interfere with the ROI-dependent TNF activation of NF-κB, but only the PKR activation pathway [74]. Lack of influenza A virus protein ROI-dependent direct AP-1 activation [99] is at variance from other studies [85,96]. However, it has been shown that additional cell transduction pathways, JNK, are necessary for influenza A virus-induced AP-1 activation [51]. Taken together, these results suggest that influenza A virus is capable of activating multiple pathways, thereby leading to an increase in the activity of these transactivation factors via pathways both with and without ROI involvement.

ADDITIONAL ROLES FOR INFLUENZA VIRUS-RELATED APOPTOSIS

Leukocytes and Neurons

Influenza virus-induced apoptosis in nonepithelial, nonfibroblastic cell systems is beginning to be appreciated and defined. Although influenza

virus infection can induce lymphoproliferation [86], one of the most important effects of highly pathogenic influenza virus infection is lymphopenia, which is likely due to apoptosis [100–102]. Understanding T and B cell colonial selection and activation during influenza virus infection is important in its own right, as well as influenza virus infection being an important model system. Appropriate literature should be consulted for detailed information [45,103–105]. For T, B, and dendritic cells, mechanistic studies suggest that extrinsic factors are involved and that more than one signal is necessary, one of which is IFN or a TRAIL family ligand, such as TNF or FAS [106–109]. Apoptosis-mediated cell death has been demonstrated in neutrophils, as has the synergistic induction of cell death by influenza A virus and bacteria [110,111]. This observation has important implications for the well-known respiratory virus-bacteria superinfections central to clinical progression of pulmonary disease [86,112]. CNS complications with influenza virus are observed clinically but are poorly understood [102,113]. In murine models, results from experiments using neurotrophic WSN suggest there may be an apoptotic basis for the neuropathogenesis, with a possible olfactory pathway component [114,115].

Macrophages

Macrophages are one of the first immune system cells to encounter viruses and/or virally infected cells and have a central role in both innate and adaptive responses to viral pathogenesis. Consequences of macrophage influenza virus infection reported to date are release of cytokines, chemokines, and ROI; induction of apoptotic cell death; and a largely nonproductive infection [64,116–118]. When considering the host defense molecules highlighted above for induction of apoptosis, IFN-alpha/beta, TGF-β, and TNF are produced in vitro and observed in vivo in both animals and humans infected with influenza virus [41,43,86,100,101]. Despite the well-established role these factors have in activating death in other cell types, their roles in macrophage apoptosis are still being explored. Cellular events in macrophages during influenza A virus-induced apoptosis appear to be in most, but not all, respects the same as those described for epithelial and fibroblastic cells [20,30]. As for other cells, the antioxidants PDTC and NAC [20] decreased or inhibited apoptosis, but other antioxidants, e.g., Trolox, were not as effective. Direct TNF or Fas-FasL extrinsic autocrine induction or enhancement of influenza virus-induced apoptosis in macrophages is possible, but has not been demonstrated. In

murine macrophage J774.1 cells influenza A virus induced high levels of TNF. TNF did not appear to mediate apoptotic cell death, as exogenous TNF failed to induce or enhance apoptosis in influenza virus-infected or uninfected cells and anti-TNF antibody failed to prevent its occurrence [119; Lowy, unpublished data]. Results consistent with these observations were reported in chorion cells where influenza A virus induced TNF, IFN-beta, IFN-gamma, and Fas. However, influenza A virus did not appear to be directly involved with chorion influenza virus-induced apoptosis, as antibodies, shown to be effective in control experiments, failed to inhibit apoptosis [120]. An alternate antiviral role has been suggested for TNF, as in vitro the anti-replication effects on influenza A virus were pronounced, even in comparison to IFN [121,122]. Also there is evidence from other biological systems that TNF can activate pro- as well as antiapoptotic pathways [12,15]. Therefore, TNF, and possibly other TRAIL family members, at least for macrophages, may have roles other than as "death cytokines" that should be considered in influenza virus pathogenesis.

Apoptosis is postulated to provide a mechanism for phagocytic cells to remove damaged or infected cells while minimizing tissue damage [19]. This process for influenza has been examined by using HeLa cells infected with A/Udorn and peritoneal macrophages harvested from mice. HeLa cells were phagocytosed by the macrophages in an apoptotic-dependent fashion, as blocking HeLa cell apoptosis with caspase inhibitors decreased phagocytosis [123]. Involvement of phosphatidylserine (PS), the best-characterized surface marker for targeting phagocytes to apoptotic cells, was examined [124]. HeLa cells displayed PS in an apoptotic-dependent fashion, and the kinetics of its increased appearance post−9 h pi correlated with increased phagocytosis that was blocked by exogenous PS. Involvement of other factors was postulated. The role of influenza neuraminidase was explored using a wild-type and temperature-sensitive mutant with reduced NA activity at the nonpermissive temperature [81]. Fewer HeLa cells were phagocytosed that were infected with virus having the mutant NA. Treatment of cells with the NA inhibitor zanamivir infected with wild-type virus also decreased phagocytosis. Based on these experiments it was concluded that efficient phagocytosis depended on both PS and NA surface display. It was also suggested that phagocytosis of infected cells might be an important mechanism for directly clearing influenza virus and infected cells in vivo. Apoptosis by infected cells may also be important in paracrine activation of macrophages to their full phagocytic capabilities [125].

SUMMARY AND CONCLUSIONS

Influenza virus-induced apoptosis is being studied in an increasing variety of experimental systems. Data for influenza A virus predominate, as do in vitro data with experimental systems primarily based on MDCK, HeLa, mouse embryonic fibroblasts, and monocyte/macrophage cells. Current evidence indicates that influenza virus has multiple entry points and can activate both the intrinsic and extrinsic apoptosis induction pathways. dsRNA and several influenza A virus proteins have been proposed as having relatively direct interactions with the apoptotic pathway. These are NS1, PB1-F2, NA, HA, M, and NP, with dsRNA and the first three proteins the most extensively studied. At present the best evidence for a direct intrinsic mechanism is for PB1-F2 via interactions with mitochondria. There does not seem to be evidence for an intrinsic mechanism based on influenza virus factors interacting with the execution phase of apoptosis. In addition, an intrinsic indirect process has not been identified, despite activation of a number of important signaling pathways.

Emphasis for the role of influenza virus-induced cell-signaling pathways is on production of host-defense molecules that act via extrinsic autocrine mechanisms. Current evidence supports the idea that viral dsRNA acts by an extrinsic mechanism, increasing PKR activity to induce downstream signaling pathways, including those regulating IFN, with its multiple antiviral mechanisms, and/or including activation of Fas-FasL. Synthesis of influenza virus NA during replication resulting in activation of influenza virus-elicited TGF-β is another example of an influenza virus-host factor–based extrinsic autocrine process. Other mechanisms for influenza virus are generally incompletely defined as to whether they directly target apoptotic-specific pathways or more general cell-signaling pathways with links to apoptotic activation and/or effector mechanisms. Fortunately, with increasing knowledge about the apoptotic pathway and availability of biochemical assays allowing enhanced probing of mechanisms, improvements in understanding further details of apoptotic pathway activation should be forthcoming. In principal, mechanisms for a particular macromolecule need not be exclusively intrinsic or extrinsic, although there are no clear examples for influenza virus. NS1 currently has a prominent place in understanding influenza A virus-induced apoptosis, having been shown to have dual activity as both a pro- and an antiapoptotic factor. As for other viruses, evidence is coming forward that influenza virus interferes with host defense mechanisms. NSI has a central role in this process due to its interaction with dsRNA and PKR activation, resulting in suppression of

proapoptotic signals and pathways. Discovery of whether these differing roles of NS1 are context dependent could be important. The impact of influenza virus suppression of NF-κB on other important factors, notably IAP induction, is not currently understood.

A complete understanding of influenza virus-induced apoptosis may not be possible until the kinetics of pro- and antiapoptosis factor production are better defined. Based on lessons from metabolic, cell signaling, and physiological systems, the existence of regulatory positive and negative feedback loops controlling pro- and antiapoptosis pathways seems possible, even likely. Increasingly it appears that both pro- and antiapoptotic programs are activated during pathological processes, including influenza virus infection. DISC factors, bcl-bax family member interactions, mitochondrial cytochrome c release, and NF-κB are important potential points for integration of apoptotic activation signals, as well as direct activation of upstream caspases. Understanding influenza virus induction of apoptosis may require a better definition of the relationship between the virus replication cycle and production or activation of pro- and antiapoptotic factors. It is possible that the early and late phase of the influenza virus replication cycle may interact with apoptosis induction controls with different outcomes. Early postinfection dsRNA could act as a proapoptotic factor, but is prevented from full effect due to the action of NS1, delaying cell death. Later, during influenza virus replication, synthesis of other viral proteins, such as HA, M, and NA, as well as increased levels of dsRNA and NS1, might result in a switch to a proapoptotic state. Availability of improved tools to manipulate influenza virus [126,127] combined with methods to probe apoptosis pathways seems certain to provide new and better opportunities to understand this complex but important pathobiological process.

REFERENCES

[1] S. Schultz-Cherry, R.M. Krug, and V.S. Hinshaw, *Sem. in Virol.*, **8**: 491–495, 1998.
[2] J. Yewdell and A. Garcia-Sastre, *Curr. Opin. Micro.*, **5**: 414–418, 2002.
[3] C.A. Benedict, P.S. Norris, and C.F. Ware, *Nature Med.*, **3**(11): 1013–1018, 2002.
[4] H. Everett and G. McFadden, *Virology*, **288**: 1–7, 2001.
[5] G.N. Barber, *Cell Death Diff.*, **8**(2): 113–126, 2001.
[6] H. Everett and G. McFadden, *Curr. Opin. Micro.*, **5**: 395–402, 2002.
[7] D.R. Green, *Cell*, **102**: 1–4, 2000.
[8] M.O. Hengartner, *Nature*, **407**(6805): 770–776, 2000.
[9] A. Roulston and R.C. Marcellus, *Ann. Rev. Microbiol.*, **53**: 577–628, 1999.
[10] H.R. Stennike, C.A. Ryan, and G.S. Salvesen, *TIBS*, **27**(2): 94–101, 2002.
[11] G. Kroemer, B. Dallaporta, and M. Resche-Rigon, *Ann. Rev. Physiol.*, **60**: 619–642, 1998.