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Dossier "Apoptosis part II"

The 2-5A system in viral infection and apoptosis

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Summary – The 2-5A system is an established endogenous antiviral pathway. Interferon treatment of cells leads to an increase in basal, but latent, levels of 2-5A-dependent RNase (RNase L) and the family of 2′-5′ oligoadenylate sythetases (OAS). Double-stranded RNA, thought to be derived from viral replication intermediates, activates OAS. Activated OAS converts ATP into unusual short 2′-5′ linked oligoadenylates called 2-5A [ppp5′(A2′p5′)2A]. The 2-5A binds to and activates RNase L which cleaves single stranded RNA with moderate specificity for sites 3′ of UpUp and UpAp sequences, and thus leads to degradation of cellular rRNA.

During apoptosis, generalized cellular RNA degradation, distinct from the differential expression of mRNA species that may regulate specific gene expression during apoptosis, has been observed. The mechanism of RNA breakdown during apoptosis has been commonly considered a non-specific event that reflects the generalized shut down of translation and homeostatic regulation during cell death. Due to the similar RNA degradation that occurs during both apoptosis and viral infection we investigated the potential role of RNase L in apoptosis.

To investigate whether RNase L activity could lead to apoptosis, NIH3T3 cells were transfected with a *lac*-inducible vector containing the human RNase L gene. Treatment of these cells with isopropylthiogalactoside (IPTG) caused loss of cell viability that was confirmed as an apoptotic cell death by morphological and biochemical criteria. Similarly, specific allosteric activation of endogenous RNase L by introduction of 2-5A directly into L929 cells also induced apoptosis. In L929 cells poly(I).poly(C) treatment in combination with interferon caused an increase in apoptosis whereas neither interferon or double stranded RNA alone altered cell viability. Therefore, increased expression or activation of RNase L causes apoptosis.

Inhibition of RNase L, specifically with a dominant negative mutant, suppressed poly (I)Ypoly(C)-induced apoptosis in interferon-primed fibroblasts. Poliovirus, a picornovirus with a single-stranded RNA genome, causes apoptosis of HeLa cells. Expression of the dominant negative inhibitor of RNase L in HeLa prevented virus-induced apoptosis and maintained cell viability. Thus, reduction or inhibition of RNase L activity prevents apoptosis.

Both apoptosis and the 2-5A system can provide defense against viral infection in multicellular organisms by preventing production and therefore spread of progeny virus. RNase L appears to function in both mechanisms, therefore, initiation of apoptosis may be one mechanism for the antiviral activity of the 2-5A system. © 1998 Elsevier, Paris

ribonuclease / apoptosis / interferon

Apoptosis is a controlled biochemical pathway distinguishable from cell necrosis by characteristics including cellular shrinkage, membrane blebbing, chromatin condensation, and intranucleosomal DNA fragmentation [1]. Apoptosis is a process that occurs during numerous biological processes including development, growth factor withdrawal, chemotherapy, radiation, stress, and viral infection.

Cellular apoptosis following viral infection may represent an antiviral mechanism that acts by rapidly eliminating the infected cell, thus preventing the release of viral progeny. This hypothesis is strongly supported by reports indicating that viruses often express viral factors homologous to pro-survival host factors to evade host cell death [2]. Bcl-2 is an endogenous membrane bound protein that can block numerous pathways of apoptosis ranging from radiation-induced to developmental apoptosis [3–6]. Adenovirus expresses E1B, highly homologous in sequence and function to the anti-apoptotic host factor Bcl-2, which allows the virus to establish a persistent infection and to potentially promote transformation via the inhibition of host cell apoptosis [7, 8]. Similarly, BHRF-1 of Epstein-Barr virus is homologous to Bcl-2 and can delay apoptosis of the infected cell [9, 10]. Another strategy used by viruses to avert premature host cell death is to generate viral factors that can inhibit host cell pro-apoptosis factors, thus extending the replication capacity of the virus. This mode of survival is observed in bacu-

lovirus by p35 and IAP and in cowpox virus by CrmA [11–13] which act as inhibitors of apoptosis putatively by blocking ICE family protease activity. Both adenovirus E1B [7, 14, 18] and human papilloma virus [15, 16] block p53 activity to prevent apoptosis. Also, viruses can trigger apoptosis in subsets of immune cells to prevent a cell-mediated immune response, as is seen in the depletion of CD4+ cells in response to HIV infection [17]. Viruses can also inhibit various cytokines and endogenous antiviral pathways to facilitate viral replication [18–21].

Cytokines are biological molecules that can stimulate natural immunity by protecting cells against viral infection. Interferons are a group of cytokines that confer antiproliferative and antiviral effects on many cell types. Prior to infection, interferons arm the cell with a defense mechanism. Type 1 interferons, comprised of two isoforms (α and β), bind to the same receptor and induce similar responses. Mononuclear phagocytes mainly produce interferon α and cultured fibroblasts mainly produce interferon β, while some cells can produce both. In normal cells, interferon is barely detectable, but upon viral infection levels of interferon α and β increase dramatically. Following induction by interferon, the host cell immediately secretes more of this cytokine which can then bind to neighboring cells. Interferon binds to specific tyrosine kinase receptors on the cell surface and induces the expression of several proteins, some of which are critical antiviral factors.

One of the systems that mediates the interferon-dependent inhibition of viral replication is the 2-5A system, comprised of 2'5' oligoadenylate synthetase (OAS), 2'- > 5'-linked oligoadenylates (2-5A), and 2'5'-dependent ribonuclease (RNase L). Double-stranded RNA (dsRNA), thought to be derived from viral replication intermediates, activates the synthetases resulting in the production from ATP into an unusual series of short, 2'-5' linked oligoadenylates collectively referred to as 2-5A (eg, ppp5'(A2'p5')2A). 2-5A allosterically activates the normally latent 84 kDa RNase L.

RNase L was initially discovered as a mediator of the 2'5'A-dependent RNA breakdown stimulated by interferon and has been implicated in the inhibition of encephalomyocarditis virus, mengo virus, reovirus, and vaccinia virus replication. RNase L is composed of nine N-terminal ankyrin repeats, a protein kinase homology region, a cysteine-rich putative zinc finger domain, and a C-terminal catalytic domain. This catalytic domain appears to be responsible for RNase L-directed cleavage of single stranded RNA regions 3' of UU and UA bases. The activation of RNase L causes the specific degradation of both viral and cellular RNA [22]. As a result, the ability of the cell to support viral replication is severely impaired. However, the precise mechanism for the antiviral effect of the 2-5A system in vivo is unknown.

Picornaviridae is one family of viruses strongly inhibited by the 2-5A system. Picornaviruses are single-stranded positive-sense RNA viruses. Upon infection, the mRNA is translated on cellular ribosomes in the cytosol into a polymerase which then generates the negative strand and forms a double-stranded RNA replication intermediate. Double-stranded RNA intermediates derived from the picornavirus, encephalomyocarditis virus (EMCV), bind to and activate OAS in interferon-treated cells [23, 24]. Interferon-treated cells after EMCV infection showed an activated 2-5A system and rRNA cleavage products characteristic of RNase L activity [25, 26]. Additionally, in the presence of an analog inhibitor of 2-5A, able to bind but unable to activate RNase L, EMCV replication was not inhibited [27], suggesting the requirement of RNase L for the antiviral response of interferon.

Ribonuclease activity frequently has been associated with apoptosis. Increases in ribonuclease activity during metamorphosis, glucocorticoid treatment, irradiation, and viral infection [28–34] have in some cases coincided with DNA fragmentation, indicating a correlation with classic biochemical markers of apoptosis. Though this biochemical event can be cited in several cases, the ribonuclease responsible for this activity remained unknown until recently [35]. Many studies have correlated 2-5A synthetase activity, RNA breakdown or ribonuclease activation to cell death or tissue regression [30–33, 36–39]. The regression of chick oviduct following withdrawal of estrogen was accompanied by an increase in 2-5A synthetase activity [40] as measured by 2-5A levels, and the breakdown of ovalbumin mRNA and 18S rRNA [41]. Similarly, 2-5A and related oligoadenylates were observed during cessation of lactation in rat mammary glands [42]. The cytocidal effect of treating the HT29 human colon carcinoma cell line with both TNF-α and interferon-γ was accompanied by an increase in 2-5A synthetase levels and apparent rRNA breakdown [43]. Furthermore, total RNase activity in the spleen

and the thymus dramatically increased with gamma-irradiation [31, 33], a potent inducer of apoptosis in lymphocytes, and glucocorticoids, that induce lymphocyte apoptosis, were found to stimulate RNA degradation in rat lymphocytes [30]. A latent RNase is activated during pupation of fly larvae, a time of massive apoptosis during metamorphosis [29]. More recently rRNA degradation has been found to occur simultaneously with apoptosis induced by a number of agents [37–39]. This rRNA degradation is reminiscent of the rRNA cleavage induced by RNase L including the selective cleavage of uridine-rich regions in both systems. Thus, RNase L was a candidate ribonucleases responsible for the messenger and ribosomal RNA degradation or deregulation that occurs in many systems undergoing apoptosis.

RNase L, a member of the interferon-induced 2-5A pathway, is required for the antiviral activity of interferon following viral infection. Initial studies on the role of RNase L in apoptosis were based on observations that ribonuclease activation occurs during apoptosis and during viral elimination and that apoptosis is proposed as an antiviral mechanism. The results from this work implicate RNase L in the mediation of the antiviral activity of interferon through the regulation of host cell apoptosis.

To study the potential involvement of RNase L in apoptosis, we examined changes in cell viability after increasing the levels of RNase L [35]. NIH3T3 cell lines were stably transfected with an expression vector containing the *lac* repressor then subsequently transfected with an expression vector containing the human RNase L cDNA under the control of the *lac* promoter. Upon addition of IPTG, the lac repressor was sequestered enabling RNase L to be transcribed. Expression levels of the human RNase L protein were measured in cells in the absence and presence of IPTG induction in which expression levels increased by several fold in the latter condition [35]. To examine RNase L activity in these cells, the RNase L activator, trimeric 2-5A [ppp5′(A2′p5′)2A] was introduced into vector control and inducible cell lines [35]. Specific 18S and 28S rRNA degradation products, characteristic of 2-5A-dependent RNase L cleavage, were detected in uninduced cells and this ribonuclease activity increased following IPTG induction. Similar results were found in two other clones examined. Degradation of 28S rRNA, a characteristic of human RNase L, was not observed in control cells expressing only the endogenous murine RNase L nor in cells after induction by IPTG in the absence of 2-5A. After induction for 24 hours, cells overexpressing RNase L were only 20% and 38% viable in comparison to uninduced cells, as measured by protein synthesis inhibition, and by trypan blue dye exclusion, respectively.

The form of death was classified as apoptosis due to positive in situ labeling of DNA fragmentation [35]. The number of positively stained apoptotic cells were quantified resulting in 0–2.2% apoptotic cells before induction of RNase L and 8.8–13.5% apoptotic cells after induction of RNase L. In the absence of IPTG, cells grew more slowly than vector control cells. The rate of [^{3}H]-thymidine incorporation was identical in the two cell lines, suggesting that the inducible cells, chronically expressing human RNase L, had a higher cell death rate rather than a slower cell division rate.

To determine whether the effect of RNase L on cell viability was due to the specific activation of the ribonuclease, 2′5′ oligoadenylate, a direct and highly specific activator of RNase L, was transfected into cells [35]. Introduction of trimeric 2-5A (ppp5′A2′p5′A2′p5′A) triggered apoptosis in fibroblasts whereas mock transfection of cells did not. Apoptosis was not triggered by the structurally related analogue-inhibitor, ppp5′A2′p5′A2′p5′U [44] which could bind to RNase L but is 10^5-fold less effective as an activator due to the missing N1/N6 domain of the third adenine ring of parent 2-5A. The induction of apoptosis was seen by in situ labeling of DNA fragmentation in fibroblasts after the introduction of 2-5A trimer but not after the introduction of the 2-5U analog inhibitor. These results correlated an increase in RNase L enzymatic activity with an induction in apoptosis.

We confirmed the significance of the interferon pathway in apoptosis by examining a well-established model of the 2-5A pathway, the fibroblast L929 cell line [35]. Double-stranded RNA had been shown to activate OAS, to increase levels of endogenous 2-5A and to cause death of interferon-treated fibroblasts and as opposed to the trimeric 2-5A was a less specific activator of RNase L. We therefore examined whether poly (I)•poly (C), a synthetic form of dsRNA, triggered apoptosis. Neither interferon nor dsRNA alone significantly reduced L929 cell viability or induced apoptosis. However, the combination of interferon and dsRNA caused an 88–91% decrease in cell viability and caused a large increase in the num-

ber of apoptotic cells. We characterized this cell death as apoptosis, using two methods – in situ labeling to detect DNA fragmentation and a fluorescent indicator to detect condensed nuclei [35]. To determine whether RNase L was required for this form of apoptosis, as opposed to the dsRNA-dependent protein kinase which had previously been reported to mediate this pathway of death [45], we transiently transfected fibroblasts with a dominant negative inhibitor of RNase L [35]. The inhibitor was a truncated version of RNase L, designated RNase L_{ZB1}, that lacked 89 C-terminal amino acids and lacked ribonuclease activity [46]. This truncated protein could function as a potent inhibitor of the catalytic activity of wild-type RNase L both in cell-free systems and in intact cells. The inhibitor of RNase L was shown to increase the sensitivity of interferon-treated fibroblasts to infection by the picornavirus family member, encephalomyocarditis virus [46]. This work suggested that RNase L was required for the antiviral effect of interferon.

Following transfection of the dominant negative mutant of RNase L into fibroblasts, a dramatic suppression of dsRNA-induced apoptosis was observed [35]. Cells expressing the RNase L inhibitor remained viable significantly longer than the vector control cells following interferon and poly (I)•poly (C) treatment. Thus, dsRNA directly induced apoptosis in interferon-treated cells and inhibition of RNase L inhibited this cell death. These results suggested that RNase L activity and the 2-5A pathway may mediate viral-induced apoptosis. We therefore examined poliovirus, a single-stranded RNA virus, classified in the picornaviridae family, which had recently been shown to induce apoptosis in HeLa cells [47]. HeLa cells were transiently transfected with *RNase* L_{ZB1}, the dominant negative mutant, and then infected with poliovirus [35]. After 45 hr, only 22% of the vector control transfected HeLa cells remained viable whereas 80% of the *RNase* L_{ZB1}-transfected cells remained viable. Thus, inhibition of RNase L activity blocked apoptosis due to poliovirus. The mechanism by which poliovirus induces apoptosis remains unknown. This data suggested that RNase L responded to PV infection by inducing cellular suicide, and in the absence of RNase L, apoptosis did not occur.

Bcl-xL blocks the process of apoptosis during neuronal development, in response to chemotherapeutic agents, and during viral infection. To determine how RNase L participates in established pathways of apoptosis, we studied the effect of Bcl-xL on apoptosis induced by nonspecific activation of RNase L and by poliovirus [35]. Transfection of cells with the *Bcl-*x_L gene did not protect cells from poly (I)•poly (C)-induced apoptosis. Either poly (I)•poly (C) induces an apoptosis pathway that is not blocked by Bcl-x_L or it activates apoptosis downstream of the Bcl-x_L regulatory point. Interestingly, and in contrast to poly (I)•poly (C)-induced apoptosis, the poliovirus-induced cell death was blocked by transfection with the *Bcl-*x_L gene. Thus, RNase L activity was required for the poliovirus induced, Bcl-x_L sensitive, pathway of apoptosis.

Our findings demonstrated that apoptosis was induced by overexpression of RNase L, specific activation of RNase L by 2-5A, and activation of the 2-5A pathway by dsRNA. Furthermore, RNase L was a regulator of dsRNA and poliovirus-induced apoptosis, implicating RNase L as a significant factor in the host defense mechanism of apoptosis in response to viral infection.

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