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Letter to the Editor

L-DNase II activation by the 24 kDa apoptotic protease (AP24) in TNFα-induced apoptosis

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Dear Editor,

The 24 kDa apoptotic protease (AP24) is a serine protease with elastase-like activity that is activated during TNFα- or UV light-induced apoptosis.[1] *In vitro*, it stimulates internucleosomal DNA fragmentation in isolated nuclei. This distinguishes AP24 from several other proteases, such as granzyme B and caspases, which require the presence of cytosolic components to activate nuclear DNA fragmentation in cell-free systems. This feature leads to the hypothesis that AP24 may play a role in transmitting apoptotic signals from the cytosol to the nucleus, where it may directly or indirectly activate endogenous endonucleases, resulting in DNA digestion.

Activation of L-DNase II (acid, cation-independent DNase) was first demonstrated in the lens during lens cell differentiation,[2] in neural apoptosis during retina development[3] or in cell culture.[4,5] L-DNase II precursor is a protease inhibitor, leukocyte elastase inhibitor (LEI).[6] This protein belongs to the superfamily of serpins that act by a mechanism of suicide inhibition. These are substrates of specific proteases and are released in a cleaved form. LEI inhibits several serine proteases such as elastase, cathepsin G and proteinase-3.[7,8] Among these proteases, cathepsin G has shown some apoptotic properties[9] and, elastase participates in apoptosis and in LEI transformation into L-DNase II, as shown in L1210 cells induced in apoptosis by staurosporine.[4]

As AP24 belongs to the elastase-like family of proteases, it was hypothesized that the missing link between AP24 and DNA degradation could be LEI/L-DNase II. This hypothesis was investigated using U937 cells, induced to die by TNFα, a model where activation of AP24 was originally described.

First, immunocytochemistry was performed, after induction of apoptosis with TNFα, using polyclonal antibody against L-DNase II. As seen in Figure 1a, control cells show a very faint stain with this antibody, while TNF-treated cells show a higher level of fluorescence, which is concentrated in the nucleus, suggesting an activation of 5 L-DNase II. To verify L-DNase II activation, total DNase II-like activity was measured in cell extracts. TNFα-treated cells showed enhanced DNase II-like activity (Figure 1b), which was suppressed with a polyclonal L-DNase II antibody (Figure 1c). This shows that the increase in DNase II activity after TNFα treatment can be attributed in large part to L-DNase II. Taken together, these results indicate that L-DNase II is activated in U937 cells that have been induced to apoptosis by TNFα. Moreover, most of the acidic endonuclease activity generated seems to be provided by L-DNase II.

Among serine protease inhibitors, the reversible inhibitor, DK120 (carbobenzoxy-Ala-Ala-borophe) efficiently inhibits DNA fragmentation, AP24 induction and cell death during TNFα-mediated apoptosis, when it is added at 2 μM 1 h before apoptosis induction, as previously shown.[1] Consequently, we pretreated U937 cells with DK120 in order to investigate its effect on L-DNase II activation. As seen in Figure 1b (TNFα + DK120), the cell death protection by DK120 (verified by flow cytometry) was associated with the inhibition of L-DNase II activation. This effect can be attributed to inhibition of a serine protease, because DK120 has no effect on L-DNase II activity (not shown). The control cells treated with DK120 showed only a slight inhibition of DNase II-like activity, which can be explained as an inhibition of basal production of DNase II-like activity (not shown). As DK120 can inhibit AP24 apoptotic activity in isolated nuclei and is considered a specific inhibitor of AP24,[1,10] these results suggest that L-DNase II is activated in U937 cells during TNFα-induced apoptosis, and that this activation is mediated by AP24.

AP24 was defined as an elastase-like enzyme. We investigated then if it had the same interaction with LEI as with elastase. To do this, 35S recombinant LEI was incubated with AP24, and the interaction between these molecules was followed at different time points. Figure 1d, shows that 35S-LEI bound to AP24 forms a SDS-resistant complex after 1 h (lane 2). At longer incubation times, this complex disappeared and the 35 kDa cleaved LEI was seen (lane 4). This cleaved form may correspond to L-DNase II.[6] Incubation with DK120 inhibited initial complex formation and delayed LEI cleavage into L-DNase II (lanes 2 and 5). This result is in accordance with the reversible inhibitory mechanism of DK120 and strongly supports the idea of a specific interaction between AP24 and LEI. These experiments, performed using an *in vitro* synthesized LEI, suggest a serpin-like activity of LEI during the enzyme's interaction with AP24. To verify this hypothesis, the inhibitory effect of LEI on AP24 activity was measured using a recombinant LEI produced by *E. coli* and purified by affinity chromatography. We showed that recombinant LEI inhibited AP24 activity in dose-dependent manner (Figure 1e), indicating that the complex previously seen with radioactive LEI is an inhibitory complex. As previously stated, the cleavage of LEI by elastase induces the DNase activity. To obtain this effect, LEI is usually incubated with elastase overnight.[6] In order to verify whether the 35 kDa band seen in the previous figure corresponds to L-DNase II, we incubated recombinant LEI with AP24 overnight and measured its endonuclease activity. Under these conditions, the digested LEI is able to relax and linearie a supercoiled plasmid

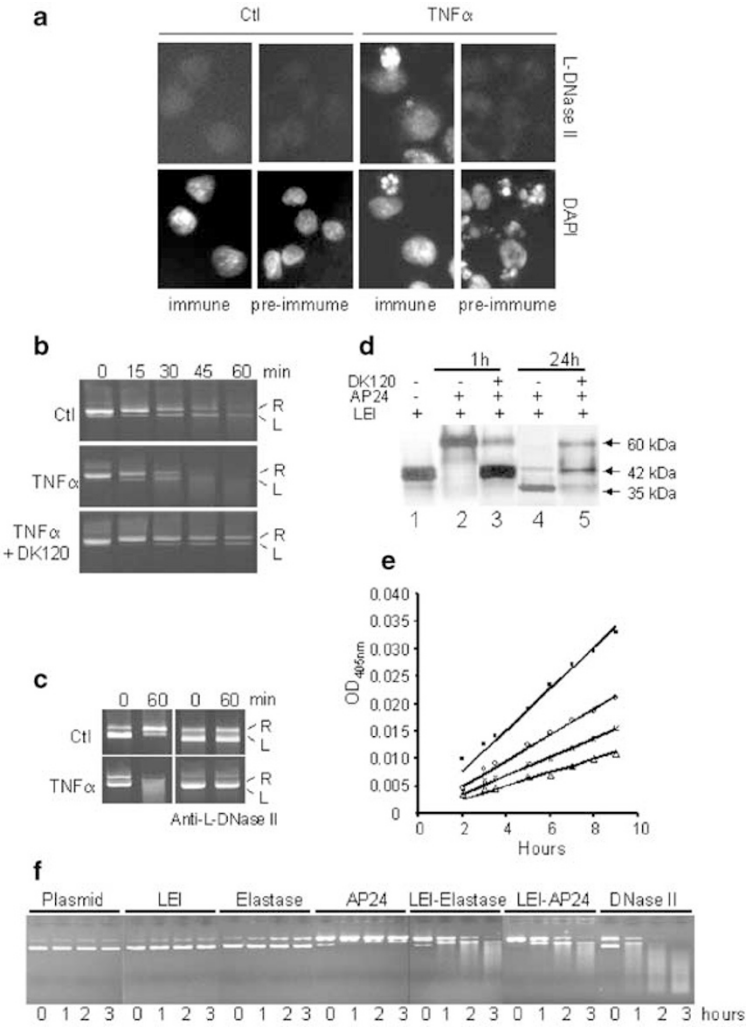

Figure 1 Activation of L-DNase II in TNFα-induced apoptosis. (**a**) U937 cells were seeded at 5×10^5 cells/ml for 90 min with or without 5 ng/ml TNFα and 0.5 μg/ml cycloheximide. Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, saturated with PBS containing 1% skim milk and then incubated for 1 h at room temperature with a polyclonal anti-L-DNase II antibody (1/100), in PBS 0.1% milk and then with a TRITC-conjugated goat anti-rabbit IgG (1/500 dilution) in PBS 0.1%. During the last wash in PBS, nuclei were stained with the fluorescent nuclear marker, 4′,6-diamidino-2-phenylindole (DAPI), and then mounted on glass slides with 50% glycerol in PBS. An increased nuclear labeling is seen in apoptotic cells. (**b**) After treatment, 3×10^7 cells were homogenized in 10 mM Tris-HCl pH 7.4 containing 1 M NaCl, 1 mM PMSF, 5 mM 10-*O*-phenanthrolin, 1 μg/ml leupeptin and 0.2 μg/ml aprotinin, disrupted with a sonicator for three 1-s intervals. The homogenate was centrifuged (10 000 × *g*, 4°C) for 30 min. The supernatant was dialyzed overnight at 4°C against 10 mM Tris-HCl pH 7.4, 1 mM EDTA in the presence of 0.1 mM PMSF, 0.5 mM 10-*O*-phenanthrolin, 0.1 μg/ml leupeptin and 20 ng/ml aprotinin. Total DNase II activity was measured by incubating 7 μg of cell extract at 37°C in a final volume of 60 μl of 10 mM Tris and 10 mM EDTA (pH 5.5) containing 2 μg of plasmid DNA. Aliquots (10 μl) were frozen at different incubation times. DK120-treated cells were preincubated for 1 h with 2 μM DK120 before adding TNFα. The effectiveness of DK120 in inhibiting apoptosis was checked by flow cytometry using a EPICS Ultra Beckman Coulter flow cytometer. (**c**) L-DNase II was measured as on panel (**b**), but inhibited by preincubation with a polyclonal anti-L-DNase II (1/10 of final volume), in the absence of plasmid, for 1 h at 37°C. The reaction was initiated by the addition of the plasmid. Rabbit nonimmune serum did not inhibit L-DNase II activity. (**d**) Recombinant LEI using the SP6 transcription promoter of the pGEM vector was carried out by an *in vitro* transcription–translation system using the rabbit reticulocyte in the presence of ^{35}S-methionine. LEI (2 μl) was incubated with 10 μl AP24 at 37°C for different periods of time in the absence or presence of 300 nM DK120. DK120 solvent has no effect on LEI/AP24 interaction. Gel electrophoresis was performed using 12% polyacrylamide slab gels. On the right of the panel, the different forms of LEI/L-DNase II are identified by an arrow: 60 kDa (complexed LEI), 42 kDa (LEI), 35 kDa (L-DNase II). (**e**) Elastase-like activity was measured with 0.25 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (MAAPV, stock solution 250 mM in DMSO) as a substrate. AP24 (20 μl) was added to 164 μl of PBS (137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.5) containing the substrate and 16 μl of various dilutions of recombinant LEI purified from *E. coli* or 16 μl of LEI buffer (50 mM Tris, 50 mM EDTA, pH 7.5) for the control activity of AP24 (black square). (**f**) Recombinant LEI purified from *E. coli* by affinity chromatography, was incubated with AP24 (1 : 1) overnight at 37°C. L-DNase II activity was measured using 20 μl of the aliquot LEI/AP24, as described above. A parallel experiment was done using 5 μg of elastase to digest LEI. Purity of LEI and AP24 preparations was verified by PAGE, followed by Coomassie blue staining

(Figure 1f), indicating that AP24 can transform LEI into L-DNase II. Since it is widely recognized that LEI can inhibit both serine proteases, human leukocyte elastase and cathepsin G, AP24 is now identified as another target for LEI.

In summary, this study shows that TNFα induces an increase in L-DNase II activity in U937 cells. Its activation depends on induction of serine protease activity during apoptosis. The use of DK120, a powerful AP24 inhibitor,

protects U937 cells from TNF-induced apoptosis and decreases L-DNase II activity, suggesting that the responsible protease in L-DNase II is AP24. If AP24 is able to interact with LEI and transform it into L-DNase II, it is reasonable to consider that this interaction might be serpin-like, as for elastase. This potential interaction between AP24 and LEI/L-DNase II was confirmed *in vitro*. It is worth noting that the complex between LEI and AP24, and its cleavage, is sensitive to DK120, which is a competitive inhibitor of AP24. Moreover, *in vitro* AP24 activity is inhibited by LEI and, after cleavage, LEI is transformed into L-DNase II, suggesting that AP24 can replace elastase in some situations and that L-DNase II is activated by AP24. It is important to note that tissue specificity may be involved in the activation of this pathway by TNFα as U937, like most white blood cells, express high levels of LEI, a feature that might facilitate the use of this pathway.

The couple AP24/LEI-L-DNase may provide another example of DNase activation by proteases. The caspase-activated DNase (CAD) exists in normal cells in an inactive form that is complexed with its inhibitor, ICAD.[11] Upon apoptotic stimuli, ICAD is inactivated by cleavage with caspase-3, and the CAD activity is liberated. In the case of L-DNase II, its activation depends on the cleavage of the precursor molecule. The original hypothesis put forward in relation to AP24 was that, in normal cells, AP24 remained inactive in a complex with an inhibitor or in a proenzyme form.[1] Our results prove that LEI can inhibit AP24. Thus, this molecule may participate in the inhibition of AP24 in resting cells. We do not know the half-life of the complex AP24/LEI, or whether it pre-exists in normal cells. Nevertheless, we can speculate that this complex is inactive in normal cells. After the induction of apoptosis, the intracellular environment could produce favorable conditions to efficiently dissociate AP24 and cleaved LEI, that is, L-DNase II. This hypothesis is currently under investigation in our laboratory.

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Séverine Altairac[1], Susan C Wright[2], Yves Courtois[1] and Alicia Torriglia[,1]*

[1] Développement, Pathologie et Vieillissement de la Rétine, INSERM U450, Association Claude Bernard, Institut Biomédical des Cordeliers, 15, rue de l'Ecole de Médecine, Paris, France;
[2] Palo Alto Institute of Molecular Medicine, Mountain View, CA, USA
* Corresponding author: A Torriglia, Développement, Pathologie et Vieillissement de la Rétine, INSERM U450, Association Claude Bernard, Institut Biomédical des Cordeliers, 15, rue de l'Ecole de Médecine, 75006 Paris, France; E-mail: torrigli@infobiogen.fr

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