Apoptosis
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A novel paraptosis pathway involving LEI/L-DNaseII for EGF-induced cell death in somato-lactotrope pituitary cells*

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We have recently reported that EGF triggers an original form of cell death in pituitary cell line (GH4C1) with a phenotype sharing some characteristics of both apoptosis (internucleosomal DNA fragmentation) and paraptosis (caspase-independence and cytoplasmic vacuolization). However, the endonuclease involved in EGF-induced DNA fragmentation has not been assessed so far. In the present work we therefore further explored the putative paraptosis involvement in EGF-induced cell death and asked whether L-DNaseII might be involved. Indeed, this endonuclease is known to mediate internucleosomal DNA fragmentation in caspase independent manner. Our Western blot, immunocytochemistry and enzymatic measurement assays show that EGF triggers a cleavage of Leukocyte Elastase Inhibitor (LEI) precursor into L-DNaseII, its subsequent enzymatic activation and nuclear translocation thus pointing to the involvement of this endonuclease pathway in caspase-independent DNA fragmentation. In addition, EGF-induced cell death can be blocked by paraptosis inhibitor AIP-1/Alix, but not with its anti-apoptotic C-terminal fragment (Alix-CT). Altogether these data suggest that EGF-induced cell death defines a novel, L-DNaseII-mediated form of paraptosis.

Keywords: AIP-1/Alix; caspase-independent pathway; L-DNaseII; paraptosis .

Introduction

Programmed cell death (PCD) is a form of cell death in which cell has an active role in its own demise in contrast to necrosis representing a passive cell death outcome. The "physiological" PCDs (i.e. those observed during development and tissue homeostasis) are classically divided into type 1 (nuclear or apoptotic), type 2 (autophagic) and type 3 (cytoplasmic) according to the morphological criteria.[1]

Apoptosis is the best-known phenotypic expression of PCD. It is related to a series of stereotypic morphological and biochemical alterations resulting from the activation of cystein-dependent aspartate-directed proteases called caspases. The canonical pathway of caspase activation is achieved through mitochondrial route via cytochrome c release involved in "executive" caspase-3 activation.[2] This pathway is associated with Caspase Activated DNase (CAD) activation leading to the typical inter-nucleosomal DNA fragmentation. More recently, another caspase-independent mitochondrial pathway has been characterized. This pathway is mediated through Apoptosis Inducing Factor (AIF) release from the intra-membrane mitochondrial space and its subsequent translocation to the nucleus. AIF triggers DNA fragmentation into the large fragments corresponding to type 1 chromatin condensation characteristic of apoptosis-like PCD.[3]

Non-mitochondrial caspase-dependent PCDs have also been described. The most studied among these pathways is death receptor-mediated classical apoptosis.[4] The existence of the additional non-mitochondrial, caspase-dependent pathways associated with endoplasmic reticulum stress-[5] and dependence receptor-[6] induced apoptosis have been reported.

Molecular mechanisms involved in autophagic and cytoplasmic PCDs remain less known. It has however been reported that Ras can trigger the autophagic PCD[7] whereas insulin-like growth factor receptor I (IGFRI) has been involved in a PCD reminiscent of cytoplasmic (type 3) PCD.[8] A similar death phenotype is initiated after activation of neurokinin-1 receptor by its ligand substance-P.[9] These latter PCDs, characterized by insensitivity to braod-range caspase inhibitors and extensive cytoplasmic vacuolation, have

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been designated as paraptosis.[8,9] More recently, it has been shown that paraptosis involves the activation of MAP kinases and could be specifically blocked by AIP-1/Alix.[10]

We have recently reported that EGF triggers a PCD of somato-lactotrope GH4C1 cells with striking similarities to paraptosis.[11] However, this PCD differs from paraptosis by the presence of caspase-independent internucleosomal DNA fragmentation. In the present work we assessed whether the endonuclease responsible for this fragmentation might be L-DNaseII. Indeed, this endonuclease appeared as a good candidate since it displays the required characteristics, *i.e.* the capacity of internucleosomal, caspase-independent DNA-cleavage.[12]

Materials and methods

Cell culture and treatment

Rat somato-lactotrope GH4C1 cell line containing undetectable lactotrope cells[13] were obtained from American Type Culture Collection (Rockville, MD). They were grown in Ham's F10 supplemented with 15% horse serum, 2.5% fetal calf serum, penicillin/streptomycin, fungizone (all from Gibco). After the passage GH4C1 cells were maintained in culture for 1 day and then treated for 48 h with 1nM EGF (Sigma-Aldrich, St Louis, MO) or 24 h with 5 μM staurosporine, concentration based on previous reports by using GH4C1 cells.[11] Staurosporine, which is a classical apoptotic trigger, was used as a control for the involvement of LEI/L-DNaseII pathway.

The constructs used for cell transfection were: FLAG-Alix wild type corresponding to mouse Alix cDNA (MMAJ5073) subcloned into pCI FLAG. Alix-CT corresponds to a fragment expanding from amino acid 468 to 869 of Alix.[14] They were obtained as a generous gift from Prof. R. Sadoul (EMI 0108, LAPSEN, Grenoble, France). The transient GH4C1 cell transfection was performed for 48 h by using TransFast^{TM} reagent (Promega, Madison, WI).

Assessment of cell death by trypan blue exclusion and DNA fragmentation

Cell death and DNA fragmentation were assessed as previously described.[11] At the end of the treatment periods (24 h, 48 h), floating cells were collected and added to the cells detached from the well bottom by trypsinization. After centrifugation, cell pellets were resuspended in phosphate-buffered saline (PBS) to which trypan blue solution was added in a final concentration of 0.04%. Both total and dead cell numbers were determined by four independent hemocytometer counts in each experiment.

DNA was extracted from both floating and adherent cells with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated from the aqueous phase with acetate:alcohol (0.5 volume of 7.5 M sodium acetate, 2 volumes of ethanol 100%). Internucleosomal DNA fragmentation was detected by using ApoDNA1 kit ladder assay kit (Interchim, Montlucon, France).

Western blot conditions

Total cell extracts were obtained by collecting the cells at the end of treatment period and lysing the cell pellets in RIPA lysis buffer (1% NP-40, 0,1% SDS, 0,5% desoxycholate sodium in PBS) supplemented with protease and phosphatase inhibitors PMSF (1 mM), Na_3VO_4 (1 mM), leupeptin (10 μg/μl), aprotinin (10 μg/μl). Fractionned cell extracts were obtained by lysing the cell pellets in lysis buffer pH 7.4 (10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). After 10 strokes in dounce homogenizer and 1300 rpm spin for 5 min, the cytoplasmic proteins were obtained in the supernatant. The nuclear proteins were obtained by lysing the nuclei pellets in inhibitor supplemented RIPA lysis buffer.

Extracted proteins were separated by SDS-PAGE, immobilized on PVDF membrane (Millipore, Billerica, MA, USA) and blotted with rabbit polyclonal anticaspase-3 antibody (H-277, Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:800 dilution or with rabbit polyclonal anti-LEI in a 1:1000 dilution.[15,16] The secondary, goat-anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) was used in a 1:5000 dilution. The amount of total protein extracts used was 30 μg/lane or 50 μg/lane, for caspase-3 or LEI detections, respectively. In subfractioning experiments, 50 μg/lane of cytoplasmic or nuclear proteins were used for LEI detection. In these latter experiments, the purity of subcellular fractions was checked using polyclonal anti-α-actin or anti-lamin B antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:1000 dilution.

Measurement of L-DNase II activity

3×10^7 cells were homogenized in ice, using a Potter homogenizer, in 1 ml of 10 mM Tris-HCl pH 7.4 containing 1 M NaCl, 1 mM PMSF, 5 mM phenanthrolin, 1 μg/ml leupeptin and 0.2 μg/ml aprotinin. Extracts were then disrupted with a sonicator (3 $\times$ 1 s). The homogenate was centrifuged (10,000 $\times$ g, 4 °C) for 30 min. The obtained supernatant was dialyzed overnight at 4 °C against 10 mM Tris-HCl pH 7.4, 1 mM EDTA in presence of 0.1 mM PMSF, 0.5 mM phenanthrolin, 0.1 μg/ml leupeptin and 20 ng/ml aprotinin. Protein concentration was evaluated with the BCA method (Pierce), using bovine serum albumin (BSA) as a standard.

Total L-DNaseII activity was measured by incubating 2 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 1 μg of supercoiled pGEM plasmid DNA. Aliquots (10 μl) were frozen at different incubation times and then loaded on a

1% agarose gel. L-DNaseII activity was specifically inhibited by a preincubation with a polyclonal anti-L-DNaseII (1:50 dilution) or rabbit non-immune serum (1/50 of final volume) in the absence of plasmid for 1 h at 37 °C. The reaction was then started by the addition of the plasmid.[17] L-DNaseII was obtained from Worthington (ref. 58P2465Y).

Agarose gel analysis of DNA

1% neutral agarose horizontal slab gels were made up in 40 mM Tris-acetic acid, pH 7.8, 20 mM NaCl and 2 mM EDTA in presence of ethidium bromide. The samples were loaded and run at 100 V until bromophenol blue reached 5 cm from the starting point of the migration front. Gels were then analyzed on a UV transilluminator (302 nm, Gel Doc) and photographed using a numeric camera.

Immunocytochemistry

Cells were seeded in 24-well plates containing a coverslip. After incubation and treatment, cells were washed twice with PBS, fixed in 4% paraformaldehyde (10 min at room temperature) and then washed twice with PBS. The cells were permeabilized with Triton 0.03% (15 min). Non-specific protein binding sites were blocked by incubation in a blocking buffer containing 1% skim milk in PBS (1 h, room temperature). Cells were then incubated with LEI polyclonal antibody at a 1:100 dilution in 0.1% skim milk in PBS. This was followed by five washes with 0.1% skim milk in PBS and incubation for 1 h with an 1/100 dilution of rhodamine iso-thio-cyanate (RITC)-conjugated goat anti-rabbit IgG. Cells were finally washed with PBS, incubated for 5 min with 4′-6 di-amindino-2-phenyl indoledichloride (DAPI) and washed twice with PBS. Immunoreactivity was visualized using fluorescence microscopy with an Aristoplan microscope (Leitz, France). RITC-conjugated secondary antibodies alone and cells stained with isotypically matched control immunoglobulin were run in parallel as negative controls.

Results

EGF-induced cell death is independent of caspase-activated endonucleases

We have recently shown that EGF treatment of somatolactotrope GH4C1 cells triggers an original PCD with heterogeneous phenotype.[11] This EGF-induced cell death is accompanied by DNA fragmentation in a caspase-independent manner.[11]

To further address the underlying mechanism of EGF-induced PCD in GH4C1 cells, we first performed a detailed kinetic study of EGF actions. As shown on the Figure 1a, EGF (1 nM) triggers a time dependent increase in the number of trypan blue permeable cells which was comparable in its extent to the increase seen in cells treated with 5 μM staurosporine (Figure 1b).

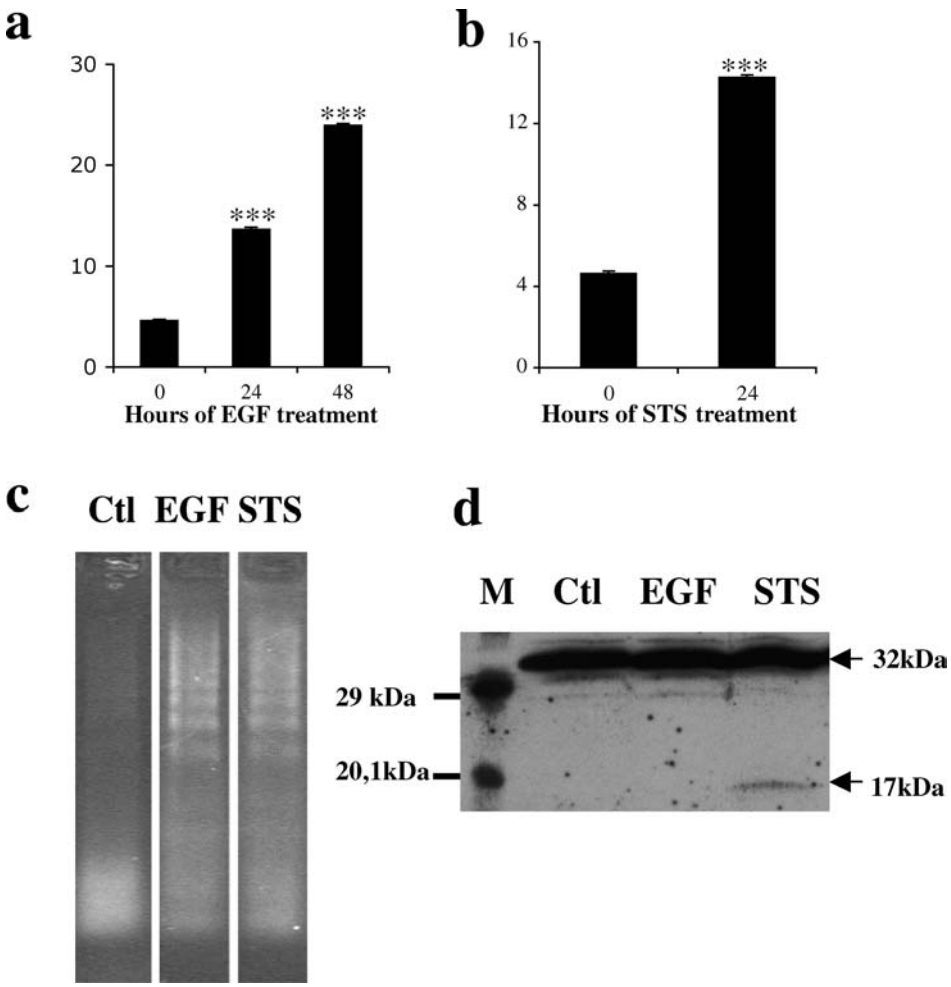
As expected, staurosporine-induced cell death is accompanied by DNA fragmentation (Figure 1c, lane STS). In accordance with our previously published data,[11] the characteristic DNA ladder could also be visualized using ApoDNA1 kit ladder assay in DNA extracts from EGF-treated cells (Figure 1c, lane EGF) but not in control cells (Figure 1c, lane Ctl). These data suggested that both cell-death triggers involve the activation of an endonuclease able to fragment internucleosomic DNA. However, only staurosporine was able to induce a caspase-3 activation as attested by cleavage of the inactive pro-caspase-3 (32 kDa) into its active, 17 kDa form (Figure 1d). The endonuclease responsible for EGF-mediated DNA fragmentation might therefore be distinct from that involved in staurosporine effects and its activation is independent of caspase-3.

EGF triggers LEI precursor cleavage

To assess a possible involvement of LEI/L-DNaseII pathway in EGF actions on GH4C1 cells, we first asked whether the expression of LEI alters as a function of EGF treatment duration. Our Western blot data obtained on soluble protein extracts coming from the whole cells left untreated (Figure 2a, lane 0) or treated for different time periods with EGF (Figure 2a, lanes 12, 24 and 48) point to a time-dependent cleavage of LEI precursor (42 kDa) into a partially-activated, intermediate form with apparent molecular mass of 35 kDa. Thus, in untreated, control cells the predominantly expressed form displays a molecular mass of 42 kDa whereas after 48 h of treatment with 1 nM EGF, the 35 kDa-form appears predominant (Figure 2a). By contrast, in staurosporine-treated cells the labeling was similar to the control (Figure 2a, STS lane 24).

To further explore the putative EGF-mediated L-DNaseII cleavage from its LEI precursor, we performed the analogous Western blot experiments on the sub-cellular fractions obtained from either control or EGF-treated cells. These experiments pointed to the presence of 42 kDa precursor form in control cytoplasmic and nuclear fractions (Figure 2b, Ctrl). The addition of EGF triggered the appearance of 27 kDa form corresponding to the fully activated enzyme in the nuclear fraction (Figure 2b: EGF), thus suggesting a full activation of L-DNaseII in the nucleus.[15] Moreover, the proportion of nuclear 27 kDa form increased as a function of the length of EGF treatment (Figure 2b: EGF). It is worth to note that the 27 kDa form is not visible in whole cell extracts (Figure 2a) since in these extracts, nuclear proteins represent only 5–10% of the total protein content. Therefore, in the case of nuclear protein extracts, the protein equivalent contained in the Western blot membrane shown on the panel (b) of the Figure 2 is 10–20 fold greater than the corresponding equivalent shown on the panel (a). An additional immunoreactive band of the apparent

Figure 1. Time course of EGF-induced cell death in GH4C1 cells (a and b) Dead cell number estimation by trypan blue exclusion test in GH4C1 cells treated for 24 h or 48 h by EGF (1 nM) or 24 h by 5 μM staurosporine (STS).***: $p < 0.001$ for all exposure times in treated- vs untreated cells in respective experimental groups (EGF and STS). (c) DNA fragmentation in GH4C1 cells treated for 24 h with 1 nM EGF or 5 μM STS and its absence in control cells (lane Ctl). (d) Western blot of soluble protein extracts obtained from cells treated with EGF, STS (24 h) or left untreated (lane Ctl) to serve as a control. The lane M on the left indicates molecular weight markers. The arrows on the right point to pro-caspase-3 (32 kDa) and active caspase-3 (17 kDa).



mass of 60 kDa was also seen (Figure 2b). This band has been frequently seen in previous studies: it represents the covalent complex between LEI and its activating protease.[16,18,19]

The quality of subcellular fractioning was checked by reprobing the immunoblots with either anti-α-actine or anti-lamin B antibodies. The presence of anti-α-actine immuno-positive band (43 kDa) in cytoplasmic, but not nuclear, fractions and conversely of anti-lamin B immuno-positive band (80 kDa) in nuclear, but not in cytoplasmic, fractions point to the absence of cross contaminations between these subcellular fractions (Figure 2c).

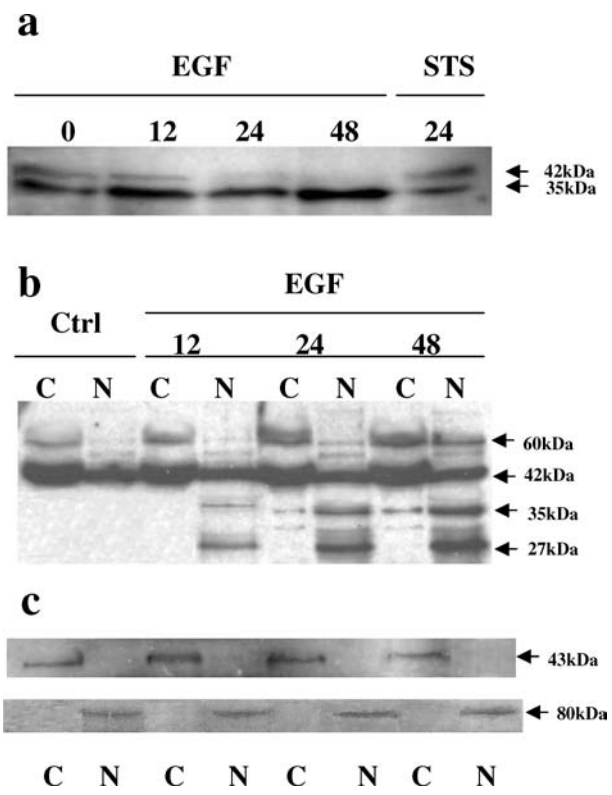
Altogether, these data suggested that EGF might induce cytoplasmic LEI precursor (42 kDa) conversion into a fully active L-DNaseII (27 kDa) and its nuclear translocation.

EGF triggers the activation of LEI/L-DNaseII pathway

To test this hypothesis we next assessed the subcellular localization of L-DNaseII and its enzymatic activity in EGF-treated cells and compared it with data obtained for control cells.

Activation of L-DNaseII was confirmed by direct demonstration of its enzymatic activation. The measurement of L-DNaseII activity in the total cell extracts showed that EGF could induce a time-dependent cleavage of the plasmid used as the endonuclease substrat whereas such cleavage was not observed in the extracts obtained from staurosporine-treated cells (Figure 3). Indeed in this later case, the L-DNaseII activity appears similar to that seen in controls (Figure 3: compare "control", STS12 h" and "STS 24 h"). It is worth noting

Figure 2. EGF triggers the cleavage of LEI. (a) Total protein extracts were obtained from GH4C1 cells treated with 1nM EGF for 12, 24, 48 h or with 5 μM staurosporine (STS) for 24 h. Then 50 μg of soluble protein extracts were analysed by Western blot with polyclonal anti-LEI antibody. (b) Fractionned protein extracts were obtained from GH4C1 cells treated with 1 nM EGF for 24 and 48 h. 50 μg of soluble protein extracts corresponding to cytoplasmic (C) or nuclear fractions (N) were analysed by Western blot with polyclonal anti-LEI antibody. (c) Absence of cross contaminations between subcellular fractions. Cytoplasmic, but not nuclear, fractions display α-actin (43 kDa) whereas nuclear, but not cytoplasmic, fractions display lamin B (80 kDa). The immunoblot shown in (b) has been stripped and then re-probed with either anti-β-actin or anti-lamin B antibodies as described in Materials and Methods.



that ionic and pH conditions used in these experiments prevent the activation of calcium and/or magnesium-dependent nucleases such as CAD but allow the activity of acid DNases such as DNaseII alpha. To confirm the specificity of L-DNaseII actions on plasmid cleavage by extracts obtained from EGF-treated cells we performed parallel experiments in which the endonuclease activity was measured after pre-incubation of the extracts with anti-L-DNaseII antibody. These experiments showed that the pre-incubation with anti-L-DNaseII could prevent plasmid degradation (Figure 3, the bottom panel).

Our immunocytochemical experiments confirmed a time-dependent translocation of L-DNaseII from cytoplasm to the nucleus (Figure 4: EGF 12 h, 24 h and 48 h versus EGF 0 h). In the same time, DAPI staining of DNA pointed to the absence of the compacted chromatine in EGF-treated cells (Figure 4, left column). By contrast, no L-DNaseII translocation was seen in staurosporine (5 μM) treated cells either 12 h or 24 h after its addition (Figure 4: STS). However, DAPI staining indicated the presence of typical apoptosis-like chromatine condensation (Figure 4: STS, left column) in staurosporine-treated cells.

Interestingly, EGF-treated cells displaying large vacuoles (Figure 5, panel "Nomarsky") consistently showed an intense nuclear L-DNaseII staining (Figure 5, "overlay": compare arrow-head (showing the huge vacuole) and arrow (showing nuclear L-DNaseII staining)- pointed cell with surroinding cells). This is attested by the absence of the red color in the nucleus of the pointed cells and was the best visible at the panel "EGF 12 h" (Figure 5: "overlay").

Altogether, these data confirmed the enzymatic activation of L-DNaseII and it subsequent translocation to the nucleus in the presence of EGF in contrast to its absence in staurosporine-treated cells used as a negative control.

EGF-induced cell death is prevented by Alix

Taken together with our previously reported data,[11] these results suggested that EGF-induced cell death might be paraptosis. Given that paraptosis can be specifically blocked by Alix,[10] we assessed the consequence of its over-expression on EGF-induced cell death.

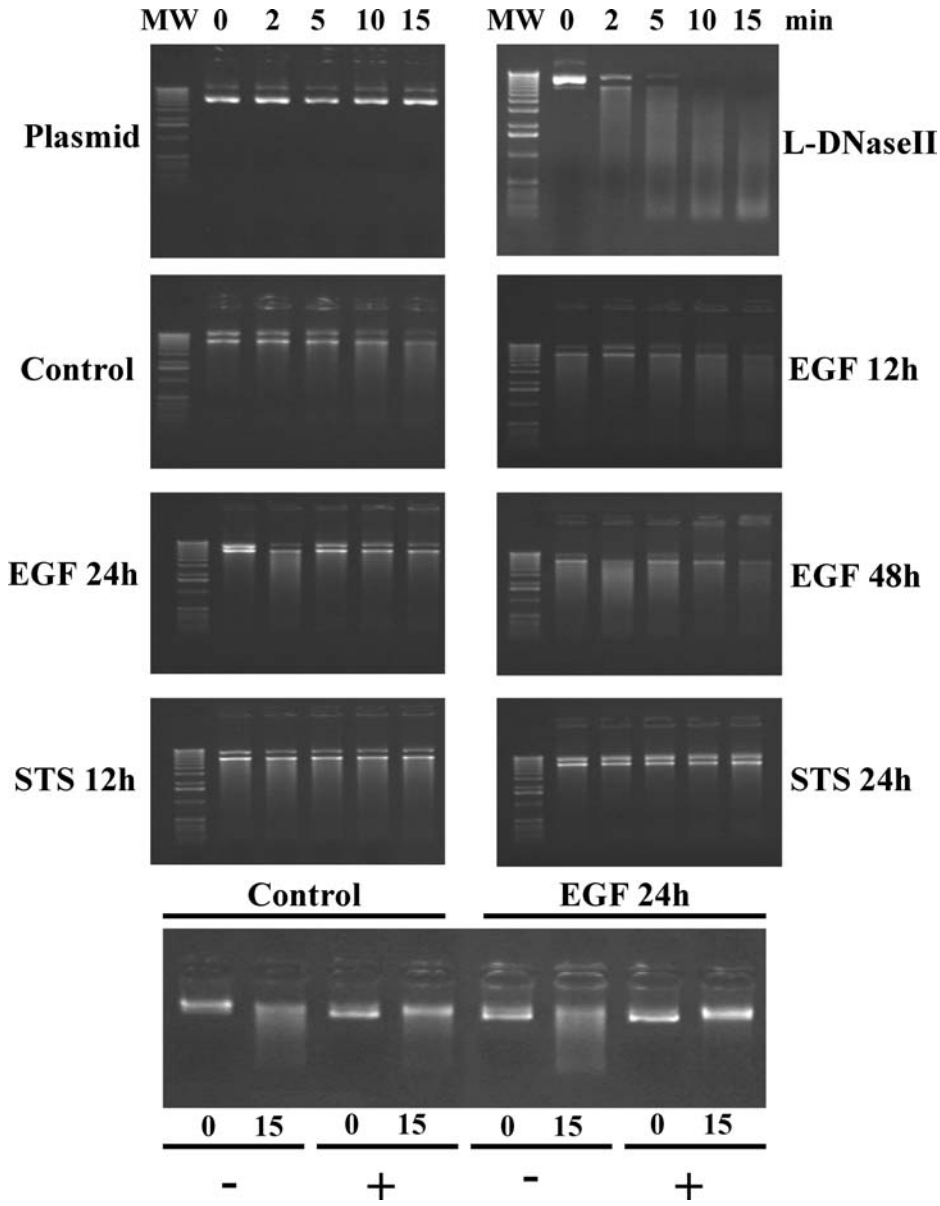
Our data clearly show that in GH4C1 cells over-expressing Alix, in contrast to cells transfected with the control "empty" vector, EGF could not induce cell death (Figure 6). The opposite was seen in Alix-CT over-expressing cells. Indeed, in the latter case EGF could still induce cell death, and this even more efficiently than in mock-transfected cells (Figure 6).

Discussion

EGF triggers a PCD in somato-lactotrope GH4C1 cells with striking similarities to paraptosis from a morphological point of view[11] but also because it can be efficiently blocked with AIP-1/Alix (this study). However, in contrast to paraptosis, this PCD is characterized by the presence of the caspase-independent internucleosomal DNA fragmentation.[11] The analysis of the pathways involved in EGF-induced internucleosomal DNA fragmentation performed in this study show that L-DNaseII represents a serious candidate for such a role.

In the last 10–15 years, cell death by apoptosis has attracted much attention although the research in the field has been greatly dominated by the analysis of caspase-dependent apoptosis. However, it is now clear that not all cell deaths can be neatly classified and that different types of overlap exists.[20–22] This seems also to be the case in the model we analyzed in this work. Indeed, the cell death studied here can be defined as paraptosis based on the capacity of Alix, but not Alix-CT, to prevent

Figure 3. EGF induces an increase in L-DNaseII activity. A 4.5 μg sample of a supercoiled plasmid (pGEM) was incubated with the extracts of cells treated with EGF (for 12, 24, 48 h) or staurosporine (for 12, 24 h) as described in Materials and methods. L-DNaseII enzymatic activity was visualized by the degradation of plasmidic DNA. The "plasmid" panel represents the plasmid incubated in the absence of cell extract, showing no spontaneous degradation. "L-DNaseII" panel corresponds to a positive control obtained by incubating the same plasmid with 1 μg of the purified L-DNaseII. The bottom panel shows the specificity control performed with extracts obtained from either control ("control")- or EGF ("EGF 24 h)-treated cells incubated with substrate plasmid in the absence ("−") or in the presence ("+") of anti-L-DNase II used in 1:50 dilution.



it. Consistently, it has been recently reported that these molecules allow to discriminate between classical (caspase-dependent) apoptosis and paraptosis (caspase-independent PCD distinct from caspase-independent apoptosis) since the former is selectively blocked by Alix-CT whereas the second is blocked exclusively by Alix.[10] These findings further extend our recently published data indicating that the EGF-mediated cell death of somato-lactotrope pituitary cells displays some characteristics of paraptosis, namely caspase-independence and the presence of large, apparently empty vacuoles.[11] However, in contrast to the original description of paraptosis,[10] the cell death observed in our model system does not involve MAP kinases pathway and can be blocked by bcl-2 over-expression.[11] Altogether, these data therefore suggest that EGF-induced programmed cell death in pituitary GH4C1 cells might represent a novel paraptosis pathway.

Our data bring also some new evidence on the functional involvement of L-DNaseII in different forms of the programmed cell death. Indeed, previous studies have

Figure 4. EGF triggers L-DNaseII translocation from cytoplasm to nucleus. GH4C1 cells were treated with 1 nM EGF for different periods (12, 24, 48 h) or with 5 μM staurosporine (STS) for 12 h and 24 h. After treatment, cells were fixed and immunostained with a polyclonal anti-LEI antibody and counter-colored with DAPI. (DAPI staining was numerically changed in red to allow a better overlay with the green emission of the LEI immunoreactivity). The arrows point nuclear labelling of L-DNaseII.

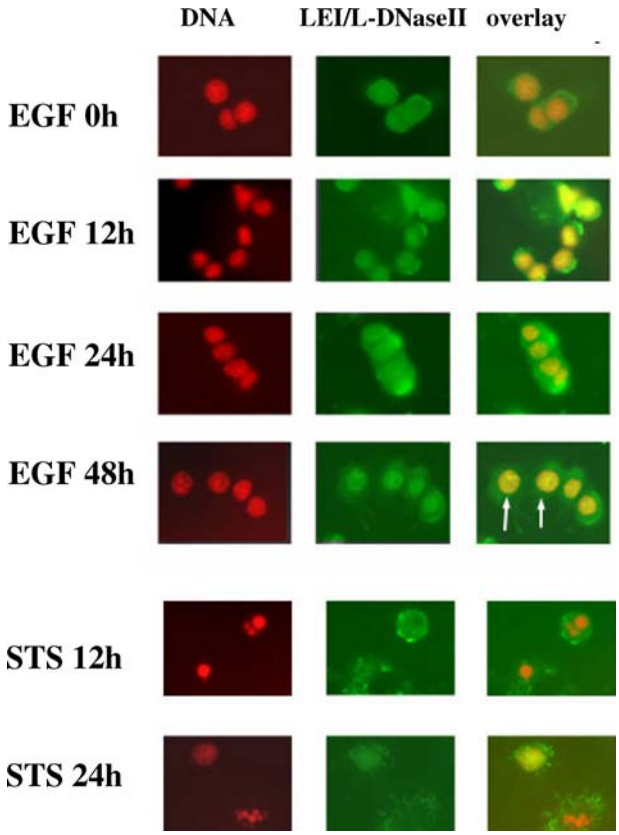


Figure 5. Correlation between EGF-induced L-DNaseII translocation into the nucleus and cytoplasmic vacuolization. GH4C1 cells were treated with 1 nM EGF for different periods (12, 24, 48 h). After treatment, cells were fixed. Nomarsky photographs of EGF-treated cells show large cytoplasmic vacuoles (pointed by arrow-heads on the panel "Nomarsky"). EGF-treated cells were immunostained with a polyclonal anti-LEI antibody and counter-colored with DAPI (DAPI staining was numerically changed in red to allow a better overlay with the green emission of the LEI immunorectivity). The arrows on the panel "overlay" point more intense nuclear labelling of L-DNaseII in the cells displaying large vacuoles.

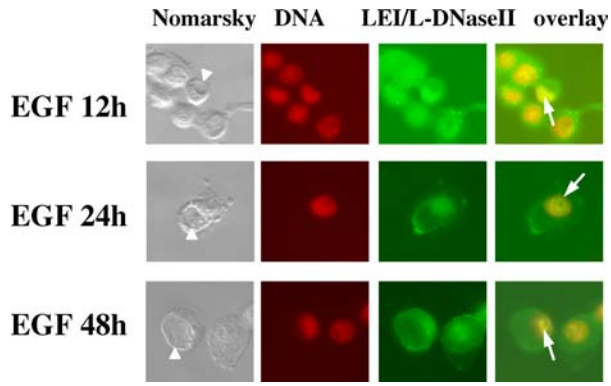
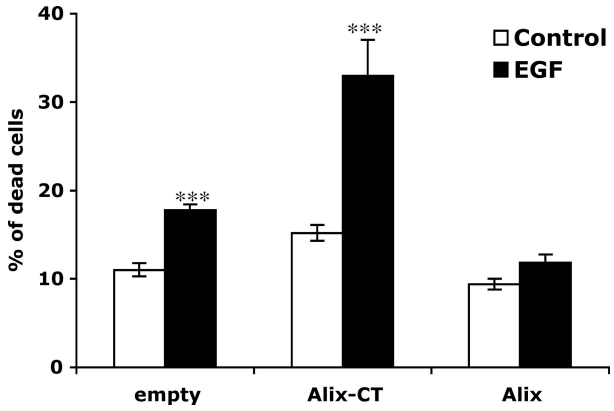


Figure 6. EGF-induced cell death is blocked by Alix but not by Alix-CT. Cell death was determined in empty- and either pCI Flag-Alix-CT or pCI Flag-Alix expression construct-transfected cells. In both experimental groups, cells were left untreated (control) or were treated with 1nM EGF for 48 h. Transfections were performed by adding 0,5 μg cDNA/well 48 h before the beginning of the EGF treatment. For this cDNA quantity and for the 2:1 charge ratio of TransFast™ to DNA, 74% of cells were transfected as determined by quantification of X-GAL coloration after a parallel cell transfection with βGAL construct inserted into pCEP (Stratagene) vector.



involved this endonuclease exclusively in the classical apoptosis.[15,17,19] We show here that the EGF-mediated LEI cleavage occurs in the absence of caspase activation and fulfils the criteria required for L-DNaseII activation. First, EGF addition triggers the cleavage of LEI into intermediate 35 kDa and fully active 27 kDa forms. Second, the nuclear translocation of LEI/L-DNaseII is observed in EGF-treated cells. Third, the enzymatic activity of L-DNaseII increases in a time-dependent manner upon EGF addition. These findings therefore suggest that L-DNaseII might be not only classical apoptosis executioner but might also participate in the execution of additional, non-apoptotic PCDs. Interestingly, it has been reported that, in contrast to what we found in GH4C1 cells, staurosporine-induced apoptosis in L1210 murine leukaemia cells involves L-DNaseII.[19] The capacity of staurosporine to induce the activation of L-DNaseII pathway appears therefore cell-type specific. It is currently not clear whether this cell-type specificity results from different profile of protease and endonuclease expressions. Indeed, it can be hypothesized that a given extra-cellular signal might trigger apoptosis only in cell types in which the level of caspase expression and their capacity to be activated are sufficient to support the triggering of this proteases cascade. According to this hypothesis, cell-death inducing signals like staurosporine might trigger classical apoptosis via either caspase (and subsequent CAD) activation when these proteases/endonuclease are available in a given cellular context or, in opposition, by activation of other proteases capable of activating endonucleases different from CAD. This

hypothesis predicts that staurosporine-mediated activation of the L-DNaseII pathway in leukaemia L1210 cells occurs in the absence of caspase activation[19] in contrast to caspase-dependent staurosporine actions seen in pituitary GH4C1 cells[11] (and present study). Consistently, it has been repeatedly suggested that if, for any reason, caspase activation cannot be achieved, cell committed to die can switch from apoptotic to other types of programmed cell death programs (reviewed in[20–23]).

The mechanism of LEI cleavage into fully activated L-DNaseII has been characterized: it involves at least two different proteases, elastase[15,19] and AP24.[17] However, the up-stream mechanisms coupling cell-death-inducing signals and elastase/AP24 activation remain poorly understood. In particular, it is yet unknown whether Ca^{2+}-dependent proteases such as for example calpain might impinge on elastase activation and consequently on LEI/L-DNaseII pathway triggering.[24] In the light of the putative mechanism underlying EGF actions in triggering this pathway, the question of a possible calpain (or other Ca^{2+}-dependent proteases) involvement appears particularly important since it has been reported that an increase in the intracellular Ca^{2+}concentrations mediates EGF effects on GH4C1 cells.[25] More generally, the results presented here taken together with the previously published data suggest a dual involvement of L-DNaseII in both apoptosis[15,17,19] and paraptosis (present study). It should be stressed that a dual involvement of CAD in both apoptosis and necrosis has also been reported in ischemic hippocampal neurons.[26]

Another important implication of the current results concerns the mechanisms by which Alix inhibits EGF-induced paraptosis. This protein has been identified as a protein interacting with the cell death-related calcium-binding protein ALG-2 which is itself associated with apoptosis.[27,28] Although the pro-apoptotic role of Alix, as well as its capacity to prevent paraptosis, remain incompletely defined, it has been shown that Alix binds to endophilins and thus might interfere with endocytosis.[14,29] Our transfection assays clearly show that Alix prevents EGF capacity to induce cell death. Taken altogether these data suggest that such protective effect of Alix might be associated with its capacity to block EGF receptor (EGFR) endocytosis via its direct interaction with endophilines. An obvious functional consequence would be an increase in the number of EGFR expressed on the cell surface. The latter hypothesis implies a hyperactivation of EGFR expressed on GH4C1 cells by a physiological concentration of EGF since we used this trophic factor in a nanomolar concentration (corresponding to the affinity of pituitary EGFR).[25,30] Future studies focused on the analysis of the functional consequences of such putative EGFR hyperactivation will certainly help understanding the molecular mechanisms linked with EGF-associated malignant transformation.

Finally, the fact that in Alix over-expressing cells there is no significant difference between cell mortality in control and EGF-treated cells point to the incapacity of Alix to induce cell death (by apoptosis) after turning down the paraptotic pathway. This observation has a great therapeutic interest. Indeed, it suggests that: i) apoptotic cell death program is shut down in the course of malignant transformation of GH4C1 cells; ii) an alternative, paraptotic program can still be triggered in these conditions. In addition, data obtained in Alix-CT over-expressing cells pointing to a synergy between Alix-CT and EGF in inducing paraptosis suggest that EGF triggers this pathway by a mechanism distinct from Alix-CT-mediated one. Given that in GH4C1 cells apoptotic pathway appears turned-down, this implies that Alix-CT facilitates EGF-mediated induction of paraptosis. These latter considerations definitively deserve full attention and further experimental support. They also focus on Alix-CT as a potential therapeutic target for tumours resistant to apoptosis-inducing therapies that might arise in the course of cancerigenesis from the selective disabling of this pathway, probably selected for its growth-advantage properties.

Conclusion

EGF induced PCD in somato-lactotrope GH4C1 cells involves the activation of L-DNaseII pathway in a caspase-independent manner. This PCD can be blocked by an over-expression of a paraptosis inhibitor AIP-1/Alix. According to its caspase independence and inhibition by AIP-1/Alix (11) and present study) and in combination with the previously reported cytoplasmic vacuolization,[11] EGF-induced PCD may be classified as paraptosis.[8,10] However, in contrast to classical paraptosis, EGF-induced PCD in GH4C1 cells involves internucleosomal DNA fragmentation but does not involve MAP kinases.[11]

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