The ability to cleave 28S ribosomal RNA during apoptosis is a cell-type dependent trait unrelated to DNA fragmentation

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Abstract

Discrete cleavages within 28S rRNA divergent domains have previously been found to coincide with DNA fragmentation during apoptosis. Here we show that rRNA and DNA cleavages can occur independently in apoptotic cells, i.e. that the previously observed correlation is likely to be coincidental. In HL-60 cells, apoptosis with massive DNA fragmentation could be induced without any signs of rRNA cleavage. The opposite situation; rRNA cleavage without concomitant internucleosomal DNA fragmentation, was found in okadaic acid-treated Molt-4 cells. Other leukemia cell lines underwent apoptosis either without (K562 and Molt-3) or with (U937) both forms of polynucleotide cleavage. In K562 cells transfected with a temperature-sensitive p53 mutant, internucleosomal DNA fragmentation but not 28S rRNA cleavage was inducible by wild-type p53 expression. The absence of apoptotic rRNA cleavage in some cell types suggests that this phenomenon is tightly regulated and unrelated to DNA fragmentation or a presumed scheme for general macromolecular degradation in apoptotic cells.

Keywords: apoptosis, rRNA cleavage, ribosome, DNA fragmentation, p53

Abbreviations: GTC, guanidinium thiocyanate; SLS, sodium lauroylsarcosine

Introduction

The ribosome consists of two major subunits (60S and 40S), each made up of rRNA backbones and a number of both firmly bound and more loosely associated proteins, together forming the translational apparatus. For unknown reasons, regions of the rRNA backbone of the large ribosomal subunits have greatly expanded in higher eukaryotes (Raué *et al*, 1988). These expansions are called divergent domains due to great variation in primary structure, even between closely related

species (Michot and Bachellerie, 1987). While these expansions could serve as anchoring points for eukaryote-specific proteins involved in translational regulation, it is also possible that these regions have functions unrelated to protein synthesis, e.g. related to apoptosis. The latter was recently suggested by the finding that subdomains within these divergent or D-domains were specifically cleaved during apoptosis (Houge *et al*, 1993). Discrete cleavages, affecting major D-domains (D2, D6 and D8), occurred after the onset of apoptosis and in parallel with DNA fragmentation in rat myeloid IPC-81 cells, rat thymocytes, human promyeloid NB4 cells and bovine endothelial cells (Houge *et al*, 1995). This alluded that both forms of polynucleotide cleavage could be linked to general nuclease activity in apoptotic cells, i.e. that apoptosis involves the controlled use of RNases and DNases (in addition to proteinases) in an ordered process of cellular degradation.

To test this hypothesis, five leukaemia cell lines (HL-60, U937, K562, Molt-3, Molt-4) were screened for the presence of DNA and rRNA cleavage during apoptosis induced by either okadaic acid, a phosphatase inhibitor, or the DNA topoisomerase-I inhibitor camptothecin. The reason for including three p53-negative cell lines (HL-60, U937 and K562) (Shiohara *et al*, 1996) was a reported covalent association between 5.8S rRNA and a C-terminal serine-residue in p53 (Samad and Carroll, 1991; Fontoura *et al*, 1992). Because (wild-type) p53 expression can induce apoptosis, it was of interest to determine if apoptotic 28S rRNA fragmentation correlated with this p53/rRNA association. However, apoptosis in these p53-negative cell lines occurred both with (U937) or without (HL-60, K562) 28S rRNA cleavage, regardless of internucleosomal DNA fragmentation being present (U937, HL-60) or absent (K562). The independency of the two parameters was further shown in another leukaemia cell line (Molt-4), where okadaic acid induced apoptosis with 28S rRNA cleavage but without DNA fragmentation. Our findings indicate that the 28S rRNA fragmentation seen during apoptosis is not part of a scheme for general macromolecular disintegration and degradation, but rather a genetically controlled trait present in some cell types and absent in others. The molecular basis for this difference is unknown.

Results and Discussion

28S rRNA cleavage was not detected during apoptosis of HL-60 cells

The human myeloid cell line HL-60 has been a popular model system for the study of cell death and differentiation (e.g. Han *et al*, 1996; Jarvis *et al*, 1996). Apoptosis could be efficiently induced by treating these cells with e.g. the phosphatase inhibitor okadaic acid, presumed to kill cells by inducing a

Figure 1 Examination of DNA fragmentation (A) and 28S rRNA cleavage (B) in normal and apoptotic HL-60 cells. A positive control, prepared from human promyelocytic NB4 cells treated for 10 h with 316 nM okadaic acid (90% apoptosis), is shown on the right of panel B. DNA (10 μ g per lane) was separated on a 1% agarose gel (A). RNA from the same cells (25 μ g per lane) was analyzed by Northern blotting and hybridisation to a combination of two 32 P-labelled oligonucleotide probes complementary to the ends of rat 28S rRNA (B), i.e. nucleotides 16-51 (5'28S probe) and 4695-4719 (3'28S probe) (see Houge *et al*, 1995 for more detailed sequence information). For clarity, the hybridisation of the separate 5' and 3' 28S oligonucleotide probes to apoptotic 28S rRNA fragments is shown in the positive control sample (NB4 cells). The cells were treated as follows: lane 1, control (3% apoptosis); lane 2, 316 nM okadaic acid for 10 h (97% apoptosis); lane 3, 316 nM camptothecin for 10 h (58% apoptosis); lane 4, 3.16 μ M camptothecin for 10 h (60% apoptosis).

state of dysphosphorylation (for a review, see Gjertsen and Døskeland, 1996), or camptothecin, a DNA topoisomerase-I inhibitor (Figure 1A). In both cases, morphologically apoptotic cells started to appear after about 3 h of treatment, and after 10 h of treatment most of the cells were apoptotic. The extent of apoptosis corresponded with the extent of DNA fragmentation (Figure 1). We then investigated for occurrence of 28S rRNA cleavage, a novel parameter for apoptosis (Houge *et al*, 1995). However, 28S rRNA from apoptotic HL-60 cells appeared completely intact, even in late apoptosis. 28S rRNA fragments were also undetectable after Northern blotting and hybridizations with 32 P-phosphate-labelled 28S rRNA-complementary oligonucleotides (Figure 1B). A similar lack of 28S rRNA cleavage was found when another phosphatase inhibitor, calyculin A (100 nM), was used to induce apoptosis (data not shown). So far, other apoptosis-inducing agents have not been tested for the induction of rRNA cleavage in HL-60 cells. However, it is likely that the findings will be similar. This is because extensive studies on rat myeloid IPC-81 cells have shown that the occurrence of rRNA cleavage is unrelated to the apoptosis-inducing agent (Houge *et al*, 1995).

The intactness of ribosomal RNA (28S and 18S) in HL-60 cells having extensive DNA fragmentation and cellular disintegration, indicated that rRNA cleavage is not a general phenomenon in apoptosis, e.g. a consequence of an increased (random) nuclease activity in apoptotic cells. Furthermore, the exceptional integrity of 28S rRNA in apoptotic HL-60 cells alludes that these cells lack a molecular mechanism for apoptotic 28S rRNA cleavage. Interestingly, the absence of both primary D2 and primary D8 cleavage indicates that the two apoptotic cleavage pathways are interconnected (see Houge and Døskeland, 1996, for a more detailed discussion of the two different options for 28S rRNA cleavage in apoptosis). In rat myeloid IPC-81 cells, apoptotic D8 cleavage occurred in an exposed area of the ribosomal rRNA, while D2 cleavage was found in stem-loop junctions inaccessible to chemical agents capable of modifying single-stranded bases in the rRNA molecule (Leffers and Anderson, 1993; Holmberg *et al*, 1994; Houge *et al*, 1995). Despite this structural difference between the D2 and D8 cleavage sites, it appears that the molecular pathways for their cleavage during apoptosis have factors in common that either are lacking or are inhibited in HL-60 cells.

Apoptotic 28S rRNA cleavage is not linked to the presence of p53 protein, DNA fragmentation or a specific apoptosis-inducing agent

The complete lack of 28S rRNA cleavage during apoptosis of HL-60 cells prompted us to study if this could be a general finding in p53-negative cell lines. A link between the apoptosis-inducer p53 (for a review, see Lee and Bernstein, 1996) and apoptotic 28S rRNA cleavage would have given a rationale for the reported covalent association between the C-terminal serine residue of p53 and 5.8S rRNA (Samad and Carroll, 1991; Fontoura *et al*, 1992). To this end, the apoptotic 28S rRNA cleavage status of the p53-negative human leukaemia cell lines K562 (undifferentiated, myeloid, e.g.

Sucaï *et al*, 1992) and U937 (monocyte-like, derived from a histiocytic lymphoma, e.g. Sugimoto *et al*, 1992) were examined. In addition, Molt-3 (lymphoblastoid T-cell leukaemia, unknown p53 status) and Molt-4 cells (lymphoblastoid T-cell leukaemia, originally derived from the same patient as Molt-3, and containing a splice site mutation in one p53 gene causing an in-frame stop codon, see Chow *et al*, 1993) were similarly studied. Apoptosis was induced using camptothecin or okadaic acid, 28S rRNA cleavage was analyzed after oligonucleotide hybridizations to Northern blots, and DNA fragmentation was analyzed on conventional 1% agarose gels (Figure 2). Neither DNA fragmentation nor 28S rRNA cleavage was found associated with apoptosis in K562 and Molt-3 cells. Note that okadaic acid could induce apoptosis in all cell lines with similar efficiency. Significant 28S rRNA cleavage in both divergent domain D2 with D2c excision (giving rise to the A-bands in Figure 2) and D8 (giving rise to the B-band in Figure 2, for details see Houge *et al*, 1995) was found in Molt-4 cells. Importantly, in the okadaic-acid treated Molt-4 cells, this 28S rRNA cleavage occurred without concomitant internucleosomal DNA fragmentation. A much fainter band having the same position as the B-band can also be seen in Molt-3 rRNA after 10 h of camptothecin treatment (Figure 2), but A-bands were not found. It has previously been reported that (multiple) cleavages in the D8 region can occur during necrosis (Houge *et al*, 1995), and this band could

possibly result from 'contamination' with a low level of late apoptotic and secondary necrotic Molt-3 cells. Somewhat more extensive 28S rRNA cleavage was found in U937 cells (Figure 2), and the cleavage patterns corresponded with what has previously been found in other leukaemia cell lines (Houge *et al*, 1995).

It has been shown in a number of cell death systems that there is no clear correlation between DNA cleavage and the percentage of apoptotic cells. This appears also to be the case in the present study, and applies not only to DNA fragmentation but also to apoptotic 28S rRNA cleavage (compare e.g. Molt-4 cells treated with okadaic acid and camptothecin, and U937 cells with Molt-4 cells, Figure 2). Interestingly, internucleosomal DNA fragmentation (as shown in Figure 1) and 28S rRNA cleavage were uncoupled in a few cases (e.g. HL-60 cells and Molt-4 cells). This makes it unlikely that dual-function nucleases (RN/DNases) causes both 28S rRNA cleavage and DNA fragmentation during apoptosis, although such nucleases have been found to be proteolytically activated in apoptosis (Fraser, 1994). Taken together, these findings indicate that the ability to cleave 28S rRNA during apoptosis is a cell-type dependent trait, variably present in different cell types (i.e. strong in U937 cells, weak in Molt-4 cells, absent in K562, Molt-3 and HL-60 cells). The molecular basis for this difference is unknown.

Although the presence of p53 appears to be no prerequisite for apoptotic rRNA cleavage (see above), it remained possible that (wild-type) p53 expression could turn on a ribosomal RNA cleavage pathway during apoptosis in an otherwise p53 negative cell line. To test this, K562 cells transfected with a murine p53 mutant (Val135) that is temperature-sensitive and adopts wild-type conformation when grown at 32°C (Soddu *et al*, 1996), were examined. In these K562p53 cells, wild-type p53 induction sensitised the cells to camptothecin and made them undergo camptothecin-induced apoptosis with some DNA fragmentation (Figure 3) (Samali *et al*, manuscript in preparation). However, the cells still showed no 28S rRNA cleavage during apoptosis (Figure 3), indicating that the induction of DNase activity by wild-type p53 expression was not linked to apoptotic 28S rRNA cleavage.

In conclusion, we have shown that 28S rRNA cleavage, like DNA fragmentation, is a parameter that is either absent or present to a variable degree during apoptosis, and that the two forms of polynucleotide cleavage can occur independently of each other. While the occurrence and degree of DNA fragmentation could depend on the apoptosis-inducing agent and be inducible by wild-type p53 expression, this was not the case for 28S rRNA cleavage. 28S rRNA cleavage still seems to be a highly specific parameter for apoptosis, apparently regulated by a pathway that is inducible or subjected to inactivation. The purpose for having such an apoptosis-specific 28S rRNA cleavage pathway is a mystery, as is the function of ribosomal divergent domains in general. However, a role related to virus defence, as previously suggested (Houge *et al*, 1995), is still conceivable. In this respect, the recent demonstration of pronounced 28S rRNA cleavage during virus induced apoptosis (Suarez *et al*, 1996) is intriguing.

Figure 2 Examination of 28S rRNA cleavage in normal and apoptotic K562, Molt-3, Molt-4 and U937 cells. Total RNA (25 µg per lane) was analyzed by Northern blotting and hybridization to ³²P-labelled oligonucleotide probes complementary to the ends of the 28S rRNA molecule (as in Figure 1, B). The treatments were as follows: lane 1, control; lane 2, 316 nM okadaic acid for 10 h; lane 3, 3.16 µM camptothecin for 10 h. DNA was isolated from the same cell samples and analyzed for internucleosomal DNA fragmentation, as shown in Figure 1. The presence of clear-cut DNA fragmentation is indicated by a plus-sign, the absence of DNA fragmentation by a minus-sign below the lanes. The percentage of apoptotic cells in each sample is listed at the bottom of the figure.

Figure 3 Examination of 28S rRNA cleavage (A) and DNA fragmentation (B) in p53-transfected K562 cells. K562 cells were transfected with the pRSVneo plasmid (K562neo cells) or the pN53cG(Val135) plasmid, the latter causing expression of a temperature-sensitive p53 mutant protein which adopts wild-type conformation at 32°C (K562p53 cells), and analyzed for DNA fragmentation and 28S rRNA cleavage as shown in Figure 1. All cells samples were treated with 3.16 μ M camptothecin for 10 h. Lane 1, K562neo cells grown at 37°C; lane 2, K562neo cells grown at 32°C; lane 3, K562p53 cells grown at 37°C; lane 4, K562p53 cells grown at 32°C. The percentage of apoptotic cells in each sample is indicated at the bottom of the figure.

Materials and Methods

Cell cultures

Apoptosis was induced in six different human leukaemia cell lines; HL-60, U937, K562, Molt-3, Molt-4 and NB4 cells. All cell lines were cultured at densities around 6×10^5 cells/ml in DMEM or RPMI supplemented with 10% foetal calf serum. Cell death was induced by adding camptothecin (Sigma, MO), a DNA topoisomerase-I inhibitor, or the phosphatase inhibitors okadaic acid or calyculin A (Calbiochem, CA) to the cultures. The undifferentiated K562 cells, originally derived from a patient with chronic myelogenous leukaemia in terminal blast crisis, were resistant to apoptosis-induction by camptothecin but were killed by phosphatase inhibitors (okadaic acid or calyculin A) at concentrations down to 10 nM. Okadaic acid can induce apoptotic changes in most cell types (Bøe *et al*, 1991).

K562 cells transfected with the plasmid pN53cG(Val135) (K562p53 cells) or the control plasmid pRSVneo (K562neo cells), both introduced by electroporation, were a gift from Silvia Soddu at Regina Elena Cancer Institute in Rome, Italy (for a more detailed description of these cells, see Soddu *et al*, 1996). Transfected cells were cultivated at densities around $5-6 \times 10^5$ cells/ml and in the presence of 200 μ g/ml G418 (Sigma). The temperature-sensitive Val135 p53 mutation has been used to study wild-type p53 function in other p53-negative cell lines, e.g. in the M1 myeloid leukaemia cell line (Yonish-Rouach *et al*, 1993). p53 adopts wild-type conformation at 32°C.

The extent of apoptosis was assessed in cell samples prepared by cytopsin followed by staining with RAPI-DIFF II (Diagnostic Development, UK), as described (Samali and Cotter, 1996). Slide preparations were mounted with DPX mountant (BDH Chemicals, UK), and the percentage of apoptotic cells (cells with condensed and fragmented nuclei) was determined. Sometimes cells were tested for trypan blue exclusion to assess the number of (secondary) necrotic cells.

Preparation and analysis of RNA and DNA

Total RNA was prepared from cell cultures using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynsky and Sacchi, 1987). Cell pellets were immediately lysed in 25 mM sodium citrate buffer pH 7 containing 4 M guanidinium thiocyanate (GTC), 0.5% (w/v) sodium lauroylsarcosine (SLS) and 1% 2-mercaptoethanol. Denaturing gel electrophoresis of RNA and capillary blotting of RNA onto nylon membranes (Northern blotting) were done as previously described (Houge *et al*, 1990). DNA was prepared and electrophoresed using standard procedures (Sambrook *et al*, 1989). Oligodeoxynucleotides, complementary to various regions of rat 28S rRNA, were 5'-end labelled with 32 P using polynucleotide kinase and [γ - 32 P]ATP and used as probes for Northern blot hybridizations, as previously described (Houge *et al*, 1995).

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