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Apoptosis of cell lines conditionally immortalized by SV40 is not associated with a pattern of gene induction similar to that observed during thymocyte apoptosis

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Abstract : Inactivation of SV40 large T antigen, in cells immortalized with conditional mutants, leads to activation of p53 and apoptosis. We have analyzed during this process the expression of genes induced by p53 or differentially expressed during apoptosis in other systems. We find an early induction of *Waf1/Cip1*. We also observed *clusterin* is induced late during the process and displays a high level of expression in non-apoptotic cells suggesting a protective role for *clusterin*. Other genes identified during thymocyte and lymphocyte apoptosis are not induced, showing that the pattern of gene induction is specific to the system studied.

Key words : SV40 / p53 / apoptosis / immortalization / gene expression

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1. INTRODUCTION

Apoptosis is an active process of gene-directed cellular self-destruction and in most cases serves a biologically meaningful homeostatic function [1-4]. It has been previously observed that rodent embryo fibroblasts conditionally immortalized by temperature sensitive mutants of SV40 large T antigen undergo apoptosis at restrictive temperature [5, 6]. Apoptosis of these cells in restrictive conditions appears to be mediated by the release of wild type p53 from large T antigen as judged by coprecipitation experiments and its ability to induce *mdm-2* expression [5]. We have choose one of these cell lines, called REtsAF, as a model system for studying p53-mediated apoptosis.

We have observed that although global transcription and translation rates are only slightly affected by the induction of apoptosis in these cells, some proteins are differentially synthesized [7]. This suggests that, as in other cases of apoptosis, some genes are differentially expressed during REtsAF apoptosis. In this report we examine in apoptotic REtsAF cells the expression of genes known to be regulated during apoptosis of other cells. Parallel experiments performed with the REtsAF-Rev1 variant which is no longer temperature sensitive and with a cell line (RELPB) immortalized by wild-type SV40 [8] allowed us to distinguish effects of the temperature shift from apoptosis related changes.

2. MATERIALS AND METHODS

2.1. *Cell lines and cell culture*

The REtsAF and RELPB cell lines were isolated at low cell density from a rat embryo fibroblast culture infected with SV40 [8]. REtsAF was obtained using a tsA58 mutant and is temperature sensitive for immortalization while RELPB was obtained with wild-type SV40 and is immortal at both 33 and 39.5°C. REtsAF-Rev1 was derived from REtsAF by selection for proliferation at 39.5°C [7]. Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum or DMEM/F12 supplemented with 2% Ultroser G, plus

penicillin (100µg/ml) and streptomycin (100 U/ml) under 5% CO_2. The cultures were screened regularly for the absence of mycoplasma.

2.2 .*RNA analysis*

Total cellular RNA was isolated from cells by the method of Chirgwin *et al.* [9]. Gene expression was assayed by the Northern blotting analysis. 10 µg of total RNA was fractionated on 1% agarose-formamide gel and transferred to nylon membrane (HybondN, Amersham) according to [10]. Probes for Calmodulin, Chondroitin sulfate proteoglycan core protein, Clusterin, Ubiquitin and GAPDH were obtained respectively by labeling of the insert of plasmid p21, p8.5, pSP64-60, pHUB14.38, and pGAPDH respectively with [^{32}P]dCTP by random primers using the Megaprime labeling systems (Amersham). Probes for RP8, Glutathione S-transferase, (made from plasmids pRP-8, pGST2) were labelled with [^{32}P]dCTP by PCR of pBluescript inserts. PCR radiolableling reactions (100 µl) contained 10 ng of supercoiled plasmid template, 100 pmol of universals primers, 5 nmol each of three nucleotides dATP, dGTP, and dTTP, 0.32 nmol dCTP, 2.5 µl of [^{32}P]dCTP (400 Ci/mmol) and 2.5 U of Taq DNA polymerase (Bioprobe). PCR was carried out for 30 cycles as follows: 94°C for 30 s for denaturing; 45°C for 1 min for annealing; and 72°C for 1 min for extension, followed by 1 cycle for extension at 72°C for 5 min. Hybridization was carried out in 5X SSPE, 0.5% SDS and 5X Denhardt's at 65°C. Washes were done twice in 2x SSPE, 0.1% SDS at room temperature and twice for 15' at 65°C in 1 SSPE, 0.1% SDS.

3. RESULTS

3.1. *Expression of genes induced during apoptosis in other systems*

We have analyzed during apoptosis of REtsAF cell lines the expression of several genes known to be induced in different models of programmed cell death. Three types of genes were studied : i) genes identified during glucocorticoid-induced thymocyte apoptosis (*RP-8*) [11, 12]; ii) during prostate regression and other cell death process (*clusterin*) [13]; and iii) during gamma irradiation-induced lymphocyte apoptosis [14] and insect muscles degeneration [15] (polyubiquitin).

Figure 1 shows the example of *RP-8* [12] and *clusterin* [13]. *Clusterin* is induced 10 hours after the shift-up to the restrictive temperature (39.5°C) whereas *RP-8* is constituvely expressed. Table 1 summarizes the results obtained with various genes. The genes encoding Calmodulin, Chondroitin sulfate proteoglycan core protein, Ubiquitin and Glutathione S-transferaseYb1, which are induced in apoptotic thymocytes or lymphocytes [11, 14, 16, 17], are not induced during apoptosis of REtsAF.

In order to determine if the induction of *clusterin* is specific to apoptotic cells we have analyzed its expression during the shift from 33°C to 39.5°C in non conditional cell lines. Figure 2 shows that, *clusterin* is expressed at a high constitutive level in the temperature insensitive variant REtsAF-Rev1 as well as in RELPB and in primary cells. This result argues against a causative role for clusterin in cell death. In order to determine if *clusterin* induction could be due to a heat-shock response, REtsAF cells grown at 33°C were keeped for 15 minutes at 42°C and then shifted-back to 33°C. In contrast to *HSP70* which is indeed induced *clusterin* is not. This result shows that induction of *clusterin* during apoptosis is not related to a heat-shock effect.

3.2. *Expression of genes known to be induced by p53*

We have previously observed that *mdm-2* is induced in REtsAF after 24 hours in restrictive conditions [5] suggesting that apoptosis is mediated by p53. The *Waf1/Cip1* gene is a potential mediator of p53 tumor suppression [18] that inhibits G1 cyclin-dependent kinases [19]. We analyzed the level of *Waf1/Cip1* mRNA in REtsAF during apoptosis. An induction of the *Waf1/Cip1* gene is observed after 4 hours in restrictive conditions (fig. 3). *Waf1/Cip1* is not detected in the temperature insensitive cell lines whatever the temperature. Thus, a specific induction of *Waf1/Cip1* is observed, supporting the idea that p53 is involved in the induction of REtsAF apoptosis. Moreover this result suggest that *Waf1/Cip1* could be a mediator of p53 leading to cell growth arrest and apoptosis.

4. DISCUSSION

A number of genes, induced during apoptosis of thymocytes or lymphocytes, are not induced during apoptosis of REtsAF. This result may indicate some cell type specificity in the pattern of induction. This pattern could also be specific to the apoptosis induction signal. Nevertheless, at least 3 genes are induced during REtsAF apoptosis: *clusterin, mdm-2* and *Waf1/Cip1*.

Clusterin, also called *TRPM-2* and *SGP-2*, is an early indicator of programmed cell death [13]. The product of this gene is a sulfated glycoprotein the function of which remains unclear. Since we found that *clusterin* is induced late during the process (about 10 hours) and displays a high level of expression in temperature insensitive cells, it is probably not involved in the cell death process itself. Rather, in agreement with other results [20], this high constitutive level of expression suggests a protective role for clusterin. Apoptosis of REtsAF cells in restrictive conditions appears to involve the release of p53 activity [5]. Thus we can ask whether the induction of *clusterin* is mediated by the transcriptional activator function of p53. This hypothesis cannot be ruled out but seems unlikely according to the high constitutive level observed in temperature insensitive cells in which p53 is inactivated by large T antigen ([5] and data not shown). Furthermore, the conserved putative cis-element which appears to be the target for specific DNA-binding factors in the *clusterin* gene [21] does not look like a p53 binding site and a computer search in the *clusterin* gene sequence [22] does not reveal any p53 binding site in other part of the gene (data not shown).

The p53 inducible gene *Waf1/Cip1* is induced during REtsAF apoptosis between 4 and 8 hours after the temperature shift. This induction is more rapid than that of *mdm-2* which is in agreement with the proposal that other factors are rate limiting for *mdm-2* expression [23]. The kinetics of induction of *Waf1/Cip1* is close to the kinetics of commitment to apoptosis [24] which suggest that *Waf1/Cip1* could be involved in commitment to apoptosis. It has been shown that p53-dependent apoptosis can occur in the absence of transcriptional activation of p53-target genes in SV40 large T antigen immortalized GHFT1 cells [25] which suggest that, in some cells, p53 can mediate apoptosis by repressing survival genes. Recently, a reevaluation of the role of *de novo* protein synthesis in thymocyte apoptosis has also suggested that inhibitors of protein synthesis may delay apoptosis rather than prevent it [26] suggesting that some of the components of the apoptotic machinery are already present before apoptosis induction. However, the identification of the genes that are up- or down-regulated during apoptosis remains of great interest. Indeed, at least three types

of genes can be regulated during apoptosis : regulators of apoptosis, effectors of apoptosis and genes the expression of which is modified as a cell response to physiological changes. Identification of these genes should give an insight into apoptosis regulation and the successive biochemical events leading to cell death [4]. Further work is needed to identify these genes and understand how the release of p53 from SV40 large T antigen leads to the biological and physiological changes occuring during the process [24].

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LEGENDS TO FIGURES

Figure 1 : *Steady state levels of RP-8 and clusterin mRNA during REtsAF apoptosis*. Total cellular RNA prepared from REtsAF at various time after 39.5°C temperature shift were analysed by Northern blotting. Nylon membrane was probed succesively with *RP8* and*clusterin* probes.

Figure 2 : *Expression of clusterin in various rat embryo cells*. Total cellular RNA prepared from REtsAF at various time after 39.5°C temperature shift were analysed by Northern blotting. The same experiment was performed with different cells that do not undergo apoptosis at 39°C: REtsAF-Rev1, a REtsAF variant that is no longer temperature sensitive; RELPB, established by the wild type SV40; primary rat embryo fibroblasts . Northern blotting of total cellular RNA from REtsAF at various time after a heat-shock (15' at 42°C) is also shown. Nylon membrane was probed succesively with *clusterin*, *HSP70* and *GAPDH* probes.

Figure 3 : Waf1 in differents cell lines

Table 1: Expression of genes indentified by their diffential expression during apoptosis.

GENE(S)	INDUCED IN	EXPRESSION IN REtsAF
Calmodulin, Chondroitin sulfate proteoglycan core protein	Glucocorticoids induced thymocyte apoptosis [11, 16]	not detected
RP8	Glucocorticoids or radiation induced thymocyte apoptosis [12]	not induced
Glutathione S-transferaseYb1	Prostate regression [27] and steroid induced lymphocyte apoptosis [17].	not induced
Clusterin	Prostate regression and various other cell death process [13]	induced
Ubiquitin	Radiation-induced lymphocyte apoptosis [14] and insect muscles degeneration [15]	not induced