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Studies of specific gene induction during apoptosis of cell lines conditionally immortalized by SV40

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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 analysed 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 observe *clusterin* is induced during the process and displays a high level of expression in non-apoptotic cells, suggesting a protective role for *clusterin*. Other genes associated with 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

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 cells 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 chosen 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 (100 μg/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 mg 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 [11], pSP64–60HE [12], pHUB14.38 [13] and pGAPD-13 [14], 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 [15], pGST2 [16]) were labeled with [^{32}P]dCTP by PCR of pBluescript inserts. PCR-radiolabeling 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 5 × SSPE, 0.5% SDS and 5 × Denhardt's at 65°C. Washes were done twice in 2 × SSPE, 0.1% SDS at room temperature and twice for 15 min at 65°C in 1 SSPE, 0.1% SDS.

3. Results

3.1. Expression of genes induced during apoptosis in other systems

We have analysed 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: (1) genes identified during glucocorticoid-induced thymocyte apoptosis (*RP-8*) [11,15]; (2) during prostate regression and other cell-death processes (*clusterin*) [17]; and (3) during γ irradiation-induced lymphocyte apoptosis [18] and insect muscle degeneration [19] (*polyubiquitin*). Fig. 1 shows the example of *RP-8* [15] and *clusterin* [17]. *Clusterin* is induced 10 h after the shift-up to the restrictive temperature (39.5°C) whereas *RP-8* is constitutively expressed. Table 1 summarizes the results obtained with various genes. The genes encoding calmodulin, chondroitin sulfate proteoglycan core protein, ubiquitin and glutathione S-transferase Yb1, which are induced in apoptotic thymocytes or lymphocytes [11,18,20,21], are not induced during apoptosis of REtsAF.

In order to determine if the induction of *clusterin* is specific

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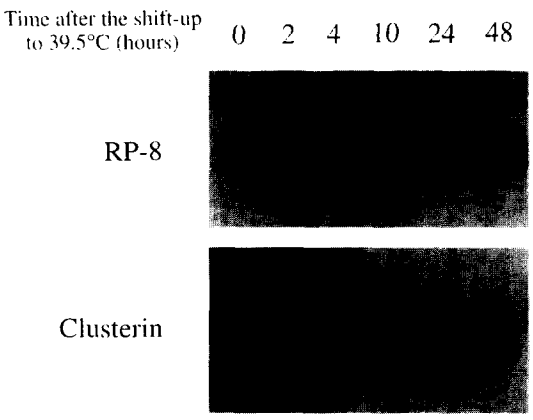

Fig. 1. Steady-state levels of RP-8 and clusterin mRNA during REtsAF apoptosis. Total cellular RNA prepared from REtsAF at various times after the shift to restrictive conditions were analysed by Northern blotting. Nylon membrane was probed successively with *RP8* and *clusterin* probes.

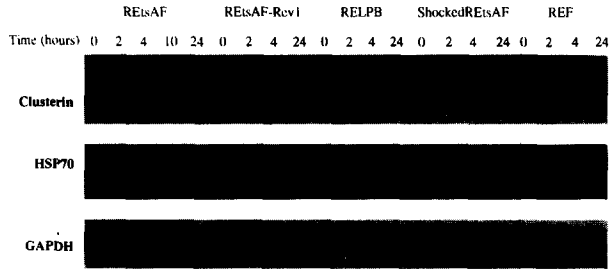

Fig. 2. Expression of clusterin in various rat embryo cells. Total cellular RNA prepared from REtsAF at various times after the shift to restrictive conditions were analysed by Northern blotting. The same experiment was performed with different cells that do not undergo apoptosis at 39°C: REtsAF-Rev1,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

to apoptotic cells, we have analysed its expression during the shift from 33 to 39.5°C in non-conditional cell lines. Fig. 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 min 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 h in restrictive conditions [5], suggesting that apoptosis is mediated by p53. The *Waf1/Cip1* gene is a potential mediator of p53 tumor suppression [22] that inhibits G1 cyclin-dependent kinases [23]. We analysed the level of *Waf1/Cip1* mRNA in REtsAF during apoptosis. An induction of the *Waf1/Cip1* gene is observed after 8 h 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 *Waf1/Cip1* coud be a mediator of p53 leading to REtsAF 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 gene induction. This pattern could also be specific to the apoptosis induction signal. Nevertheless, at least 3 genes previously found to be associated with apotosis or regulated by p53 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 [17]. The product of this gene is a sulfated glycoprotein the function of which remains unclear. Since we found that *clusterin* also 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 [24,25], the high constitutive level of expression in REtsAF-Rev1 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 [26] does not look like a p53-binding site and a computer search in the *clusterin* gene sequence [27] does not reveal any p53-binding site in other parts of the gene (data not shown).

The p53-inducible gene *Waf1/Cip1* is induced in REtsAF between 4 and 8 h after the temperature shift. This induction is more rapid than that of *mdm-2*, in agreement with the pro-

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 [11,20]	Not detected
RP8	Glucocorticoids or radiation induced thymocyte apoptosis [15]	Not induced
Glutathione S-transferase Yb1	Prostate regression [32] and steroid induced lymphocyte apoptosis [21]	Not induced
Clusterin	Prostate regression and various other cell death process [17]	Induced
Ubiquitin	Radiation-induced lymphocyte apoptosis [18] and insect muscles degeneration [19]	Not induced

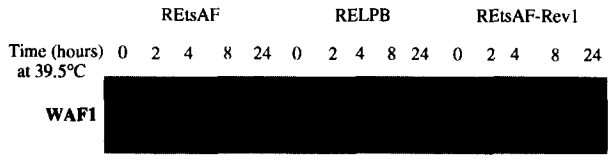

Fig. 3. Expression of Waf1 in various cells. Total cellular RNA prepared from REtsAF at various times after the shift to restrictive conditions were analysed by Northern blotting. The same experiment was performed with REtsAF-Rev1; RELPB and REF. Nylon membrane was probed with a *Waf1* probes.

posal that other factors are rate limiting for *mdm-2* expression [28]. Induction of *Waf1/Cip1* suggest that it could be at least involved in the arrest of DNA replication occurring in REtsAF after the shift up to the restrictive temperature [7]. However, it is not yet clear if it plays a direct role in apoptosis induction. We have observed that growth arrest induced at 33°C in REtsAF by growth factor deprivation or antimitotic drugs (data not shown) leads to apoptosis. Thus, it is possible that growth arrest induced by *Waf1/Cip1* also participates in REtsAF apoptosis induction. However in others systems, p53-dependent apoptosis can occur in the absence of transcriptional activation of p53-target genes [29], 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 [30], 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 downregulated during apoptosis remains of great interest. Indeed, at least three types of genes can be regulated during apoptosis: inducers 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 to understand how the release of p53 from SV40 large T antigen leads to the biological and physiological changes occurring during the process [31].

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