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Susceptibility to p53 dependent apoptosis correlates with increased levels of Gas2 and Gas3 proteins

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Abstract

p53 dependent apoptosis is a critical regulator of tumorigenesis. In this paper we demonstrate that BALB/c cells transformed with a LT mutant perturbing pRb but not p53 functions (LT-2809) show unrestrained cell division under low serum condition which is actively counterbalanced by apoptosis. BALB/c cells transformed with a LT mutant perturbing p53 but not pRb functions (LT-K1), show similar unrestrained cell division but no evident signs of apoptosis when grown in low serum. Such apoptotic response of LT-2809 cells is characterised by increased expression of Gas2 which becomes proteolytically processed. Similarly Gas3 expression is markedly increased in LT-2809 cells with respect to LT-K1. Since both Gas2 and Gas3 have been previously associated with the apoptotic response at growth arrest, our observations suggest that they could also contribute to the regulation of cellular susceptibility to p53 dependent apoptosis.

Keywords: Go; p53; LT-SV40; gas genes; apoptosis

Abbreviations: Gas, growth arrest specific protein; *gas*, growth arrest specific gene; LT, Large T antigen; BrdUrd, bromodeoxyuridine; pRb, retinoblastoma tumor suppressor protein

Introduction

Apoptosis or active cell death, plays a key role in the regulation of different physiological and pathological processes (Thompson, 1995). A strict link has emerged between apoptosis and neoplasia: alterations in genes suppressing or inducing apoptosis have been detected in different tumors thus confirming that tissue homeostasis is based on the active balance between cell division and cell death (Green and Martin, 1995; White, 1996). Furthermore, resistance of some cancer cells to chemiotherapic treatments relays on genetic lesions affecting both positive and negative regulators of the cell death program (Lowe *et al*, 1994; Fisher, 1994).

The tumor suppressor gene p53 is an important sensor that links DNA damage to apoptosis (Lane, 1992; Lowe *et al*, 1993). p53 gene is frequently mutated or lost in various human cancers, thus evidencing that aberrations in its response pathways are relevant for tumorigenicity (Cariello *et al*, 1994).

A natural instrument to study p53 dependent apoptosis is the transforming protein of the simian virus 40 large-T-antigen (LT) (Zheng *et al*, 1994). This protein can stimulate cell growth by affecting both p53 and pRb oncosuppressors.

In a transgenic mouse model for brain tumorigenesis it has been demonstrated that an SV40 LT fragment perturbing pRb but not p53 functions, induces slow-growing tumors (Saenz-Robles *et al*, 1994) characterized by high apoptotic index (Symonds *et al*, 1994). On the contrary tumors induced by wild-type SV40 LT are much more aggressive and lead to rapid death of the mice (Chen and Van Dyke, 1991).

p53wt can induce apoptosis in cells with oncogenic lesions, such as loss of functional p105 pRb or expression of viral oncoproteins that perturb pRb function (Morgenbesser *et al*, 1994; Debbas and White, 1993; Wu and Levine, 1994; Symonds *et al*, 1994).

Therefore it is possible that p53 can induce apoptosis in response to different stimuli and not solely in response to DNA damage.

Non-transformed cells in culture respond to limiting amounts of serum by exiting the cell cycle thus entering a quiescent state or Go (Zetterberg *et al*, 1995): if serum starvation is eventually prolonged an apoptotic program can be activated (Brancolini *et al*, 1995). It is well established that cell transformation can induce increased susceptibility towards apoptosis as triggered by low serum conditions (Evans *et al*, 1992; Rao *et al*, 1992; Mymryk *et al*, 1994).

For these reasons we decided to analyse the response to serum starvation in BALB/c 3T3 cell lines transformed with wild-type LT SV40 and various LT mutants containing mutations that affect its ability to form complexes with pRb or p53 (Kalderon and Smith, 1984; Zhu *et al*, 1991, 1992). Since growth arrest induced by serum starvation in fibroblasts is characterized by increased expression of the *gas* genes (Schneider *et al*, 1988) we have analyzed the expression levels of some of the Gas proteins in these cell lines. Here we show that BALB/c cells transformed with a SV40 LT mutant unable to complex p53 (LT-2809) (Zhu *et al*, 1992) are highly sensitive to apoptosis as induced by serum starvation. Cell death induced by serum starvation in this cell line is accompanied by increased expression of Gas2 and Gas3 proteins even though cell division is not blocked. Previous experiments have demonstrated that both these proteins play a role in apoptosis. The carboxy-

terminal domain of Gas2 is proteolytically processed during apoptosis by an ICE like protease. Removal of this domain triggers a potent cell shape and microfilament re-organizing activity in Gas2 (Brancolini *et al*, 1995). On the other side *gas*3 overexpression, by itself, is able to trigger apoptosis in NIH3T3 cells (Fabbretti *et al*, 1995).

The above described relationships between *gas* genes and the apoptotic fate have also been confirmed in the LT-2809 cells: Gas2 is in fact proteolytically processed at its carboxy-terminal domain, during apoptosis by serum starvation. On the other hand *gas*3 is able to induce apoptosis in BALB/c LT-K1 cells (Kalderon and Smith, 1984) when ectopically overexpressed. Altogether, these results indicate a possible function of the Gas proteins in increasing cellular susceptibility to environmental signals that can determine apoptosis.

Results and discussion

Proliferation rate of BALB/c cell lines transformed by different LT mutants under low serum conditions

A panel of BALB/c 3T3 cell lines transformed with wild-type SV40 Large T antigen and various mutants that affect its ability to form complexes with pRb and p53 oncosuppressors were used in the present study.

Figure 1a shows a schematic representation of such LT mutants: LT-K1 mutant contains a point mutation in the LT protein at aa107 converting a Glu to Lys (Kalderon and Smith, 1984) which renders it unable to bind pRb while mutant LT-2809 has a linker insertion mutation at aa409 thus encoding an LT protein unable to interact with p53 (Zhu *et al*, 1992).

As a first step we analyzed the ability of the different BALB/c cell lines to enter the quiescent state in response to serum starvation. Exponentially growing cells were shifted to 0.5% FCS 24 h after seeding in 10% FCS and the percentage of cells in S-phase was measured by analyzing the incorporation of BrdUrd in newly synthesized DNA on coverslips for each time point as shown in Figure 1b.

BALB/c NEO control cell line responds to low serum by leaving the cell cycle into Go phase: BrdUrd incorporation becomes drastically reduced, after 48 h from serum starvation. On the contrary BALB/c cell lines transformed by LT-wt and by the different mutants LT-K1 and LT-2809, are unable to enter Go following serum starvation and more than 30% of the cells are still in S-phase 48 h after serum starvation.

This analysis demonstrates that BALB/c cells transformed with LT wild-type or LT mutants able to complex p53 but not pRb (LT-K1) or pRb but not p53 (LT-2809) are deficient in arresting cell division cycle in response to low serum as compared to the control cell line BALB/c NEO. It is well established that the rate of cell proliferation depends on cell division (cycling cells) on one hand and cell death on the other hand (Thompson, 1995). Therefore we decided to analyse the proliferation rate of the different BALB/c cell lines cultured in 0.5% FCS. Figure 1c shows such analysis where the rate of cell proliferation is represented as increase in cell number. For this purpose only viable cells, as determined by trypan blue exclusion were considered in these experiments. Cell proliferation is clearly observed in BALB/c LT-wt and in BALB/c LT-K1 cell lines when compared to BALB/c NEO cell line thus confirming that the continued cell division (as represented by BrdUrd incorporation in Figure 1) leads to a sustained cell proliferation. As expected we did not observe an increase in cell number in BALB/c NEO cell lines since cell cycle is arrested under low serum condition. In the BALB/c cell line LT-2809, despite continued cell division, the

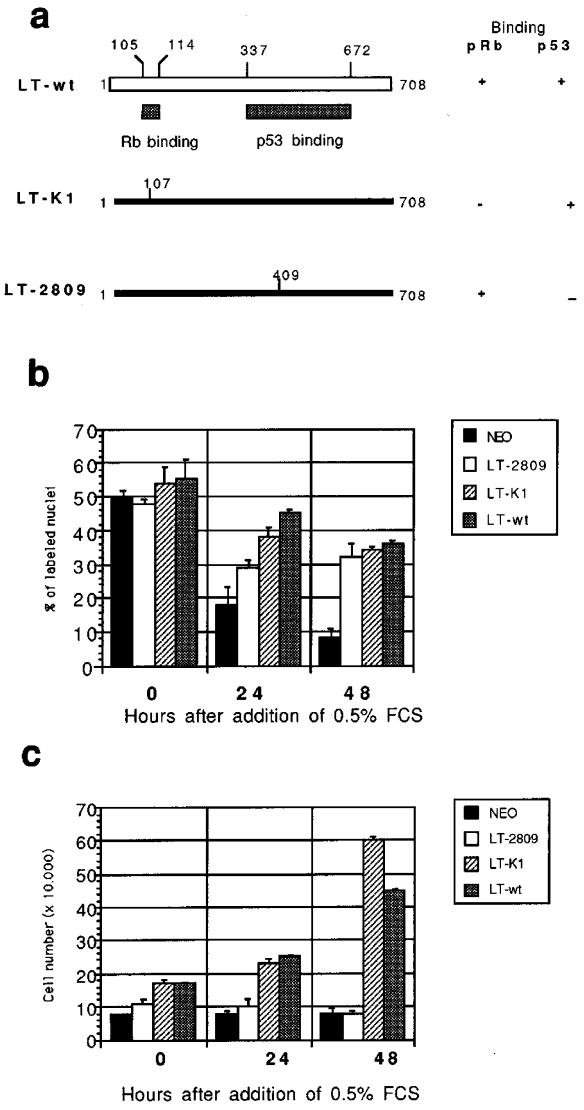

Figure 1 Proliferation and growth rates of BALB/c cell lines transformed by different LT mutants grown under low serum conditions. (**a**) Schematic representation of SV40 LT mutants used. Only the domains required to bind pRb and p53 are represented. (**b**) BrudUrd incorporation is shown in diagram analysis. Data represent arithmetic means ± SD for three independent experiments. (**c**) BALB/c cells transformed by different LT mutants 24 h after seeding were grown in 0.5% FCS for the indicated times. For each time point analyzed the relative number of viable cells were determined by trypan blue dye exclusion. Data represent arithmetic means ± SD for three independent experiments.

increase in cell number is dramatically impaired under low serum condition with a net proliferation profile similar to the BALB/c NEO cell line.

Cell death in BALB/c cell lines transformed by different LT mutants grown under low serum conditions

The previous results suggested that cell division under low serum condition in BALB/c LT-2809 cell line could be counterbalanced by apoptosis. Cell death via apoptosis is characterized by well defined morphological changes such as nuclear condensation, membrane blebbing and cell collapse finally leading to rounding up and detachment from adhesion substratum (Martin *et al*, 1994).

We therefore decided to investigate if, under low serum conditions, apoptosis can be observed in BALB/c LT-2809 cell line. After 48 h in 0.5% FCS a large number of BALB/c LT-2809 cells floating in the medium and showing apoptotic features could be observed at the phase contrast microscope (Figure 2a). On the contrary such features were clearly reduced in BALB/c cell lines transformed with the LT-wt or the mutant LT-K1.

To confirm that BALB/c LT-2809 cells die by apoptosis a detailed confocal analysis was performed using biotinylated wheat germ agglutinin (WGA) to stain the plasmamembrane, in order to evidence the membrane blebbing combined with propidium iodide to visualize the nuclear architecture. In Figure 2b a typical BALB/c LT-2809 cell showing altered morphology in response to low serum conditions is represented. Both membrane blebbing (see arrow Figure 2b/A) and alteration of the nuclear architecture (Figure 2b/B) are detectable, thus demonstrating that BALB/c LT-2809 cells cultured in low serum undergo apoptosis.

From these evidences we can conclude that in the BALB/c LT-2809 line cell division, in the presence of reduced amount of serum, is efficiently counterbalanced by apoptosis thus reducing net cell proliferation. Our observations are germane to *in vivo* studies using different LT mutants or transgenic mice with homozygous inactivation of p53 (Saenz-Robles *et al*, 1994; Symonds *et al*, 1994). These studies clearly demonstrated the relationship between pRb inactivation and p53 dependent apoptosis, thus showing that growth deregulation through pRb loss is accompanied by apoptotic cell death as orchestrated via p53.

Gas2 and Gas3 proteins levels in BALB/c cell lines transformed by different LT mutants

Growth arrest by low serum in mouse and human fibroblasts is characterized by the increased expression of the gas (growth arrest specific) genes (Schneider *et al*, 1988). We have recently shown that Gas2 and Gas3 are involved in regulating different aspects of apoptosis. Gas2, after a specific proteolytic processing, regulates microfilament changes during apoptosis (Brancolini *et al*, 1995) while Gas3 can trigger apoptosis when overexpressed in NIH3T3 cells (Fabbretti *et al*, 1995).

We therefore analyzed Gas2 and Gas3 proteins levels in these cell lines. Figure 3 shows the levels of expression of Gas2 and Gas3 proteins in serum starved and exponentially growing BALB/c LT mutants cell lines, as analyzed by Western blot.

Gas2 is detectable in growing BALB/c NEO and BALB/c LT-2809 and its level significantly increases after serum starvation. In BALB/c LT-wt and BALB/c LT-K1 cells Gas2

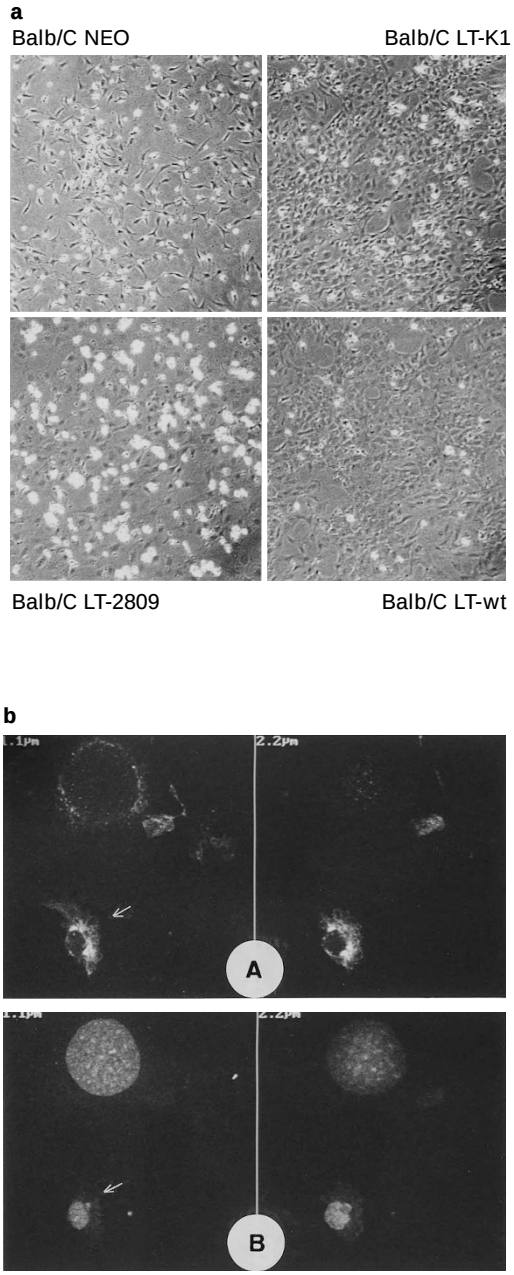

Figure 2 Cell death in BALB/c cell lines transformed by different LT mutants grown under low serum conditions. (**a**) The different BALB/c cell lines were grown for 48 h in 0.5% FCS and phase-contrast photographed. (**b**) Confocal analysis showing membrane blebbing (**A**) and altered nuclear architecture (**B**) in serum starved BALB/c LT-2809 cells (see arrow). Images represent two different focal planes (1.1 μM and 2.2 μM from the basal regions of the cell).

protein is expressed at reduced, almost undetectable, levels in both growing and serum starved cells. Gas3 expression is detectable, albeit at lower levels when compared to BALB/c NEO and BALB/c LT-2809 cells, also in BALB/c LT-wt and BALB/c LT-K1 cells. All the tested cell lines respond to serum starvation by increasing Gas3 protein levels, even though the relative level in BALB/c LT-wt and BALB/c LT-K1 cells is clearly reduced (comparable to 'growing' BALB/c NEO and BALB/c LT-2809).

The same extracts were also probed for tubulin expression as control for the total protein content. Increased expression of Gas proteins is a well established marker of reversible growth arrest by serum starvation and density inhibition (Brancolini *et al*, 1992; Fabbretti *et al*, 1995). Therefore it is not surprising that expression of Gas2 and Gas3 is reduced in BALB/c LT-wt and BALB/c LT-K1 cells since these cells are unable to enter a growth arrest state. However Gas2 and Gas3 protein levels are significantly increased in serum starved BALB/c LT-2809 despite continued proliferation. Indeed, as previously shown, BALB/c LT-2809 cells activate an apoptotic program during serum starvation. In this context we suggest that increased levels of Gas2 and Gas3 proteins in BALB/c LT-2809 cells could be instrumental to the apoptotic response.

Proteolytic processing of Gas2 during apoptosis triggered by serum starvation in BALB/c LT-2809 cells

Gas2 is a component of the microfilament system (Brancolini *et al*, 1992) which is proteolitically processed at its carboxy-terminal domain by an ICE-like cystein proteases (Nicholson, 1996), during apoptosis in NIH3T3 cells. Removal of the C-terminal domain unveils a potent cell shape and microfilament re-organizing activity in Gas2 (Brancolini *et al*, 1995).

Since Gas2 expression is increased in BALB/c LT-2809 cells during apoptosis triggered by serum starvation we decided to analyse whether Gas2 is proteolytically processed during apoptosis in this cell line. For this analysis both adherent as well as non adherent (floating dead cells) present in the medium were combined and Western analysis was performed. Figure 4 shows that a band with increased mobility is detectable in BALB/c LT-2809 cells grown for 24 h in 0.5% FCS (see arrow). This band increases in intensity after 48 h of serum starvation, thus marking the described induction of apoptosis. The same extracts were also probed with antibodies specific to the amino-terminus and carboxy-terminus of Gas2. Since the band with higher mobility is detectable only by using antibodies against the amino-terminus of Gas2, it represents the proteolytic processing of the carboxy-terminus of Gas2 previously demonstrated to occur during apoptosis. Here again tubulin shows the same size and expression levels in the different lanes. In conclusion Gas2 increased expression during apoptosis of BALB/c LT-2809 indicates that susceptibility to apoptosis parallels accumulation of pre-apoptotic effectors, as previously suggested (Arends *et al*, 1993; Willye, 1995).

Overexpression of *gas*3 in BALB/c LT-K1 transformed cells leads to apoptosis

*gas*3 represents the most evident relationship between *gas* gene expression and apoptosis. *gas*3 encodes a tetraspan

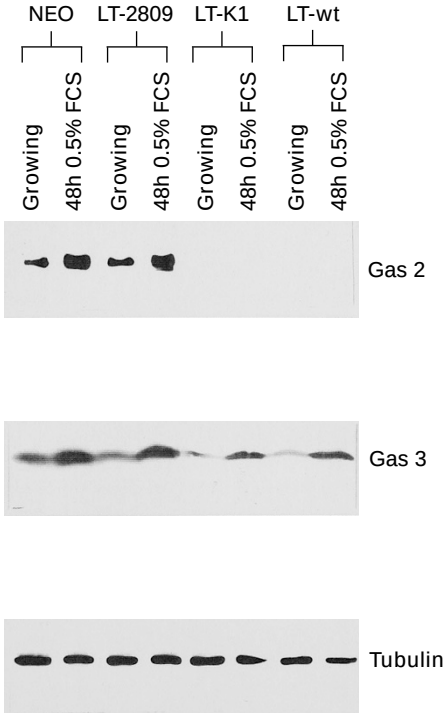

Figure 3 Western blot analysis of Gas proteins levels in BALB/c cell lines transformed by different LT mutants under different growth conditions.

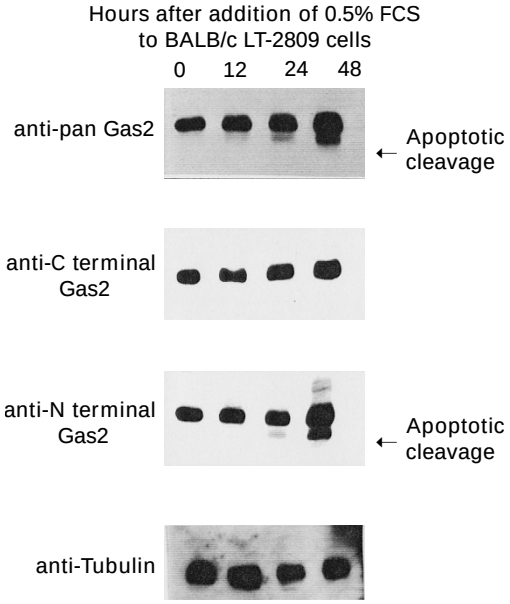

Figure 4 Proteolytic processing of Gas2 during apoptosis triggered by serum starvation in BALB/c LT-2809 cells. Both adherent and non-adherent cells were combined before Western analysis. Different antibodies against the C-terminus or the N-terminus of Gas2 were used (Brancolini *et al*, 1995).

membrane protein highly expressed in differentiated Schwann cells where it is responsible for a set of inherited peripheral neuropathies (Suter and Snipes, 1995). Recent studies demonstrating expression of *gas*3 in different adult mouse and human tissues (Fabbretti *et al*, 1995) and its widespread distribution during mouse embryogenesis (Baechner *et al*, 1995) have suggested a more general biological function of Gas3 not only restricted to myelin formation. This hypothesis has been strengthened by the identification of *gas*3 related genes expressed in different cellular systems (Taylor *et al*, 1995; Marvin *et al*, 1995).

We thus decided to analyse whether increased expression of *gas*3 can also induce apoptosis in cells not showing drastic increase in its level as in the case of BALB/c LT-K1. Cells grown on coverslip were microinjected with pGDSV7-m*gas*3 (50 ng/μl), fixed after 24 h, and processed for immunofluorescence to detect cells overexpressing Gas3. Figure 5 shows confocal microscopic analysis of a representative cell double stained for Gas3 (Figure 5A) and nuclei (Figure 5B). BALB/c LT-K1 cells overexpressing Gas3 show a dramatic alteration of cell and nuclear morphology reminiscent of apoptosis. Overexpression of a control gene such as the human transferrin receptor (Figure 5D and C), under the same experimental conditions never resulted in such effects.

Moreover overexpression of Gas3 in BALB/c LT-K1 cells leads to a dramatic decrease in the number of injected cells that are recovered for analysis (less than 10%) as compared to the control gene (data not shown). This effect is the expected consequence of the complete detachment from adhesion substrate in the *gas*3 overexpressing cells, as triggered by apoptosis. Similar results were obtained when *gas*3 was overexpressed in BALB/c LT-WT cells (data not shown).

These results indicate that *gas*3 overexpression in BALB/c LT-K1 cells triggers apoptosis. As a consequence we can speculate that the different Gas3 protein levels, higher in serum starved BALB/c LT-2809 than in serum

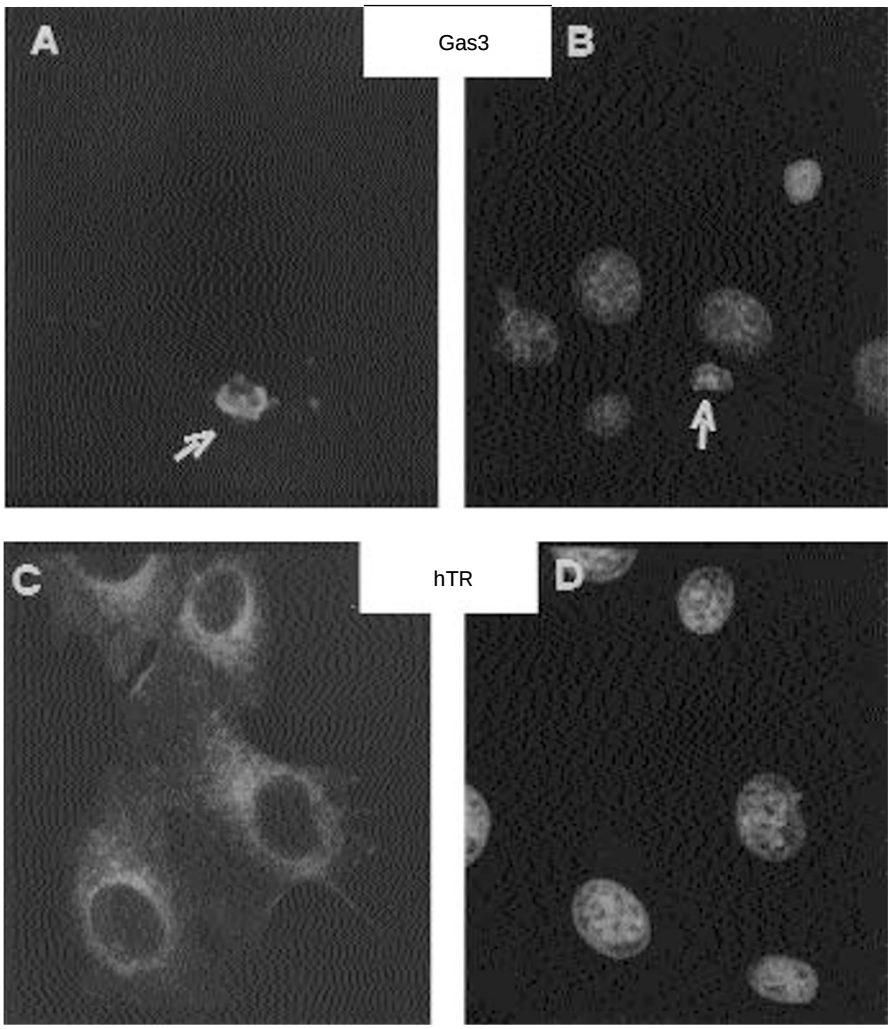

Figure 5 Confocal generated overlay showing cellular and nuclear morphology in *gas*3-microinjected cells. Exponentially growing BALB/c LT-K1 cells were microinjected with pGDSV7-m*gas*3 (50 ng/μl) (A, B) or with pGDSV7-hTR (50 ng/μl) (C, D). After 24 h cells were fixed and processed for immunofluorescence to visualize Gas3 and nuclei (A, B) or hTR and nuclei (C, D).

starved BALB/c LT-K1 cells, should similarly contribute to adjust the different apoptotic thresholds as previously evidenced in these cell lines.

The mechanisms by which p53 induces apoptosis with respect to G1 arrest are still undefined. Although both transcription dependent (Miyashita and Reed, 1995) and independent (Caelles *et al*, 1994) functions of p53 should be responsible for inducing apoptosis, a critical parameter is the 'cellular context' (Bates and Voudsen, 1996). Obviously this is determined both by environmental conditions and by the cellular genetic program. Gas gene expression is part of the genetic program associated with growth arrest as a response to limited growth factors and high cellular density in non-transformed fibroblasts.

Gas2 and Gas3 are both involved in the apoptotic process, possibly having dual functions both as regulators of the growth arrest state and as regulators/effectors of cell death (Brancolini *et al*, 1995; Fabbretti *et al*, 1995). The presence of functional p53 in LT-2809 cells is instrumental for the full induction of Gas2 and Gas3 expression under low serum condition: increased Gas2 and Gas3 expression is however uncoupled to proper growth arrest possibly due to neutralization of pRb function.

In this cellular context full induction of Gas2 and Gas3 without proper growth arrest could therefore contribute to the noticed increased susceptibility to apoptosis.

Materials and Methods

Cell lines and culture conditions

BALB/c cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). The SV40 large T antigen (LT) and all of the SV40 mutants were stably expressed in BALB/c 3T3 cells. BALB/c K1 cells express SV40 LT-K1, which affects the binding to Rb. BALB/c 2809 express SV40 LT-2809 mutants that fail to bind p53. For serum starvation, medium was changed to 0.5% FCS 24 h after seeding. For DNA synthesis assays cells growing on coverslips were incubated for 2 h in the presence of 50 μM BrdUr. After this time cells were fixed for 20 min in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. DNA was then denaturated by treatment for 10 s with 50 mM NaOH. After neutralization the coverslips were incubated with mouse monoclonal antibody against BrdUrd for 1 h at 37°C. The second antibody was rhodamine conjugated rabbit anti-mouse immunoglobulin antibodies. Total nuclei were visualized with Hoechst 33342 (1 μg/ml). The percentage of cells in S-phase was calculated as the ratio between positive for TRITC and total cells (Hoechst 33342-stained).

Immunofluorescence microscopy

Immunofluorescence analysis was performed as previously described (Brancolini *et al*, 1992). Briefly cells fixed in 3% paraformaldehyde were washed with PBS/0.1 M glycine, pH 7.5 and then permeabilized with 0.1% Triton-X100 in PBS for 5 min. Coverslips were treated with biotinylated WGA (Boehringer) to stain the plasmamembrane for 1 h in a moist chamber at 37°C. They were then washed with PBS three times, followed by incubation with FITC conjugated streptavidin (Jackson) for 1 h at 37°C. Nuclei were labelled with propidium iodide (50 μg/ml in PBS). Cells were examined by epifluorescence with a Zeiss laser scan microscope (LSM 410) equipped with a 488 λ argon laser and a 543 λ helium neon laser.

Immunoblotting

Proteins were extracted from actively growing cells and after 48 h from serum starvation. For Gas3 detection PNGase F treatment of cellular lysates was performed as previously described (Fabbretti *et al*, 1995). Nitro-cellulose sheets were saturated for 2 h in Blotto-Tween 20 (50 mM Tris-HCl ph 7.5, 500 mM NaCl, 5% non-fat dry milk and 0.1% Tween 20) and incubated over night at room temperature with the specific antibodies. Blots were then rinsed three times with Blotto-Tween 20 and reacted with peroxidase conjugated goat anti-rabbit (Southern biotechnology) for 1 h at room temperature. The blots were then washed four times in Blotto-Tween 20, rinsed in phosphate buffer saline and developed with an ECL kit, as recommended by the vendor (Amersham).

Microinjection

Microinjection was performed using the Automated Injection System (Zeiss-Germany) as previously described (Brancolini *et al*, 1995). Cells were injected with 50 ng/μl expression vector for 0.5 s at the constant pressure of 150 hPA. After 24 h cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed in PBS/0.1 M glycine at pH 7.5 and then permeabilized in PBS 0.1% Triton-X 100. The coverslips were treated with the first antibody to detect Gas3 or the hTR for 1 h in a moist chamber at 37°C. They were washed with PBS three times, followed by incubation with FITC-conjugated anti-rabbit (Southern), to detect Gas3 or with FITC-conjugated anti-mouse (Southern) to detect hTR, secondary antibodies. Propidium iodide was used to visualize nuclei. Finally cells were examined with a Zeiss laser scan microscope (LSM 410).

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