Ha-Ras sensitizes transformed mouse skin cells to Anisomycin-induced apoptosis

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Abstract **Efforts have been made to develop a chemoprevention that selectively triggers apoptosis in malignant cancer cells. Here, we demonstrated that a mutated Ha-Ras activity is required in Anisomycin-induced apoptosis in transformed keratinocytes. Anisomycin stimulates JNK activity and apoptosis in oncogenic Ha-Ras positive cells, but not in normal keratinocytes. This effect was demonstrated in stably transfected cells with dominant negative Ha-Ras, that protected transformed cells, and oncogenic Ha-Ras that sensitized non-transformed cells to Anisomycin-induced apoptosis. Lastly, the treatment of cells with inhibitors of the JNK displayed resistance to Anisomycin induced apoptosis. These data suggests that the oncogenic Ha-Ras is important for Anisomycin-induced JNK activation and apoptosis in transformed keratinocytes.**

Keywords: Ha-Ras; Anisomycin; Apoptosis

1. Introduction

Ras genes have been shown to be a major participant in the development and progression of a series of human tumors [1]. It has been shown recently that oncogenic Ras has a key role in tumor maintenance and resistance to apoptosis [2]. The influence of *ras* oncogene or wild type Ras expression on sensitization to agent inducing apoptosis has been poorly studied and has led to conflicting results. *Ras* expression was shown to cause increased sensitivity to apoptosis induction by TNF-α [3], and over expression of normal *ras* genes act as pro-apoptotic proteins [4]. On the other hand, *ras* expression inhibits drug- and UV-induced apoptosis [5], and is functionally involved in FGF-dependent suppression of apoptosis [6]. The mitogen-activated protein kinase (MAPK) family of proteins belongs to distinct and evolutionarily conserved signal transduction pathways that are activated by extracellular stimuli. In particular, c-Jun N-terminal kinase (JNK) and p38 pathways are activated by stress agents and correlate with induction of apoptosis by these agents [7–9]. Anisomycin (Ani) has been shown to activate efficiently a cellular stress response involving the activation of JNKs [10]. Furthermore, it was demonstrated that this stress response originates from an interaction of Ani with ribosomal 28S RNA and that translationally active ribosome are involved [11]. Using normal and transformed mouse keratinocytes as model system, we have investigated the role of oncogenic Ha-Ras on Ani-induced apoptosis. Our observations demonstrate that Ani induces apoptosis preferentially in oncogenic Ha-Ras transformed cells, while cells expressing normal Ha-Ras were unaffected.

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Abbreviations: Ani, Anisomycin; JNK, cJun N-terminal kinase; Ha-Ras, Harvey-Ras

2. Materials and methods

2.1. Cells lines culture, and treatment conditions

The origin of the cell lines used in this study has been described elsewhere [12]. Cells were cultured in Ham's F-12 medium supplemented with aminoacids and vitamins (Gibco Ltd., Paisley, Scotland), 10% fetal bovine serum (FBS) and 80 μg/ml gentamicine. Cultures were maintained on plastic at 37 °C in 5% CO_2 humidified atmosphere.

For Ani treatments (Calbiochem, San Diego, CA) cells were plated on six wells plates at a density of 3.5×10 cells, and DMSO dissolved Ani was added at indicated concentration and times, the control cells were treated with the similar volume of vehicle DMSO. Curcumin (Sigma, St. Louis, MO) and SP600125 a JNK inhibitor (Calbiochem) were added 1 h before Ani treatment.

2.2. Internucleosomal DNA fragmentation assay and DNA cell cycle analysis

DNA was extracted from cells as described previously by Song et al. [13]. Fragmented DNA samples were separated by electrophoresis on 1.5 agarose gel and visualized with ethidium bromide.

2.3. DNA cell cycle analysis

Cells were plated at 5×10^5/plate in 60 mm dishes and treated for indicated times and harvested with Trypsin/EDTA, and fixed in 70% ethanol. Cells were washed two times with ice-cold PBS, and resuspended in 25 μg propidium iodide/ml and 50 μg RNase A/ml in PBS. Samples were incubated at 37 °C for 1 h. and DNA profiles were analyzed by FACS using a FACScan cytometer (Becton Dickinson).

2.4. Transfected cells

MCA3D transfected with active form of Ha-Ras (Q61L) or the empty vector, and PDV cells transfected with dominant negative Ha-Ras gene (Ras N17) or empty vector were previously described [14].

2.5. Activation of JNK and Ras expression

A cell monolayers were lysed in 300 μl of lysis buffer (100 mM sodium phosphate, pH 7.2, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 mM NaF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF). Samples were separated on a reducing 10% SDS–PAGE, and blotted onto PDV membrane (Immobilon P, millipore, Bedford, MA). Activation of JNK was assayed by immune-detection with antibody (Santa Cruz, CA) that recognized the activated (phosphorylated) form of JNK. Duplicated filters were probed with antibody (Santa Cruz) that recognized both phosphorylated and unphosphorylated forms of JNK to verify equal loading. To determinate de expression level of Ha-Ras in PDV cells transfected with Dominant negative Ha-Ras, parental and transfected cells were seeded in 60 mm diameter plates (2×10^6 cells) and subject as

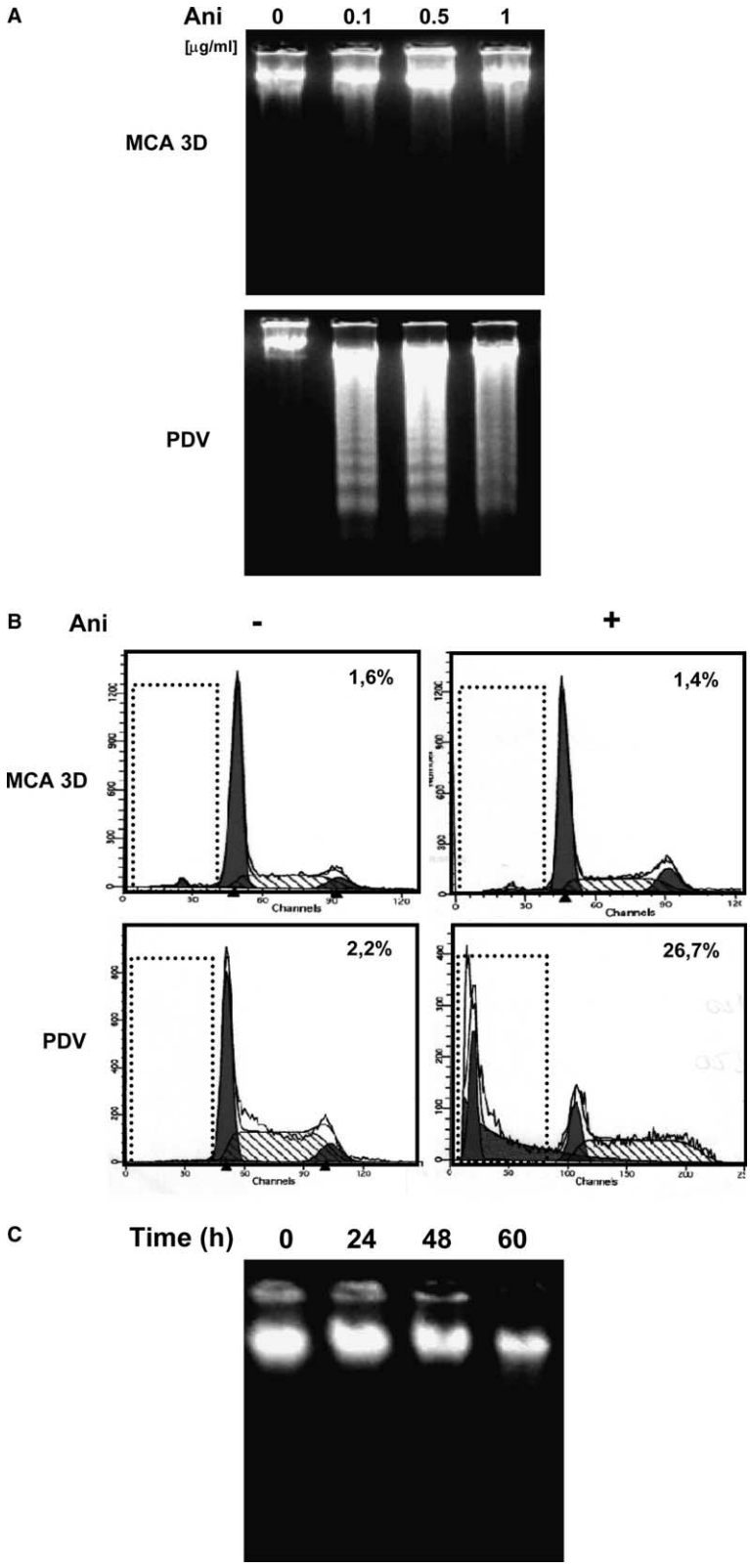

Fig. 1. Anisomicin-induced internucleosomal DNA fragmentation in a Ha-Ras mutated PDV cells but not in normal Ha-Ras MCA3D cells. MCA-3D and PDV cells (5×10^5) were seeded in six plate wells and treated with Anisomicin at 37 °C. (A) Cells were treated with increased amounts of Anisomicin for 16 h and assayed for internucleosomal DNA fragmentation. (B) Cells were treated with Anisomicin (0.5 μg/ml) for 8 h and subject to DNA cell cycle analysis and % of apoptosis was observed as population cells containing a hypodiploid DNA contents in area into doted box included in figures. (C) MCA3D cells were subject to long time of treatment with Anisomicin (1 μg/ml) and assayed as (A). Figures represent at least three independent experiments.

above. Samples were separated in 12.5% SDS–PAGE and blotted with anti-Ha-Ras antibody (Santa Cruz), and reblotted against β-tubulin (Sigma) to normalized Ha-Ras expression.

3. Results

3.1. Ani treatment induces apoptosis in Ha-Ras transformed cell lines but not in immortalized MCA3D cell line

We selected for this study two epidermal cell lines: MCA3D a normal immortalized keratinocytes with normal Ha-Ras; and PDV cells a transformed keratinocytes with two normal allele of normal Ha-Ras and one allele of oncogenic Ha-Ras, for review see [15]. We compared the relative ability of Ani to induce apoptosis in both cells lines. The results show (Fig. 1A) that Ani treatment caused an internucleosomal chromatin cleavage in PDV cells in a dose dependent form, but this effect was not observed in MCA3D. Quantification of apoptosis by propidium iodide staining and FACs analysis (Fig. 1B) showed that 8 h of treatment with Ani induces DNA fragmentation observed by population cells containing a hypodiploid DNA contents. In contrast, no increase in DNA fragmentation was observed in MCA3D cells. To determine the MCA3D resistance to Ani induced apoptosis, we treated the cells at different long periods of time with 1 μg/ml (Fig. 1C). MCA3D displayed a resistance to apoptosis induced by Ani, and a slight DNA fragmentation was observed over 48 h of treatment.

3.2. Role of JNK on Ani-induced apoptosis

Ani has been shown to strongly activate a cellular stress response involving the activation of SAPK/JNK [10], and we studied the possibility that this MAP kinase plays a role in the apoptosis sensibility of cells. We compared the relative ability of Ani to activate JNK (Fig. 2A) in MCA3D and PDV cells. When 0.5 μg/ml of Ani was added to PDV cell culture, JNK activation was observed at 15 min of treatment and maintained in the following periods of time, as determined by extent phosphorylation of this MAP kinase. When MCA3D was treated in the same way as PDV then no activation of JNK was observed upon treatment with Ani, although unphosphorylate form of JNK was present. To asses whether JNK mediated Ani-induced apoptosis, we used curcumin and SP600125, both inhibitors of JNK pathway [16,17]. The results (Fig. 2B and C) demonstrated that curcumin and SP600125 protects against Ani-induced apoptosis in Ha-Ras oncogenic positive cells. Pretreatment of the cells with curcumin or SP600125 suppressed strongly the DNA fragmentation and population cells containing a hypodiploid DNA induced by Ani. We therefore, tested whether curcumin inhibits Ani-mediated phosphorylation of JNK by Western blot analyses. As shown in Fig. 3D, pretreatment with curcumin inhibited Ani-induced JNK-activation in PDV and cells.

3.3. A dominant negative Ras mutant gene protected PDV cells from Ani-induced apoptosis

Oncogenic Ha-Ras has been shown to be related to cellular malignance, and to play an important role in tumoral progression [1]. We therefore investigated the possibility that Ha-Ras activation could explain the sensibility of tumorigenic cells to Ani induced-apoptosis. We treated with Ani the PDV cell clone transfected with a vector containing a dominant negative RasN17 mutant gene, named RN7, and observed that a RasN17 transfection conferred resistance to apoptosis that shown as non DNA fragmentation or hypoploid DNA contents (Fig. 3A and B). Clone RN7 expressed a high level of Ha-Ras (endogenous and dominant negative Ha-Ras transfected gene) compared with control cells transfected with empty vector (Fig. 3C).

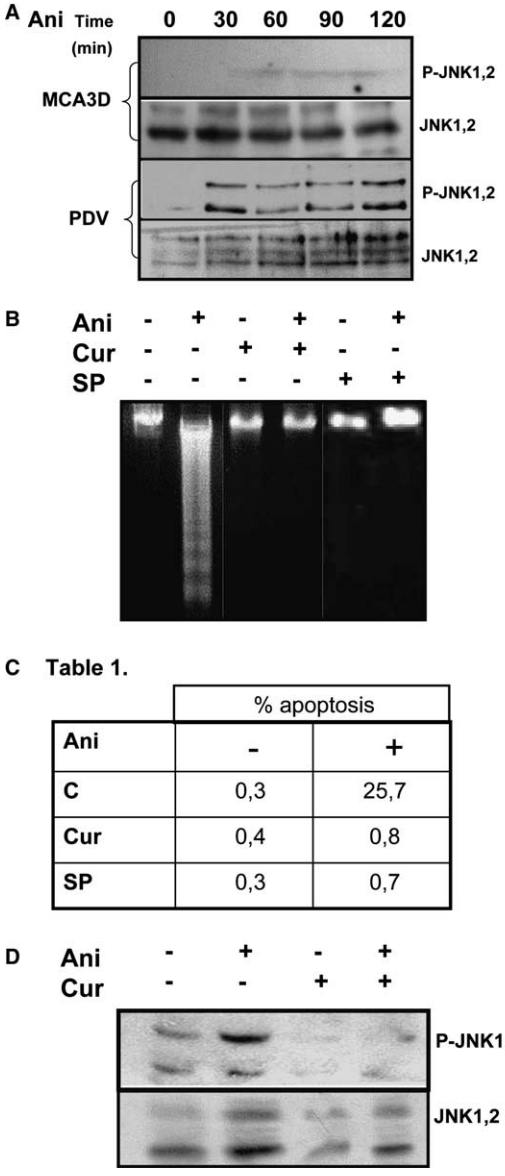

Fig. 2. Anisomicin stimuli increases JNK-mediated phosphorylation in PDV cells but not in MCA3D cells. (A) MCA3D and PDV cells were seeded at 2.5×10^5 cells/well in six wells plates. Cells were treated with 0.5 μg/ml Anisomicin from 15 to 90 min, and phospho-JNK and total JNK were determined by Western blot analysis. (B) Cells were pretreated with curcumin (Cur, 10 μM) or JNK inhibitor SP 600125 (SP, 10 μM) for 1 h, after this 0.5 μg/ml of Anisomicin was added for additional 16 h, and analyzed by internucleosomal DNA fragmentation. (C) Cells were treated with inhibitors as above, except Anisomicin treatment was for 8 h and subject to DNA Cell cycle analysis and expressed as Table 1. (D) Cells were pretreated 1 h with Cur for 1 h, and 0.5 μg/ml of Anisomicin was added for additional 1 h. JNK phosphorylation was determined by Western blot. Results are representing three independent experiments.

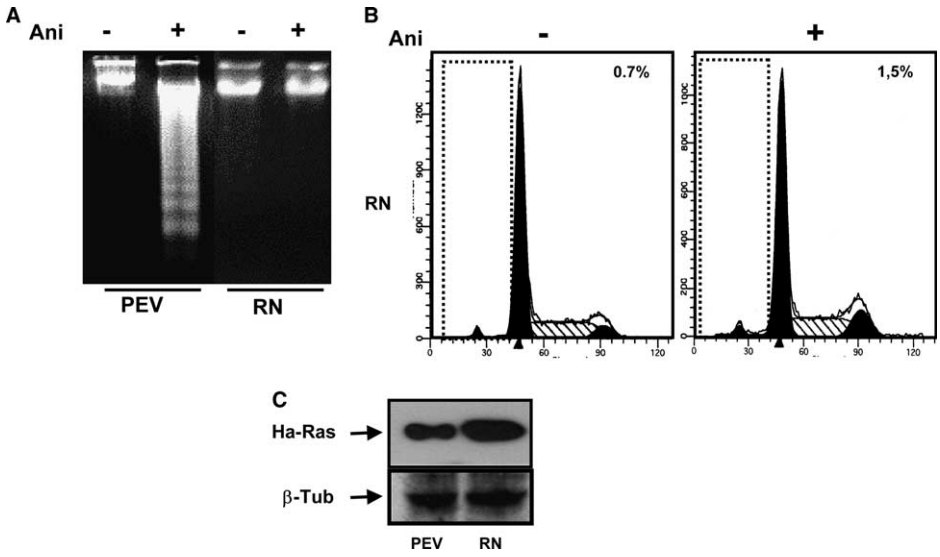

Fig. 3. A Ha-Ras inhibition block Anisomicin-induced apoptosis. Cells were seeded at 2.5×10^5 cells/well. (A) Control cells or a RN clone stably transfected with dominant negative Ha-Ras mutant (N17) were treated with Anisomicin (0.5 μg/ml) for 16 h to internucleosomal DNA fragmentation assay. (B) PDV RN clone was treated wit Anisomicin (0.5 μg/ml) for eight hours and DNA cell cycle analysis was performed. (C) Western blot for Ha-Ras expression in control cells and RN clone. Results are representing three independent experiments.

3.4. Active mutant of Ha-Ras confers sensitivity to Ani induced apoptosis and JNK activation in MCA 3D cells

When we transfected MCA3D with an active form of Ha-Ras (3D-R) a strong morphological changes were observed, transfected cells lost the epithelial phenotype and acquired more fibroblastic characteristics. When treated 3D-R with Ani cells displayed typically morphological changes a hallmarks of apoptosis (Fig. 4A), showing a DNA laddering, and enhanced the hypodiploid DNA contents compared with a MCA3D transfected with a empty vector, interestingly MCA3D transfected with active Ha-Ras showing more elevated basal level of apoptosis than cells transfected with empty vector (Fig. 4 B and C). The treatment of 3D-R with Ani by 30 min induced a significant activation of JNK while displaying an important basal activation levels than 3D cells transfected with empty vector that did not responding to drug treatment (Fig. 4D).

4. Discussion

Apoptosis is mainly the most potent defense against cancer development, and efforts have been made to develop a chemoprevention and therapeutics strategies that selectively trigger apoptosis in malignant cancer cells. Mutated ras genes are found in about 30% of all human cancers [1], and allow the possibility to differentially sensitized tumoral cells to drug-induced apoptosis.

Paradoxically, Ha-Ras can either inhibit or promote apoptosis, with the outcome probably dependent upon the cell type and the presence of other pro-apoptotic or anti-apoptotic signals [18]. While the mechanisms by which Ha-Ras provides protection against apoptosis are being elucidated, and although several reporters had shown that Ha-Ras sensizate cells to apoptotic stimulus [3,19–22], less is understood about how Ha-Ras can sensitize cells to different apoptotic stimuli.

Our results indicate that oncogenic Ha-Ras transformed PDV keratinocytes cells are highly sensitive to Ani-induced apoptosis. These transformed cells produce squamous carcinoma tumors upon injection in skin of nude mice, and acquire more malignant phenotype in Ha-Ras dependent fashion to TGF-β1 treatment [14,23]. In contrast, Ani-treatment had no effect in immortalized and non-tumorigenic MCA3D keratinocytes that have a normal Ha-Ras [15]. Sustained Ha-Ras activity is necessary for induction of apoptosis by Ani, as demonstrated by the protection against apoptosis performed by inhibition of Ha-Ras activity by dominant negative N17-Ras (Fig. 3A and B). Furthermore, Ha-Ras activity is also sufficient for induction of apoptosis by Ani, as shown by the sensitivity conferred by transfection of activated Ha-Ras (Q61L) into non-transformed cells (Fig. 4A–C).

JNK is a critical signaling component in the regulation apoptosis [24], and activation of JNK by Ani that bind to or alter the structure of 28S ribosomal RNA was proposed as ribotoxic stress response in eukaryotic cells [11], and this ribotoxic stress could sensitize tumors cells to chemotherapy [25]. We found that Ani activated JNK in oncogenic Ha-Ras transformed PDV cells, and normal immortalized MCA3D keratinocytes cells were very little stimulated (Fig. 2A). The JNK activation was necessary for the induction of apoptosis by Ani in PDV cells, observed by using both JNK inhibitors curcumin and SP600125 (Fig. 3B and C). The mechanism by which Ha-Ras could sensitize cells to Ani-induced JNK activation remains not elucidate, as does the reason for which activated JNK is involved in triggering apoptotic mechanism.

Recent studies showed that JNK translocates to mitochondria after genotoxic stress and inhibits the anti-apoptotic function of proteins belonging to Bcl2 family members [26], thereby allowing the release of mitochondrial apoptogenic proteins to cytosol as well cyto-*c* and Smac/Diablo [27], and subsequent activation of caspases to promote apoptosis [28].

In resume, our data supports the conclusion that activation of JNK is a major component of the mechanism of sensitization by oncogenic Ha-Ras of keratinocytes transformed cells to Ani-induced apoptosis. However, further analysis is neces-

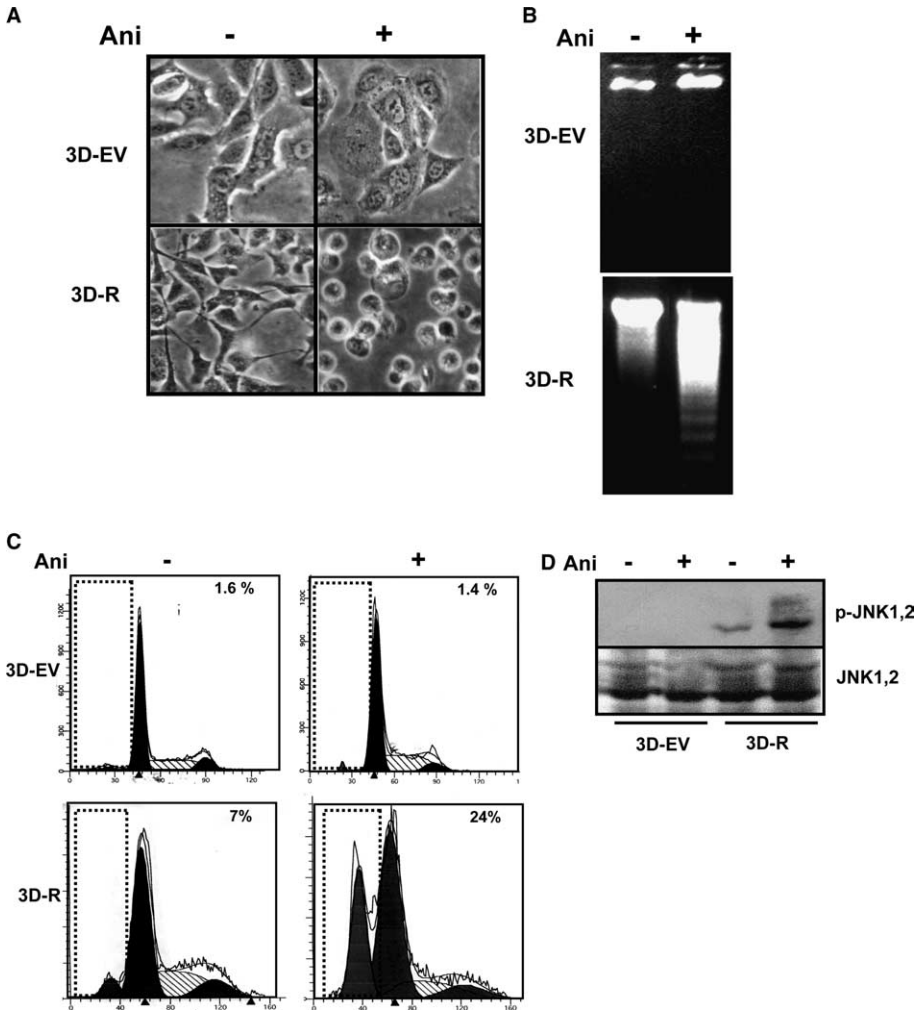

Fig. 4. Oncogenic Ha-Ras confer sensibility to Anisomicin-induced apoptosis to resistant normal MCA3D cells. MCA3D cells stably transfected with empty vector (3D-EV) or Q61L Ha-Ras mutant (3D-R) were seeded at 2.5×10^5 cells/well. (A) Morphological changes of cells after 16 h of Anisomicin (0.5 μg/ml) treatment (magnification 40×). (B) Internucleosomal DNA fragmentation assay after 16 h with 0.5 μg/ml of Anisomicin. (C) DNA cell cycle analysis after six hours of 0.5 μg/ml of Anisomicin treatment. (D) Western blot for JNK phosphorylation after 1 h of Anisomicin treatment (0.5 μg/ml). Figures are representing at least two independent experiments.

sary to understand the aspects associated with Ha-Ras sensitizes cells to translation inhibition are important, and their identification will be critical for designing strategies for the treatment of tumoral disease.

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