Selective activation of oncogenic Ha-ras-induced apoptosis in NIH/3T3 cells

H-S Liu, C-Y Chen, C-H Lee and Y-I Chou

Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, Republic of China

Summary A Ha-ras transformant ‘7-4’, derived from mouse NIH/3T3 fibroblasts, was used to study the relationship between overexpression of activated Ha-ras and cell apoptosis. This cell line contains an inducible Ha-ras<sup>mut</sup> oncogene, which was under the regulation of the Escherichia coli (E. coli) lac operator/repressor system. We demonstrate that overexpression of activated Ha-ras oncogene by exogenous isopropyl-β-D-thiogalactoside (IPTG) under serum-depleted conditions can stimulate cell apoptosis. Cell cycle analysis showed that most of the 7-4 cells with Ha-ras overexpression accumulated at S-phase and that the expression level of p34<sup>cdc2</sup> kinase was decreased, suggesting that p34<sup>RAS</sup> may be involved in 7-4 cell apoptosis. Overexpression of bcl-2 transgene in these cells blocked Ha-ras-induced apoptosis, and this blockage was confirmed downstream of Ha-ras gene expression. Cycloheximide blocked the apoptosis of 7-4 cells in a dose-dependent manner, indicating that specific protein regulating apoptosis may be synthesized through Ha-ras overexpression. Ha-ras overexpression-triggered apoptosis was also prevented in the 7-4 derivatives that express either dominant-negative ras<sup>Avi2</sup> or dominant-negative ras<sup>1-CB8</sup> to suppress Ha-ras signal transduction at different stages, indicating that overexpression of activated Ha-ras can induce cell apoptosis and that ras<sup>1</sup> pathway activity is required for this process.

Keywords: Ha-ras; bcl-2; ras<sup>1</sup>; apoptosis

Three ras proto-oncogenes (H-, K- and N-ras) in the ras family are found in the mammalian genome (Downward, 1992). The activated ras oncogenes (such as the viral v-ras oncogenes) that are derived from normal cellular proto-oncogenes and activated by the mutations at amino acid position 12 and several other sites are the most frequently identified oncogenes in human neoplasia, indicating that ras oncogenes play an important role in the process of carcinogenesis (Barbacid, 1987). Extensive studies have demonstrated that cell proliferation and differentiation are dependent upon Ras. This includes the ras<sup>1</sup> product and the mitogen-activated protein (MAP) kinase. In recent studies, several investigators have suggested that the role of activated ras oncogenes in the process of carcinogenesis is to provide cells with not only a selective proliferation function but also an antipapoptotic function (Sakai et al, 1994). Arends et al (1994) reported that transfectants with moderate or high oncogenic Ha-ras<sup>mut</sup> expression showed reduced apoptosis. Several reports have also suggested that expression of oncogenic ras has the ability to prevent apoptosis and increase cell survival to anti-cancer drugs or ionizing radiation at doses that demonstrate apoptosis (Arends et al, 1993; Sakai et al, 1994; Jimenez et al, 1995). Conversely, some reports have recently demonstrated that Ha-ras oncogene overexpression could induce apoptosis, which includes promoting the sensitivity of murine fibroblast 10T1/2 cells to apoptosis induced by tumour necrosis factor, inducing apoptosis of Jurkat cells after suppression of protein kinase C (PKC) activity (Chen and Faller, 1996) and triggering apoptosis of REF cells in the presence of the IRF-1 gene (Tanaka et al, 1994). Moreover, it is found that Ha-ras is an effective promoter of apoptosis through the Raf pathway, whereas c-myc was found to be overexpressed under serum-deprived conditions (Kauffmann-Zeh et al, 1997).

Furthermore, Fas ligation-induced apoptosis in immune homeostasis needs normal ras activation (Gubbins et al, 1995). In the ras superfamily, overexpression of normal rho-p21 or activated R-Ras induces apoptosis in NIH/3T3 cells after serum deprivation (Jimenez et al, 1995; Wang et al, 1995). Thus, either normal or activated ras genes from the ras family or the ras superfamily may be involved in apoptosis. The mechanisms of their action may differ from one cell system to another. It is possible that the effects depend on the status of other genes known to regulate apoptosis (such as bcl-2, c-myc and p53) (Fernandez et al, 1995).

In this report, a NIH/3T3 derivative designated 7-4 containing an inducible Ha-ras<sup>mut</sup> oncogene regulated by an Escherichia coli lac repressor was used to demonstrate that Ha-ras overexpression can indeed trigger apoptosis by manipulating the inducer of isopropyl-β-D-thiogalactoside (IPTG). This apoptosis was further confirmed by dominant-negative ras<sup>Avi2</sup> and ras<sup>1-CB8</sup> studies.

MATERIALS AND METHODS

Cell culture

Mouse fibroblast NIH/3T3 cells and their transgenic derivatives (7-4, 7-4-2 and dominant-negative ras and ras<sup>1</sup> cells) were maintained in α-modified Eagle medium (α-MEM; Gibco-BRL, USA) containing 10% calf serum (Gibco) and incubated at 37°C in a carbon dioxide incubator. The 7-4 cells contain plasmids pSVlacOra and p3’SS (Stratagene, USA) (Liu et al, 1992; Ducoeur et al, 1993); and 7-4-2 cells, a derivative of 7-4 cells, contain the third plasmid pCAJ-bcl-2 (Tsujimoto, 1989). IPTG (Gold biotechnology, USA), a non-metabolizable lactose analogue, was added to induce expression of Ha-ras transgene in 7-4 and 7-4-2 cells.
Ha-ras oncogene was overexpressed in 7-4 and 212 cells by IPTG induction under serum-deprived conditions. The cells were maintained in 0.2% calf serum-containing medium in the absence or presence of 2.5 mM IPTG for 48 h. Total RNA was then extracted and electrophoresed on a 1% glyoxal gel. The probes used were an \([\alpha-32P]dCTP\)-labelled 4-kb BamHI fragment of Ha-ras DNA from plasmid pSVlacOaras and a 2-kb BamHI fragment of β-actin DNA from plasmid pHFBA-1 (Liu et al., 1992). β-Actin is used as an internal control.

**Table 1** Ha-ras gene overexpression caused accumulation of 7-4 cells at S-phase under 0.2% serum condition

| Cell cycle | NIH/3T3 | 7-4 | 7-4 IPTG |
|------------|---------|-----|---------|
| G1/G0      | 88%     | 48% | 51%     |
| S          | 9%      | 48% | 90%     |

NIH/3T3 and 7-4 cells were cultured in 0.2% serum-containing medium for 24 h. IPTG (2.5 mM) was then added and the cells were harvested at 24-h intervals. This table shows the result of 72 h post-IPTG treatment. A total of 5000 cells were analysed using flow cytometry.

**DNA transfection**

Cells (2 × 10⁶) in a 60-mm plate were co-transfected with the desired reporter plasmids (1.5–3 μg per plate) and pSV5lacZ β-galactosidase reporter gene (0.5 μg per plate) as the internal control using the Lipofectin method (Gibco-BRL) for 5 h. β-Galactosidase activity was determined to calculate the transfection efficiencies. The cells were harvested and analysed 48 h after transfection.

**Microassay for cell viability**

The microassay for cell viability was performed as described previously (Ito, 1984). Briefly, cells were seeded in the 96-well flat-bottom microplates (Nunc, Denmark) at appropriate concentrations (1–1.5 × 10⁴ cells per 100 μl per well) and incubated at 37°C in a carbon dioxide incubator for 4 h for cell attachment. The medium was then replaced with fresh medium. Eight wells were used for each treatment. At the end of the incubation, the medium was removed and the cells in wells were then fixed with 10% formalin (50 μl per well) for 10 min, after which they were stained with 0.05% crystal violet (30 μl per well) for 30 min. The stained microplates were rinsed with tap water and then air dried. For the determination of cell viability, colorimetry was used. The elution fluid containing 50% ethanol and 0.1% acetic acid (150 μl per well) was added to each well, and the eluted blue dye in each well was quantified by optical density measured at 590 nm (OD₅₉₀) with a Dynatech MR5000 microplate reader (Dynatech laboratories, VA, USA). The percentage of inhibition was calculated according to the following equation:

\[
\text{Per cent} = \left( \frac{\text{OD}_{590}\text{(sample)} - \text{OD}_{590}\text{(medium control)}}{\text{OD}_{590}\text{(cell control)} - \text{OD}_{590}\text{(medium control)}} \right) \times 100
\]

**Northern blot analysis**

Thirty micrograms of total RNA prepared from the cells was loaded into the wells on a 1% agarose gel containing 5 M glyoxal. The RNA was fractionated by electrophoresis at 80 V, 80 mA, for 2 h, transferred to a Hybond-N transfer membrane in 25 mM sodium phosphate, pH 6.5, and hybridized with the probes labelled with \([\alpha-32P]dCTP\) to a specific activity of 4 × 10⁶ dpm μg⁻¹ by random priming (Sambrook et al., 1989). The blot was exposed to Kodak X-OMAT AR film with the intensifying screen before development (Liu et al., 1992).

**Western blot analysis**

Fifteen micrograms of total protein from the cells was denatured in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol) and loaded into duplicated 12% SDS-polyacrylamide gels. After electrophoresis at 100 V for 3 h in SDS–PAGE running buffer (25 mM Tris-HCl, pH 8.8, 250 mM glycine, 0.1% SDS), one of the gels was stained with 0.5% colormetric Coomasie brilliant blue (Sigma, USA) as a quantitative control and the other was transferred to the polyvinylidene difluoride (PVDF) membrane (Stratagene) and blocked with 5% skimmed milk in PBST (100 mM sodium chloride, 80 mM disodium hydrogen phosphate, 20 mM sodium dihydrogen phosphate, 0.2% Tween 20, pH 7.5) solution at 4°C overnight. After washing with PBST and phosphate-buffered saline (PBS) buffer, the membrane was hybridized with the monoclonal anti-Bcl-2 primary antibody (Dako, Japan) at 37°C for 1 h. The membrane was then washed and hybridized with the monoclonal anti-mouse IgG conjugated with horseradish peroxidase at 25°C for 1 h (KPL, USA). After washing, the membrane was exposed to radiographic film (Fuji, Japan) for 10 min after the enhanced chemiluminescence (ECL) detection reagents (Amersham, USA) were added.

**Flow cytometric analysis**

The cells harvested in PBS solution were centrifuged at 130 g for 5 min to remove the PBS; the cells were resuspended in 50 μl of Heps-buffered saline (HBS) solution [100 mM sodium chloride, 2.7 mM potassium chloride, 60 mM glucose, 10 mM Heps, 0.1% bovine serum albumin (BSA), pH 7.3]. Cells were stained with 5 μl of mercocyanine 540 (1 mg ml⁻¹; Sigma) and incubated for 10 min at room temperature. Samples were then analysed using the fluorescence-activated cell sorter (FACScan, Becton Dickinson, USA). Each result from 5000 cells was analysed by the analysis program CellFIT (McEvoy et al., 1988).

**DNA fragmentation analysis**

The harvested cells (1 × 10⁶ per 100-mm plate) were washed with PBS and pelleted by centrifugation at 240 g twice. The cell pellets were then treated with lysis buffer (1% NP-40 (Sigma) in 20 mM
ETD, 50 mM Tris-HCl, pH 7.5) for 10 s and centrifuged for 5 min at 3100 g twice. The supernatant was brought to 1% SDS with RNAase A (5 μg ml⁻¹) and kept at 56°C for 2 h followed by digestion with 2.5 μg ml⁻¹ proteinase K (Boehringer Mannheim) for at least 2 h at 37°C. After addition of 0.5 vol 10 mM ammonium acetate, the DNA was precipitated with 2.5 vols 100% ethanol, resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and separated by electrophoresis on a 1% TAE-agarose gel at 30 V for 8 h. The DNA fragments were visualized by ethidium bromide staining (Hermann et al., 1994).

**CAT assay**

A 50-μg cell lysate was incubated at 65°C for 10 min to denature the deacetylase activities. Twenty microlitres (3.5 mg ml⁻¹) of acetyl coenzyme A (Sigma) and 2 μl (sp. act. = 56 mCi mmol⁻¹) of [³⁵C]chloramphenicol (Amersham, UK) were added to the lysate and incubated at 37°C for 1 h. To stop the reaction, 1 ml of ethyl acetate (BDH, UK) was added to each sample and 0.9 ml of the upper phase was transferred to the new Eppendorf tube and dried by a speed vac (Eylca, Japan). The pellet was resuspended in 10 μl of ethyl acetate and dotted on the 25-mm thin-layer chromatography silica plate (Merck, Germany). The samples were spread by the chloroform–methanol mixture (95:5) (BDH, UK). The percentage of [³⁵C]chloramphenol acetylated was quantified by cutting the acetylated and non-acetylated spots and measuring the amount of radioactivity using the scintillation counter (LS 5000 TA, Beckman, USA).

**Luciferase and β-galactosidase activities assay**

The luciferase and β-galactosidase activities were determined by a Dual-Light luciferase and β-galactosidase reporter gene assay system (Tropix, USA). Briefly, equivalent amounts of protein lysates (the final volume was 10 μl) were mixed with buffer A, containing the reagents necessary for the luciferase reaction. Light signal from the luciferase enzyme present in the extract was measured immediately by a luminometer (Lumat, LB 9501, Germany) after the addition of buffer B containing luciferin and Galacton-plus. After another 30-min incubation, the light signal from the accumulated product of β-galactosidase and Galacton-plus reaction is initiated by the addition of a light emission accelerator and measured using the luminometer.

**RESULTS**

**Ha-ras transgene was overexpressed in the transformants in the presence of IPTG under serum-deprived conditions**

The Ha-ras⁺112 oncogene (GGC to GUC) cloned from the human T24 bladder carcinoma cell line (driven by an SV40 promoter with the E. coli lac repressor-binding operator) was introduced into
NIH/3T3 cells. Two cell lines, designated 212 and 7-4, have been established. The activated Ha-ras oncogene in these two cell lines can be overexpressed by IPTG induction under normal conditions (Liu et al, 1992). To ensure that the Ha-ras oncogene could also be overexpressed in the cells by IPTG induction under serum-deprived conditions, the assessment of basal and induced expression of the Ha-ras transgene in these cells was performed by Northern blot analysis. Very faint to undetectable Ha-ras signals in uninduced responsive cells (Figure 1, lanes 1, 3 and 5) and intense bands in induced cells (Figure 1, lanes 2 and 4) were detected 48 h after

Figure 3. DNA fragmentation was detected in 7-4 cells while Ha-ras transgene was overexpressed by IPTG induction. The cells (1.5 x 10⁶ per 150-mm plate) were maintained in the medium containing 0.2% calf serum for 24 h, 2.5 mM IPTG was then added to the same medium. Cells were harvested and DNA was extracted at 24, 48 and 72 h. (A) Without IPTG; (B) with IPTG; M, 1-kb DNA ladder marker.

Figure 4. Apoptotic 7-4 cells were evidently stained by acridine orange. The cells (1 x 10⁶ per slide) were maintained in the medium with 0.2% calf serum for 24 h, IPTG was then added to overexpress Ha-ras oncogene. The cells were fixed with 100% methanol and labelled with 1% acridine orange 48 h after IPTG induction (200x). (A) 7-4 cells without IPTG; (B) 7-4 cells with IPTG.
Figure 5  bcl-2 transgene overexpression in 7-4-2 cells blocked apoptosis and altered the morphology. The cells (1.5 x 10^6 per 150-mm plate) were maintained in medium containing 0.2% serum for 24 h, IPTG was then added as the onset of the time course investigation in the same medium. (A) Without IPTG; (B) with IPTG; M, 100-bp DNA ladder marker; (C) for morphological observation, subconfluent cells (1 x 10^6 per 100-mm plate) were cultured in 0.2% serum containing medium for 2 days in the presence of IPTG, and then were photographed (100×)

IPTG induction. The increase in band intensity in 212 and 7-4 cells is 25 x and 15 x, respectively, compared with their basal expression. Western blot analysis shows identical results (data not shown). The levels of Ha-ras overexpression and transforming characteristics of 212 and 7-4 cells are similar. Therefore, only 7-4 cells were used for the following analysis.

7-4 cells died of apoptosis while the Ha-ras oncogene was overexpressed by IPTG induction under serum-deprived conditions

The effects of Ha-ras overexpression on the cell cycle, morphological changes, viability and apoptosis of 7-4 cells were investigated. For cell cycle analysis, the cells were initially synchronized by 0.2% serum starvation for 24 h and then treated with IPTG and analyzed at 24-h intervals for 3 days in the 0.2% serum-containing
medium. The percentage of 7-4 cells in G1/G0 phase was 48% in the absence of IPTG and dropped to 5% in the presence of IPTG. Correspondingly, the percentage of 7-4 cells in S-phase was 48% in the absence of IPTG and raised to 90% in the presence of IPTG at 72 h, indicating that the major 7-4 cell population accumulated in S-phase while the Ha-ras gene was overexpressed. In contrast, the percentage of cells in G2/M phase is constant at all the times tested (Table 1).

Morphological observation shows that NIH/3T3 and 7-4 cells exhibited similar flat and polygonal morphology at 24 h after treatment. The morphology of NIH/3T3 cells remained the same at all the times investigated. The morphology of uninduced 7-4 cells became more spindle-like and formed slender processes at 48 and 72 h after induction. However, IPTG-induced 7-4 cells started aggregating and showed contracted, rounded morphology in 48 h. These cells formed small foci (containing 15–30 cells) and some of them started floating and died between 48 and 72 h (Figure 2).

To clarify whether the death of major 7-4 cells is apoptotic, the cells maintained in 0.2% serum-containing medium with or without IPTG induction for 48 and 72 h were analysed using DNA fragmentation analysis and acridine orange staining. Evident DNA fragmentation was detected in 7-4 cells maintained in 0.2% serum-containing medium for 48 and 72 h, while Ha-ras was overexpressed by IPTG induction (Figure 3B). IPTG per se or serum deprivation alone without Ha-ras overexpression could not stimulate apoptosis of all the cells tested (Figure 3A). Moreover, many 7-4 cells were rounded, and in about 80% of the cells (797 ± 5 / 1×10⁶ cells) the chromatin was condensed and heavily stained by acridine orange in the presence of IPTG for 72 h. However, 7-4 cells without IPTG induction showed normal flat morphology with very faint acridine orange staining. Less than 20% of the cells were apoptotic (178 ± 6 / 1×10⁶ cells) (Figure 4).

**Apoptosis of 7-4 cells triggered by Ha-ras overexpression could be prevented by bcl-2 overexpression or cycloheximide treatment**

To test whether bcl-2 transgene overexpression could block Ha-ras oncogene-induced apoptosis, the 7-4-2 cells that stably expressed the human bcl-2 gene (driven by an SV40 early promoter) were investigated. The expression of bcl-2 transgene at transcriptional and translational levels in 7-4-2 cells was confirmed (data not shown). DNA fragmentation in 7-4-2 cells could not be detected, suggesting that bcl-2 overexpression could block Ha-ras-induced apoptosis (Figure 5B, lanes 4–6). Neither IPTG nor serum starvation was sufficient to induce cell apoptosis without Ha-ras overexpression (Figure 5A). Moreover, 7-4-2 cells also showed spindle-like morphology. In contrast, 7-4-2 cells with significant DNA fragmentation started to contract and become round at 48 h and aggregated together within 72 h (Figure 5C).

While protein synthesis inhibitor cycloheximide (CX) was applied to 7-4 cells, Ha-ras-induced apoptosis was delayed in a CX dose-dependent manner, indicating that de novo protein synthesis is required for 7-4 cell apoptosis (Figure 6). Moreover, CX per se without IPTG induction could not trigger 7-4 cell apoptosis (Figure 6, lane 1).
The expression level of kinase p34<sup>cdc2</sup> was decreased while 7-4 cells were grown in serum-deprived conditions accompanied by Ha-ras overexpression

To understand the possible factors involved in the aberration of the 7-4 cell cycle and subsequent cell apoptosis, the kinases cyclin A, B and p34<sup>cdc2</sup> regulating the cell cycle from G2/M to M-phase were observed at the translational level.

The treatment of the cells is the same as described above. Briefly, cell lysates were extracted at a 24-h interval until 72 h after IPTG induction, and the expression levels of p34<sup>cdc2</sup>, cyclin A and B were investigated. Figure 7A shows the expression levels of p34<sup>cdc2</sup> and cyclin B using western blotting. Evidently, the p34<sup>cdc2</sup> protein levels were inversely decreased, whereas the time for IPTG treatment was increased under 0.2% serum-containing medium. Comparatively, the expression levels of cyclin B were unchanged. Similar to cyclin B, the expression level of cyclin A was unchanged as well (data not shown). The falling of p34<sup>cdc2</sup> expression either due to Ha-ras overexpression or due to serum deprivation or due to both was determined by administration of IPTG. Figure 7B demonstrates that serum deprivation alone could cause significant falling of p34<sup>cdc2</sup> (Figure 7B, lanes 1, 2 and 4),

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and Ha-ras overexpression (1.95- to 2.8-fold) further enhanced this suppression by 1.8- to 2-fold (Figure 7B, lanes 2 and 3, 4 and 5). Moreover, the phosphorylation of p34\(^{dc2}\) was not affected by Ha-ras overexpression under serum-deprived conditions (unpublished data). The decrease in p34\(^{dc2}\) at the translational level seems to play an important role in disruption of the cell cycle.

**Dominant-negative ras and dominant negative raf-1 blocked Ha-ras overexpression-induced apoptosis at different signal transduction stages**

To ensure that Ha-ras indeed triggers the apoptosis of 7-4 cells, the dominant negative ras [in plasmid pZIPneoAsn17 with a mutation at amino acid 17 (serine to asparagin)] was transfected into 7-4 cells to block Ras activity (Feig and Cooper, 1988). Three stable transfectants Ras 1, Ras 2 and Ras 3 with higher levels of dominant negative ras expression show lower AP-1 activity (Figure 8A), lower efficiency of colony formation (Figure 8B) and slower cell growth rate (Figure 8C) compared with the parental 7-4 cells, indicating that dominant-negative ras effectively blocked Ras activity. AP-1 consists of the members of the jun and the fos families, which can be activated by RAS; therefore, its activity can be used as the indicator of Ras activity. Moreover, the degree of DNA fragmentation and the population of apoptotic cells were all evidently decreased in these dominant-negative ras cell lines (Figure 9A and B), indicating that ras indeed plays a major role in 7-4 cell apoptosis.

Raf-1 is an effector of activated Ras and its downstream transducers are MAPK, Erk and Elk (Kaufmann-Zeh et al., 1997). To unveil whether ras-triggered apoptosis is through the raf-1 signalling pathway, the dominant negative raf-1 gene (in plasmid pRafC4B, with a deletion at 3' end kinase domain) was transfected into 7-4 cells (Bruder et al., 1992). Three cell lines Raf E, Raf F and Raf M stably expressing the raf-1 gene were established (data not shown). To determine the activity of Raf-1, its downstream substrate Elk activity was measured by the PathDetect Elk-trans-reporter

![Figure 10](image)

*Figure 10* Dominant negative raf-1 expressed in the derivatives of 7-4 cells blocked Ha-ras overexpression induced Elk activity, and lowered the efficiency of colony formation and growth rate. (A) For Elk activity analysis, 7-4 and Raf E, F and M cells (1 x 10\(^6\) per 100-mm plate) were co-transfected with pFAEElk (1.5 \(\mu\)g), pFRLuc (1.5 \(\mu\)g) and pSGSlacZ (0.5 \(\mu\)g), which was used as an internal control for transfectant efficiency. All the cells were maintained in the medium with IPTG for 48 h, then harvested and analysed for luciferase and \(\beta\)-galactosidase activities (see Materials and methods). (B) The procedures for colony formation and cell growth analysis are the same as described in Figure 8 B and C.

![Figure 11](image)

*Figure 11* DNA fragmentation and apoptotic population were suppressed in 7-4 derivatives expressing dominant negative raf-1. Cell maintenance and treatment were the same as described in Figure 9. (A) DNA fragmentation analysis. (B) Quantification of apoptotic cells.
that activator Raf and apoptosis, between DISCUSSION indeed of ion-induced progression ordinating cellswith tion expressed (Rubin et al, 1994) is conceivable that activated Ha-ras may alter the interactions between regulatory accessory factors such as Ras-GAP and Ras-GEF, thereby leading to derepression of one or more members of the GTP-binding ras superfamily (Bokoch and Der, 1993), such as ran/TC4 (Basu et al, 1992). The product of ran/TC4 is necessary for the function of RCC1, which is a gene product involved in coordinating the end of the S-phase and chromosome condensation (Ren et al, 1993). It is also known that inappropriate expression of normal or activated ras can interfere with normal cell cycle progression (Feramisco et al, 1984; Mulcahy et al, 1985). Denko et al (1994) have shown that selective induction of the activated Ha-ras transgene with IPTG is sufficient to disrupt at least one cell cycle control point by stimulating serum-deprived 212 cells to progress from G1 arrest into S-phase. All the data indicate that activated ras can facilitate the transition from G1 to S-phase of the cell cycle. This phenomenon is consistent with our observation of 7-4 cells that accumulated in S-phase while activated Ha-ras was overexpressed by IPTG.

The disruption of the normal cell cycle that resulted in the death of the cells has been reported (Rubin et al, 1993). Meikrantz et al (1994) classified the death of S-phase-arrested cells in an asynchronous population into multiple forms of apoptosis. The correlation between S-phase arresting and apoptosis may explain that 7-4 cells with a major population of S-phase-arrested cells showed high mortality, significant DNA fragmentation and strong acridine orange staining. Such cell cycle disruption may result from either expression at the wrong time or aberrant expression of normal cell cycle kinases, including activation or inactivation (Rubin et al, 1993). Chen and Pan (1994) reported that in Xenopus oocytes, Ha-ras oncogene overexpression suppressed the activity but not the expression level of p34\(^{oc2}\), and subsequently led the cell to rest in M-phase. In our study, Ha-ras overexpression greatly suppressed p34\(^{oc2}\) expression level and led the cells to accumulate in S-phase.

Many factors may be involved in the process of Ha-ras-induced apoptosis, but the relationships largely remain unknown (Whyte and Evan, 1995). The possible mechanisms of Ha-ras overexpression-induced apoptosis in 7-4 cells are as follows: (a) overexpression of Ha-ras oncogene inactivates cell cycle-related kinases (such as the kinase p34\(^{oc2}\)) (Girard et al, 1991); (b) Ha-ras overexpression shuts off the expression of 'survival' genes, such as bcl-2 in the absence of appropriate growth factors under serum depleted condition and guides cells to death; (c) the unknown factor that triggered the apoptotic pathway was specially activated in 7-4 cells (such as IRF-1) (Tanaka et al, 1994).

Overexpression of the bcl-2 gene blocked Ha-ras-induced apoptosis but had no effect on Ha-ras oncogene expression, indicating that its prevention of apoptosis is downstream in the Ha-ras signalling pathway (unpublished data). Moreover, Bcl-2 and p21ras molecules can be co-immunoprecipitated in Jurkat cells, and phoshorylation of the Bcl-2 protein is involved in the prevention of cell apoptosis (Chen and Faller, 1996). However, the precise mechanism by which the bcl-2 gene prevents Ha-ras-induced apoptosis remains to be determined.

Our results from (a) selective overexpression of activated Ha-ras oncogene by IPTG; and (b) specific blockade of Ras activity by dominant negative ras or dominant negative raf clearly demonstrate that overexpression of activated Ha-ras indeed induces 7-4 cells apoptosis under serum-deprived conditions. Activated Ha-ras-induced apoptosis was also detected in the other transformant 212 cells. But the degree of apoptosis was less severe compared with 7-4 cells, indicating that factors other than Ha-ras activation may also be involved. However, our observation that 7-4 cells proceeded with apoptosis without c-myc overexpression (unpublished data) is different from Kauffmann-Zeh’s report that both Ha-ras and c-myc are overexpressed in the apoptosis of the fibroblasts (Kauffmann-Zeh et al, 1997). This contrasting result indicates that c-myc overexpression is not an inducing factor but an end-point response of Ha-ras-induced apoptosis.

The effect of serum starvation on long-term viability of the cells was observed by measuring the percentage of dye excluding cells that were stained with 0.04% trypan blue at a 24-h interval. We found that, 72 h after starvation, the percentage of viable 7-4 cells is 88% in the absence of IPTG and drops to 50% in the presence of IPTG compared with NIH/3T3 cells (100%), indicating that NIH/3T3 and 7-4 cells could stand long-term starvation (unpublished data). Ha-ras gene overexpression makes 7-4 cells more vulnerable to serum deprivation and induces them to apoptosis.

The uncertainty whether nutrient deficiency caused by serum starvation may induce cell apoptosis was clarified by growing the cells in the medium supplemented with 10% AC-2 (Valio, Finland; colostrum, a serum substitute). We found that NIH/3T3 and a lung carcinoma cell line H2981 could grow normally in this kind of medium (unpublished data). In contrast, 7-4 cells with Ha-ras overexpression maintained in the same conditions underwent apoptosis, indicating that serum depletion causing nutrient deficiency is not a determining factor of apoptosis. It is consistent with Kulkarni’s report that serum starvation only caused minor cell apoptosis (Kulkarni and McCulloch, 1994). The amount of certain growth factors such as IGF-1 in either 0.2% serum or 10% AC-2 may be critical to determine cell survival or death. A detailed study is under way.

Dominant negative ras-f1 blocked the Raf-1-MAPK pathway and prevented apoptosis of 7-4 cells, indicating that Ha-ras-induced apoptosis needs Raf-1-MAPK pathway activity, which is consistent with Kauffmann-Zeh’s observation that Raf-1-MAPK is an apoptotic pathway. It seems that the Raf-MAPK pathway is a common pathway for both proliferation and apoptosis. We hypothesize that in NIH/3T3 fibroblasts while Ha-ras was overexpressed, serum starvation as an exogenous stress will turn on the apoptotic signal through

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Ha-ras and ras-1 signalling pathways. The fate of the cells will then be executed by downstream specific nuclear transactivators, which are formed after Ha-ras activation and activated through dimer formation (such as the pairing of the members of jun and fos families) that activate either survival genes or apoptotic genes. Ha-ras overexpression activated downstream from the expression of apoptotic-related genes was sustained by a protein synthesis inhibitor cyclolheximide study, which delayed 7-4 cell apoptosis.

In conclusion, our study demonstrates that Ha-ras overexpression without c-myc overexpression can trigger cell apoptosis. This apoptosis may use the Raf-1-MAPK pathway to synthesize specific nuclear factors and subsequently to turn on apoptotic-related genes. These events were confirmed by the studies of dominant-negative ras, ras-1, cyclolheximide and bcl-2 gene overexpression.

The relationship between Ha-ras oncogene overexpression and cell apoptosis observed in our laboratory will shed light on the understanding of Ha-ras oncogene-related apoptosis and aid in unveiling the factors that lead tumour cells with the expression of activated Ha-ras oncogene to programmed cell death.

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