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Differential Induction of Apoptosis in Oncogene-Transformed NIH 3T3 Cells by Methylmethanesulfonate

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ABSTRACT. Cellular oncogenes have been shown to play crucial roles in the cell death process induced by cytotoxic agents. In this study, we have demonstrated that v-H-ras transformed NIH 3T3 cells but not other transformants (v-raf, v-src, v-erbB-2, v-fes and v-mos) exhibited a survival advantage to treatment by a DNA-damaging agent, methylmethanesulfonate (MMS). Subsequently, the biochemical and morphologic criteria of MMS-treated cells were examined. It was found that MMS induced v-H-ras transformants to go through necrosis, but it induced other transformed cells to undergo apoptosis. The levels of glutathione (GSH) within each transformant as well as in NIH 3T3 cells, were determined. The results showed that GSH levels within ras transformants were 2- to 7-fold higher than the levels in other transformants and normal NIH 3T3 cells. By using the GSH synthesis inhibitor buthionine sulfoximine, GSH levels were artificially reduced. This depletion, however, made ras transformed cells more sensitive to MMS killing, but the mode of cell death was still necrosis. Western blot analysis demonstrated that the anti-apoptotic protein Bcl-2 was constitutively expressed in ras transformed cells but not in NIH 3T3 or other transformed cells. The level of Bcl-2 was correlated with the resistant phenotype of ras transformants during MMS treatment. These observations suggest that GSH and Bcl-2 levels may cooperatively confer the resistant phenotype of ras transformants in response to MMS. In addition, the mode of cell death may possibly be determined at least in part by Bcl-2 protein. BIOCHEM PHARMACOL 52;3:481–488, 1996.

KEY WORDS. apoptosis; necrosis; glutathione; Bcl-2 protein; NIH 3T3 cells; ras-transformed NIH 3T3 cells

The process of cell death can be classified typically into two types: apoptosis and necrosis. Apoptosis differs from necrosis in that its early stages show chromatin condensation and DNA fragmentation; in addition, cytoplasmic organelles remain intact [1]. The dying cells fragment into small, membrane-enclosed apoptotic bodies, which are taken up rapidly by resident phagocytic cells. The process of apoptosis often depends on RNA and protein syntheses in the dying cells, indicating positive participation of cells in the death processes [2]. In contrast, necrotic cells undergo rapid cytoplasmic swelling with gross disruption of organelle function prior to cell lysis [1]. Numerous cancer cells have been reported to undergo apoptosis when treated with antitumor agents such as etoposide (VP-16), camptothecin, cisplatin, and vincristine [3–6].

Some specific oncogenes and oncosuppressors may play crucial roles in the regulation of cell proliferation and apoptosis. For example, NIH 3T3 cells transfected with and overexpressing c-H-ras genes became more resistant to anticancer drugs including cisplatin, Adriamycin®, melphalan, and CPT-11 [7]. Also, Burt et al. [8] demonstrated that both v-H-ras and v-raf oncogenes could transform rat liver epithelial cells, resulting in an increase of cellular resistance to treatment with genotoxic agents such as Adriamycin, vinblastine, and 2-acetylaminofluorene. Generally, this drug resistance is accompanied by an elevated expression of P-glycoprotein and glutathione-S-transferase P [8]. The suppression of endogenous H-ras function by a dominant negative v-H-ras mutant resulted in apoptosis of K562 leukemia cells [9]. This directly indicates that ras itself or the ras-associated pathway may have the ability to suppress some apoptosis induced by exogenous stimuli. However, the precise mechanism(s) by which ras oncogenes provide a survival advantage to cells remains unclear.

It is noteworthy that a core signaling unit has been identified recently, consisting of Ras–Raf and Raf-regulated kinases such as MAPKK/MEK-MAPK§, which integrates signals originating from protein tyrosine kinases and protein kinase C at the membrane [10–12]. This signaling pathway has been thought to play a role in both mitogenic and

transforming activities in various oncogene-transformed cells, like *ras*, *raf*, *mos* and *fes* [13–15]. Based on these observations, a question is raised as to whether oncogenes, which have been suggested to associate with the core signaling transduction pathway, are related to the mechanism of resistance to toxic agent-induced cell death.

Therefore, in the present study we employed several viral oncogene-transformed NIH 3T3 cell lines, including v-H-*ras*, v-*raf*-1, v-*src*, v-*fes*, v-*erbB*-2 and v-*mos*, to examine the response to cell death following exposure to MMS, a DNA-damaging agent. We further demonstrated that GSH levels determine the capacity of drug resistance but not the mode of cell death in these cell lines. Finally, Bcl-2 protein was found to be elevated in v-H-*ras* transformed cells but not in normal or other oncogene-transformed cells, suggesting that Bcl-2 may play a role in the prevention of MMS-induced apoptosis.

MATERIALS AND METHODS
Cells and Culture

v-Ha-*ras*, v-*raf*, v-*src*, v-*mos*, v-*erbB*-2 and v-*fes* transformed NIH 3T3 cells were obtained from Dr. S-F. Yang of the Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan. The v-Ha-*ras* transformed cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine, G418 (100 μg/mL), and antibiotics. Other oncogene-transformed and parental NIH 3T3 cells were cultured in the same medium without G418.

Drug Treatment and Cell Viability

Cells were incubated with 1 mM MMS (Aldrich Co., Milwaukee, WI, U.S.A.) for varying time periods, as indicated in Fig. 1. After incubation, the cells were washed twice in ice-cold PBS, and cell viability was determined using the trypan blue exclusion assay.

DNA Extraction and Electrophoresis

After different treatments, both attached and floating cells were harvested, washed in ice-cold PBS, resuspended in 500 μL of TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA, pH 8.0), and lysed in 500 μL of lysis buffer (3% SDS, 50 mM Tris, pH 12.6) at room temperature for 10 min. DNA was extracted with phenol and chloroform before ethanol precipitation. DNA pellets were finally solubilized in TE buffer, and treated with RNase A for 40 min prior to 1.2% agarose gel electrophoresis.

Flow Cytometry Analysis

At specific time points, treated or untreated cells were trypsinized and fixed in 75% ethanol at −20° for at least 1 hr. After centrifugation at 2000 *g* for 5 min at 4°, cell pellets were resuspended in 0.5 mL of a hypotonic buffer (0.5%

Triton X-100/PBS and 0.05% RNase A) and incubated for 30 min; 0.5 mL of propidium iodide solution (50 μg/mL) was added and the pellets were allowed to stand on ice for 15–30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after laser excitation of the fluorescent dye by a FACSort flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

Electron Microscopy

Cells were fixed with 2% glutaraldehyde and 2% paraformaldehyde in PBS for 15 min. Following several rinses in PBS, cells were postfixed in 1% osmium tetroxide, dehydrated in alcohol, and embedded in an Epon-Araldite mixture. Thin sections were cut and doubly stained with uranyl acetate and lead citrate before being examined in a Joel 2000 EXII electron microscopy at 100 kV.

Determination of Total Intracellular GSH Level

About 10^7 cells were harvested by scraping off the bottom of the dish with a rubber policeman. Cell pellets were washed with ice-cold PBS, resuspended in 300 μL of A buffer (125 mM KH_2PO_4, 6.3 mM EDTA; pH 7.5), subjected to rapid freeze–thaw lysis four times, and reacted in a sonicator (Branson, 2200) for 5 min. The supernatant that was obtained by centrifugation at 2000 *g* for 5 min was divided into two parts: 50 μL and 250 μL. The cellular protein content in the 50 μL of supernatant was determined using the Bio-Rad protein assay. The GSH level was measured in the remaining 250 μL to which 100 μL of 12% 5-sulfosalicylic acid (Sigma, St. Louis, MO, U.S.A.) was added and then allowed to stand on ice for 2–3 hr to allow protein precipitation. Cellular protein was removed by centrifugation at 14,000 *g* for 15 min, after which 200 μL of supernatant was obtained. The NADPH reagent (0.525 mM) and 5,5'-dithiobis-2-nitrobenzoic acid (1.5 mM) were purchased from Sigma and prepared in B buffer (187.5 mM KH_2PO_4, 6.3 mM EDTA; pH 7.5). GSH was measured by the method of Nakagawa et al. [16], utilizing an enzymatic recycling assay based on GSH reductase. To measure the GSH, 400 μL of 5,5'-dithiobis-2-nitrobenzoic acid, 400 μL of NADPH, 200 μL of supernatant, and 3 μL of GSH reductase were added serially in a 10 × 45 × 4 mm plastic cuvette. The absorbance of the mixture at 412 nm was monitored on a Hitachi (U-3210) recording spectrophotometer and recorded at 20-sec intervals for 3 min. Results represent the means ± SEM of values from three separate experiments.

Western Blotting

Cellular lysates were prepared as described by Kuo and Yang [17]. A 50-μg sample of each lysate was subjected to electrophoresis on 15% SDS–polyacrylamide gels for detection of Bcl-2 p26. The samples were then electroblotted on nitrocellulose paper. After blocking, blots were incubated
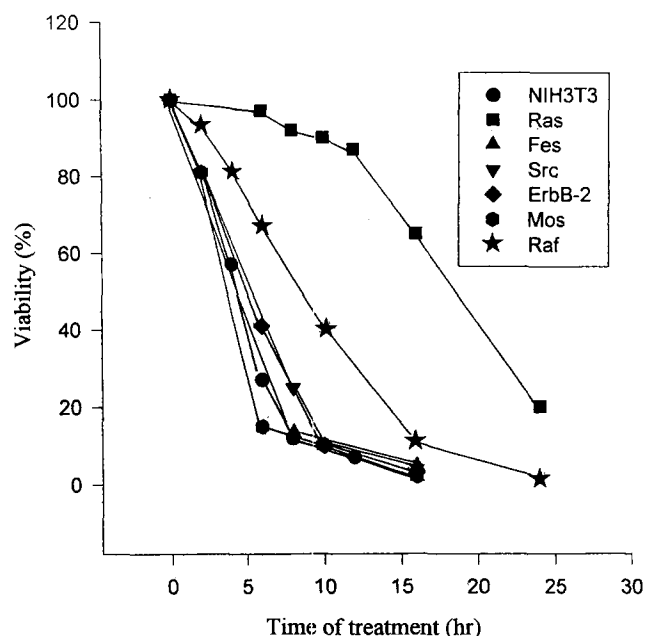
FIG. 1. Sensitivity of oncogene-transformed and parental NIH 3T3 cells to MMS killing. Cells were plated in a density of 1×10^6 cells/100 mm dish in the presence of 1 mM MMS for different time periods. Viable cells were measured by a trypan blue exclusion assay. Each point represents the mean value of duplicate experiments.

with anti-Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) antibody in PBST (PBS–0.1% Tween 20) for 1 hr followed by two washes (15 min each) in PBST, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL, U.S.A.) for 30 min. After washing, the blots were incu-

bated for 1 min with western blotting reagent ECL (Amersham), and chemiluminescence was detected by exposure of the filters to Kodak X-Omat films for 10 sec to 10 min.

RESULTS

The resistance of transformed cell lines and normal NIH 3T3 cells to MMS-mediated cytotoxicity was determined by trypan blue exclusion. These cell survival curves (Fig. 1) revealed a survival advantage for v-H-ras and v-raf transformed cells relative to other transformed cells and normal NIH 3T3 cells when exposed to 1 mM MMS for various time periods. Furthermore, the ras transformed cells were more resistant than the raf transformants to MMS killing.

The mode of cell death induced by MMS in these cells was determined via assaying DNA fragmentation, apoptotic peak, and nuclear chromatin condensation, respectively. As shown in Fig. 2A, NIH 3T3 cells treated with 1 mM MMS showed typical patterns of internucleosomal DNA laddering within 8–16 hr, but smearing at 24 hr. The same pattern of internucleosomal DNA ladder was also evident in the oncogene-transformed cells v-src, v-raf, v-erbB-2, v-mos and v-fes by 10 hr of treatment with MMS (Fig. 2B). In contrast, v-Ha-ras transformed cells retained an intact genomic DNA pattern after treatment with 1 mM MMS for 10 hr (Fig. 2C, lane 3). Even when 80% of the ras transformants were killed by exposure to MMS for 24 hr, the pattern of genomic DNA appeared as a slight smearing rather than laddering (Fig. 2C, lane 6).

Another method of assessment of apoptosis was based on the assay of DNA strand breaks in individual cells by flow cytometry. Generally, the results obtained by this method
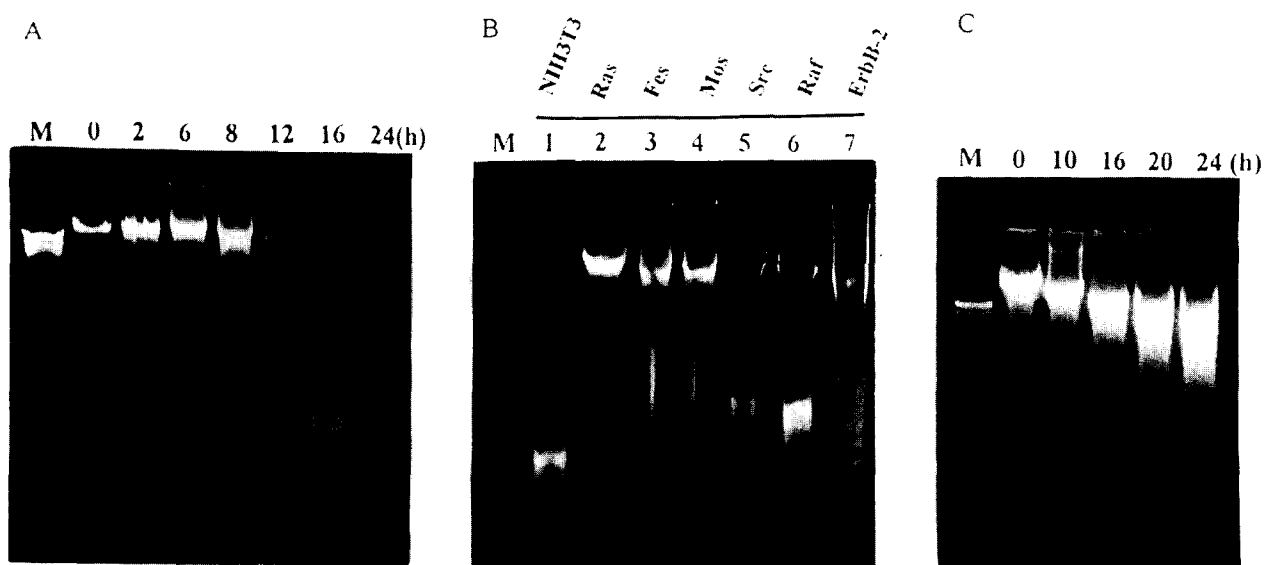

FIG. 2. Internucleosomal DNA fragmentation in various oncogene-transformed and parental NIH 3T3 cells treated with MMS. (A) NIH 3T3 cells were exposed to 1 mM MMS for different periods of time as indicated in the figure; (B) different oncogene-transformed cells were treated with 1 mM MMS for 10 hr; (C) ras transformed NIH 3T3 cells were exposed to 1 mM for 0–24 hr. DNA from different treatment protocols was extracted and electrophoresed through 1.2% agarose gel. DNA bands were visualized by staining with ethidium bromide.
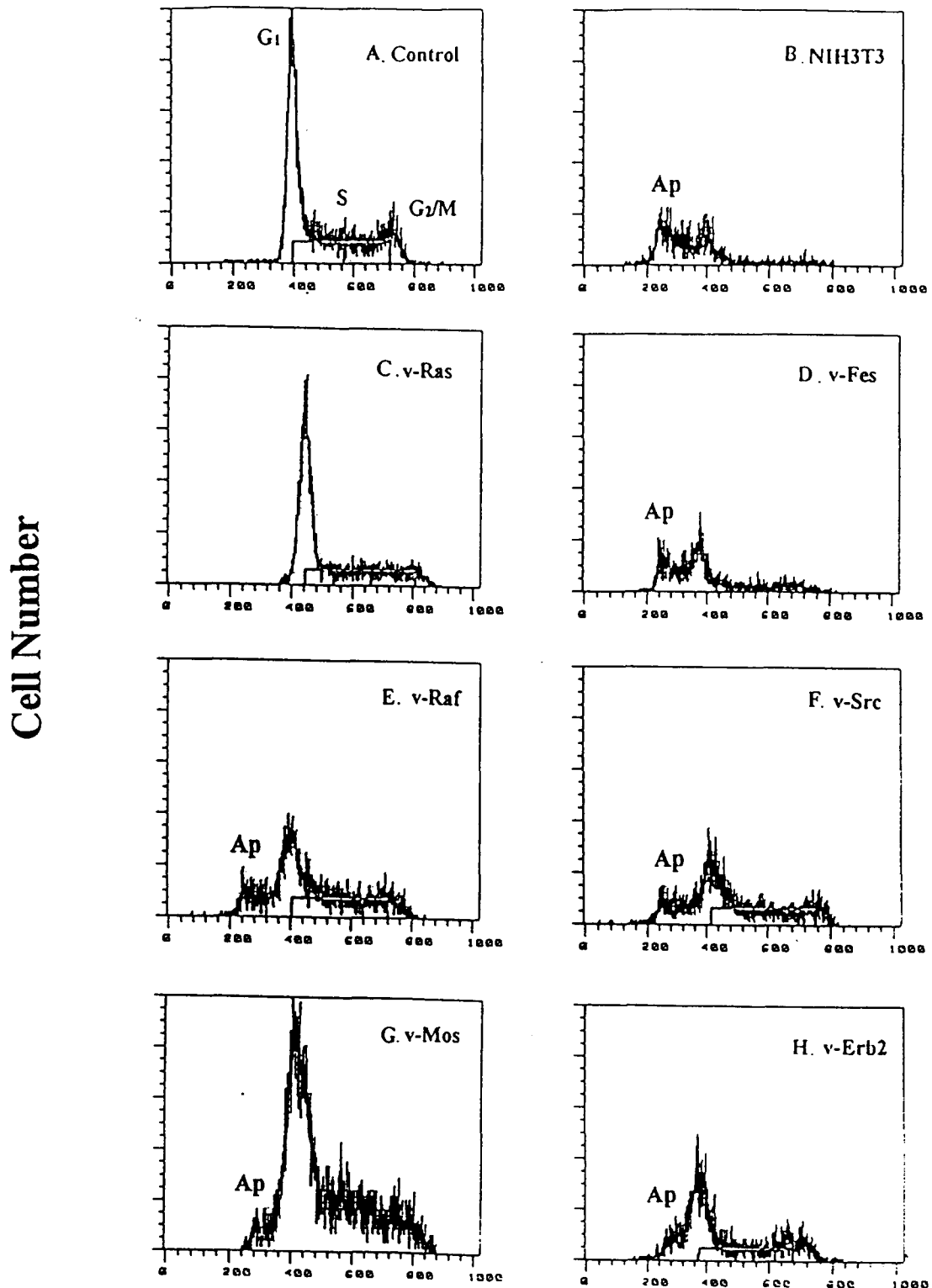
PI-DNA fluorescence

FIG. 3. Cell cycle analysis of oncogene-transformed and normal NIH 3T3 cells following treatment with MMS. Various oncogene-transformed and their parental NIH 3T3 cells were cultured in the presence or absence of 1 mM MMS for 12 hr. After removal of MMS, cells were fixed and stained with propidium iodide (PI) as described in Materials and Methods, and the DNA content was analyzed by flow cytometry. Ap represents the peak of apoptotic bodies.
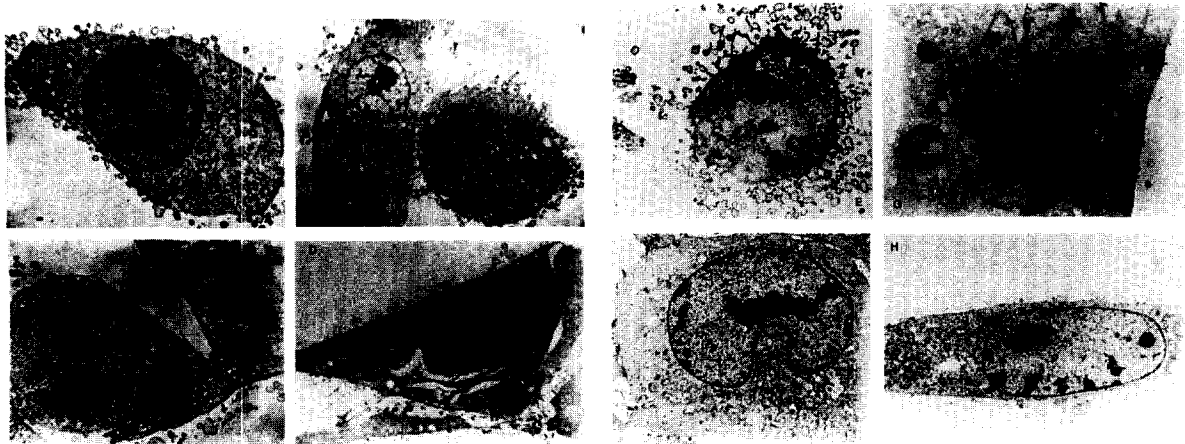
FIG. 4. Morphology of apoptosis occurring in various oncogene-transformed and normal NIH 3T3 cells following treatment with MMS. (A) Control untreated NIH 3T3 cells, (B) NIH 3T3 cells, (C) ras transformants, (D) fes transformants, (E) mos transformants, (F) raf transformants, (G) src transformants, and (H) erbB-2 transformants were exposed to MMS for 10 hr. After removal of MMS, cells were fixed and examined as described in Materials and Methods. Original magnification: ×600.

were in agreement with the data based on the DNA laddering, analyzed on agarose gel electrophoresis. Transformed cells such as v-fes, v-raf, v-src, v-mos, v-erbB-2 (Fig. 3, D–H) and normal NIH 3T3 cells (Fig. 3B) were found to show a typical sub-G_1 peak (apoptotic peak, Ap) at 12 hr of MMS treatment. However, the sub-G_1 peak was not observed in MMS-treated v-H-ras transformed cells (Fig. 3C).

Consistent with the results obtained from DNA fragmentation and flow cytometry, cell morphology examined by electron microscopy demonstrated that MMS-treated v-H-ras transformed cells did not show any morphological changes (Fig. 4C) when compared with untreated cells (Fig. 4A). Under the same treatment, the morphological features

of apoptosis, including compaction and segregation of nuclear chromatin, discreteness of nucleus and cytoplasm as well as blebbing of cell membrane, were evident in other transformed (Fig. 4, D–H) and normal NIH 3T3 cells (Fig. 4B).

Based on these observations, we suggest that activated ras gene but not other oncogenes abrogates the apoptotic cell death induced by the DNA-damaging agent MMS. Although other oncogenes have been found to associate directly or indirectly with the RAS–RAF–MEK–MAPK signaling transduction pathway, they still undergo apoptosis upon MMS treatment. To determine the possible mechanism by which ras transformed cells can increase resistance to MMS-induced apoptosis, we first examined the levels of GSH within each transformant and normal NIH 3T3 cells. The results demonstrated that the amount of GSH in the v-Ha-ras transformant was 2- to 7-fold higher than that of other oncogene-transformed cells and NIH 3T3 cells (Fig. 5). BSO an irreversible inhibitor of γ-glutamylcysteine synthetase, was used to reduce the GSH levels in ras transformed cells. After a 16-hr incubation with 50 μM BSO, GSH in ras transformed cells could be reduced to a level similar to that of the parental NIH 3T3 cells. However, the depletion of GSH by BSO did not produce any cytotoxic effect in the ras transformants (data not shown). Furthermore, we found that GSH-depleted ras transformed cells became more susceptible to MMS treatment (Fig. 6A), but the cells were induced to go through necrosis rather than apoptosis, as determined by agarose gel electrophoresis (Fig. 6B).
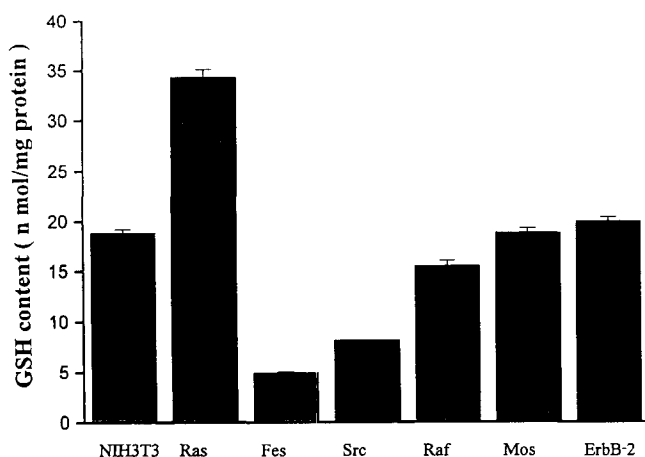
The bcl-2 gene, first identified by its deregulation in human follicular lymphoma [18], is known to prevent apoptosis induced by many, but not all, experimental conditions [19, 20]. Thus, we examined whether bcl-2 is expressed in v-H-ras transformed cells. Western blot analysis shows that bcl-2 protein was moderately expressed in ras transformed cells but not in NIH 3T3 cells (Fig. 7) or other transformed cells (data not shown). In addition, the level of Bcl-2 pro-

FIG. 5. Determination of GSH content within oncogene-transformed and normal NIH 3T3 cells. Cells (1×10^7) were harvested and washed with ice-cold PBS, and then were lysed by rapid freeze-thaw. The supernatant was obtained by centrifugation, and further means were used to measure the content of GSH. The detailed procedure is described in Materials and Methods. Values are the means ± SEM obtained from at least three separate experiments for each cell line.

A

B
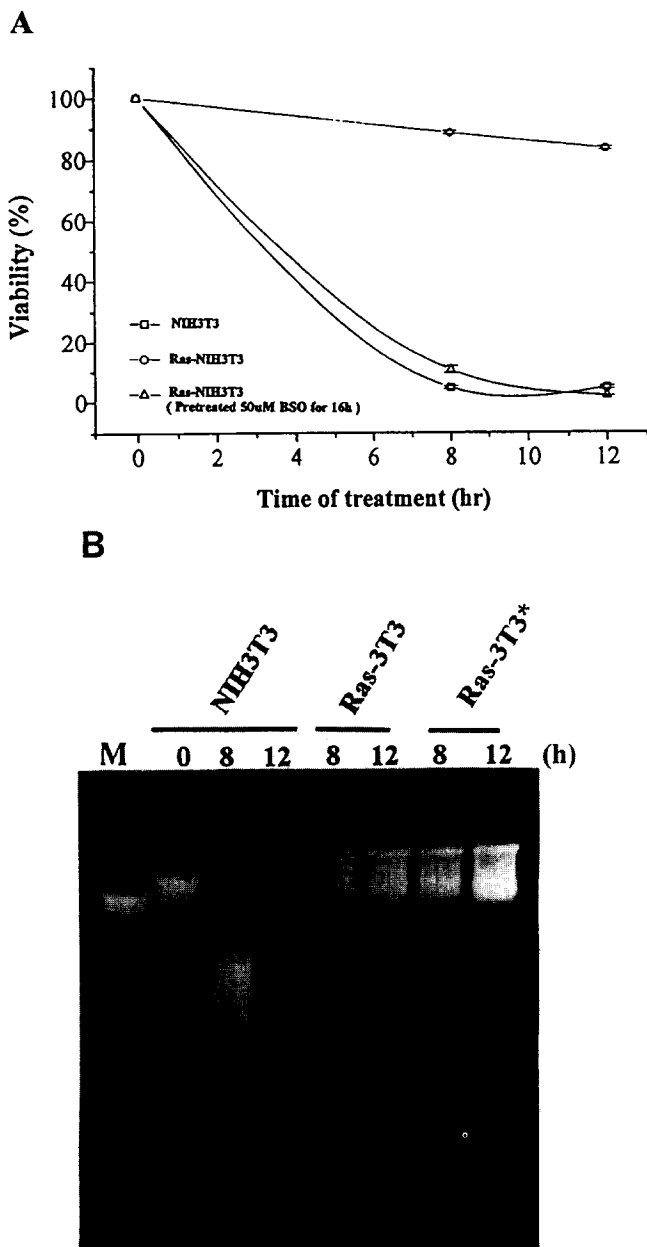

FIG. 6. Effects of GSH depletion on the resistant phenotype of ras transformants. (A) Increment of susceptibility of ras transformants to MMS-induced cell death by depletion of GSH. v-H-ras NIH 3T3 cells were pretreated with BSO for 16 hr, and then exposed to 1 mM MMS for various time periods. Viable cells were counted by means of the trypan blue exclusion assay. Values are the means ± SEM obtained from three independent experiments. (B) Analysis of DNA fragmentation in GSH-depleted ras transformants following MMS treatment. v-H-ras transformed and its parental NIH 3T3 cells were exposed to 1 mM MMS for various time periods as indicated. Ras-3T3* represents ras-transformed cells preincubated with BSO for 16 hr. The methods of DNA extraction and examination are described in detail in Materials and Methods.

teins in ras transformants remained unchanged during 10 hr of MMS treatment, correlating with the resistant phenotype of ras transformants. The results presented here strongly indicate that bcl-2 may play an important role in

determining the resistance and mode of cell death of ras transformants in response to MMS.

DISCUSSION

The results presented here clearly demonstrate that of the oncogene-transformed cells tested only the ras transformant exhibited a remarkable resistance to MMS-induced cell death. Constitutive activation of the MEK–MAPK signaling pathway has been reported to occur in all of the oncogene-transformed cells used here. It seems likely that the resistant characteristics acquired by the ras transformant may not relate to the core MEK–MAPK signal transduction pathway. Recently, Rodriguez-Viciana et al. [21] demonstrated that an important enzyme, phosphatidylinositol-3-OH kinase (PI(3)K), is a downstream target for p21^{ras}. The cellular targets of the products of PI(3)K are not defined clearly, although protein kinase C family members may be included [22]. Moreover, the two-hybrid system in yeast was used to screen a complementary DNA library for genes encoding Ras-binding proteins; four different genes, in addition to Raf, were detected [23]. Based on these observations, we suggest that some other signal transduction pathway(s) that uniquely exists in the ras transformant may be involved in such drug resistance.

GSH has been shown to play a critical role in cellular defense against a variety of injurious agents [24]. Also, several types of cancer cells have been found to increase their resistance to antineoplastic agents via elevating the GSH levels [25]. We demonstrated here that ras transformant displayed a 2- to 7-fold increase of GSH level as compared with other oncogene-transformed and normal cells; however, depletion of GSH made the ras transformant more susceptible to MMS killing. This led us to suggest that the resistant features of ras transformant to MMS should be determined mainly by the GSH content. These observations are consistent with those of earlier studies that showed an increase of GSH in various ras transformed cell lines [26]. GSH is synthesized by the consecutive actions of
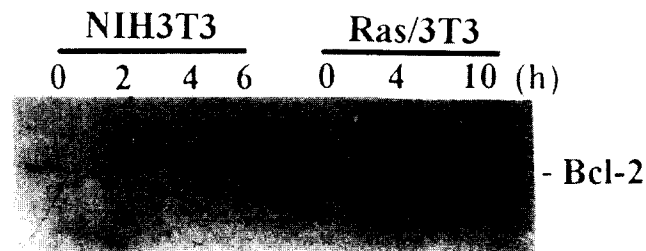

FIG. 7. Immunoblot analysis of Bcl-2 in ras-transformed and normal NIH 3T3 cells. v-H-ras-transformed and its parental NIH 3T3 cells were exposed to 1 mM MMS for various time periods as indicated in the figure. Cell lysates (50 μg) were subjected to electrophoresis by SDS–PAGE and western blotted to nitrocellulose paper. The Bcl-2 was labeled by sequential incubation with monoclonal antibody to mouse Bcl-2 horseradish-conjugated goat anti-mouse IgG. Bound antibodies were detected by enhanced chemiluminescence.

γ-glutamylcysteine synthetase as well as GSH synthetase, and is utilized by several enzymes including the GSH transhydrogenase and GSH peroxidase. Which signal pathway in the *ras* transformant may participate in the alteration of the GSH pool remains elusive.

As demonstrated in the present study, MMS induced apoptosis in parental NIH 3T3 and oncogene-transformed cells, including v-*src*, v-*erbB*-2, v-*mos*, v-*fes* and v-*raf*, but not in v-H-*ras*-transformed cells. The depletion of GSH made *ras* transformants become more sensitive to MMS-mediated necrotic cell death but not to apoptosis. This implies that some other cellular targets but not GSH regulate the mode of cell death of *ras*-transformed cells in response to MMS. It has been demonstrated that the overexpression of *bcl*-2 can prevent cell apoptosis induced by various injuries, including those caused by DNA damaging agents [27], growth factor withdrawal [19, 28], γ-irradiation, and a wide variety of chemotherapeutic drugs [27, 29]. The results presented here show that Bcl-2 proteins were indeed expressed in the *ras* transformant but not in other transformants. Thus, we strongly suggest that the defect of apoptosis in the *ras* transformant is due, at least in part, to the expression of *bcl*-2. Corroborating our findings, it has been demonstrated that activation of the *ras* pathway results in up-regulation of *bcl*-2 expression in 32D cells [30]. In addition, Fairbairn *et al.* [31] have shown recently that the overexpressed *bcl*-2 oncogene in murine hematopoietic cells results in the delay of MMS-mediated apoptotic cell death. We suggest that other possible mechanisms or molecules may also be included in regulating the apoptosis. For example, it is well documented that cells that harbor a wild-type p53 will experience a G_1 arrest and subsequently undergo apoptosis upon treatment with a DNA-damaging agent. In contrast, mutant p53 enables cells to escape apoptosis [32, 33]. Whether or not alteration of p53 occurs in the *ras* transformant is now under investigation.

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