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A Novel Mutant from Apoptosis-resistant Colon Cancer HT-29 Cells Showing Hyper-apoptotic Response to Hypoxia, Low Glucose and Cisplatin

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Solid tumors usually have regions of hypoxia and glucose deprivation. Human colon carcinoma HT-29 cells show an apoptosis-resistant phenotype in response to microenvironmental stresses. In this study, we isolated a novel mutant of HT-29, designated as HA511, that showed a high apoptotic response to hypoxia, glucose deprivation and treatment with the chemical stressors tunicamycin and glucosamine. The mutant HA511 cells exhibited nuclear condensation and fragmentation and activation of CPP32 (caspase-3) protease under the stress conditions, while the parental HT-29 cells did not. We found that apoptosis occurred in HA511 cells after prolonged cell cycle arrest at the G1 phase, while in the parental cells a progression to S phase occurred after the G1 arrest. Upon exposure to an anti-Fas antibody, HA511 cells underwent apoptosis, whereas the parental cells proliferated without substantial cell death. Furthermore, HA511 cells were preferentially hypersensitive to cisplatin. We found no alteration in expression of GRP78, anti-apoptotic protein Bcl-X_L, or p53, of which the gene was mutated in HT-29 cells. The mutant HA511 cells could provide useful information on the mechanism of apoptosis of solid tumors.

Key words: Glucose-regulated stress — Hypoxia — Apoptosis — Fas — Cisplatin

Solid tumors have regions of hypoxia (low oxygen), low levels of glucose and other nutrients, and low pH because of insufficient blood supply.[1, 2] Cancer cells in such aberrant environments can survive and show drug resistance through a stress response characterized by induction of GRPs, a major family of stress protein.[3, 4] Indeed, previous studies, including ours, have shown that GRP induction in solid tumor cells was closely correlated with the development of resistance to multiple anticancer drugs.[5–8] This resistance induction was found in various solid tumor-derived cell lines, regardless of their origin.[5–8] In agreement with the observations, hypoxia has been shown to predict poor disease-free survival.[9] Thus, the stress conditions within solid tumors are one source of cellular drug resistance and a major obstacle to successful cancer therapy.

Apoptosis is a genetically programmed cell death mechanism that can be activated by various stimuli, including chemotherapeutic agents and radiation.[10, 11] The physiological stress conditions of hypoxia and glucose starvation can also induce apoptosis.[12, 13] During malignant progression, solid tumor cells are continuously exposed to apoptosis-inducing stress conditions, resulting in a selection of cells with deficient or diminished apoptotic response to those conditions.[12] Indeed, induction of apoptosis under experimental stress conditions is minimal in most solid tumor-derived cells, and the cells showed cell cycle arrest in the G1 phase.[14–16] Further, many solid tumors have been shown to possess mutation in apoptosis-directing p53 and overexpression of antiapoptotic proteins, such as Bcl-2 and Bcl-X_L.[17–19] The genetic alterations of *p53* and *Bcl-2/Bcl-X_L* could confer resistance to apoptosis induced by various stimuli, including hypoxia[12, 20, 21] and antitumor drugs.[22, 23] Thus, solid tumor cells acquire the apoptosis-resistant phenotype that, in turn, contributes to induction of drug resistance by the stress conditions.

To overcome the stress-associated resistance of solid tumors, we have looked at the mechanisms of apoptosis hypersensitivity as well as apoptosis resistance under stress conditions. In this report, we describe an apoptosis-hypersensitive mutant from an apoptosis-resistant cell line, human colon cancer HT-29.

MATERIALS AND METHODS

Chemicals and antibodies Tunicamycin and glucosamine were obtained from Sigma Chemical Co. (St Louis, MO) and Wako Pure Chemical Industries (Osaka), respectively. Aprotinin, BrdU, EMS, DAPI, phenylmeth-

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[4] The abbreviations used are: GRP, glucose-regulated protein; EMS, ethylmethanesulfonate; BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenyindole; MCA, 4-methylcoumaryl-7-amide; FITC, fluorescein isothiocyanate; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Z-EVD-CH_2DCB, benzyloxycarbonyl-Glu-Val-Asp-CH_2OC(O)-2,6-dichlorobenzene.

ylsulfonyl fluoride, propidium iodide, and RNase A were obtained from Sigma. DEVD-MCA and YVAD-MCA were from Peptide Institute Inc. (Osaka). Rat anti-GRP78 polyclonal antibody was established in our laboratory using recombinant hamster GRP78 (StressGen, Victoria, BC, Canada) as the antigen. Anti-Fas antibodies, clone CH-11 (for induction of apoptosis) and clone UB-2 (for detection of cell surface Fas antigen), were obtained from MBL (Nagoya). Anti-Bcl-X_L and anti-CPP32 antibodies were from Transduction Laboratories (Lexington, KY). Anti-Bcl-2, FITC-conjugated anti-BrdU, anti-p53 and anti-PARP antibodies were from Boehringer Mannheim (Mannheim, Germany), Becton Dickinson (Braintree, MA), Calbiochem-Novabiochem (Tokyo) and Pharmingen (San Diego, CA), respectively. Cisplatin and etoposide were from Bristol-Myers Squibb Co., Ltd. (Tokyo). Camptothecin was from Yakult (Tokyo). Taxol was a gift from the National Cancer Institute (NIH, Bethesda, MD). All other chemicals were of analytical grade.

Cells and culture conditions The human colon carcinoma cell line HT-29 was obtained from Dr. R. Shoemaker of the National Cancer Institute (NIH, Bethesda, MD). HT-29T9 cells were previously cloned by limiting dilution,[24] and HA511 cells were established in this study (see below). The cells were maintained in RPMI 1640 medium (Nissui Co., Ltd., Tokyo) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin and were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed using exponentially growing cells and were repeated at least twice.

To produce stress conditions, tunicamycin and glucosamine were added directly into the culture medium to final concentrations of 1 μg/ml and 10 m*M*, respectively. Hypoxic conditions were achieved using an anaerobic chamber and BBL GasPac Plus (Becton Dickinson, Cockeysville, MD), which catalytically reduces oxygen levels to less than 10 ppm within 90 min.[21] Glucose deprivation was achieved by changing the medium to a glucose-free RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum.

Isolation of HA511 mutant cells We initially attempted to isolate a mutant defective in G1 arrest under chemical stress conditions (Fig. 1). HT-29T9 cells were mutagenized with EMS, as previously described.[25] The mutagenized cells were treated with 1 μg/ml of tunicamycin for 20 h. After this, more than 70% of the cells were arrested at the G1 phase (see Fig. 2A). Following removal of tunicamycin, the cells were treated for 6 h with 40 ng/ml of nocodazole, a reversible mitotic inhibitor, to concentrate cells that had not arrested at the G1 phase (about 30% of the total population) into the M phase. During the 6 h of nocodazole treatment, the 70% of cells that had undergone G1 arrest caused by tunicamycin could not enter the M phase. After the nocodazole treatment, the M phase-trapped cells were collected by gentle pipetting and were allowed to grow in fresh medium. To avoid isolating a tunicamycin-specific mutant that has a mechanism such as decreased accumulation of this stressor into cells,[26] we used another chemical stressor, glucosamine. The tunica-

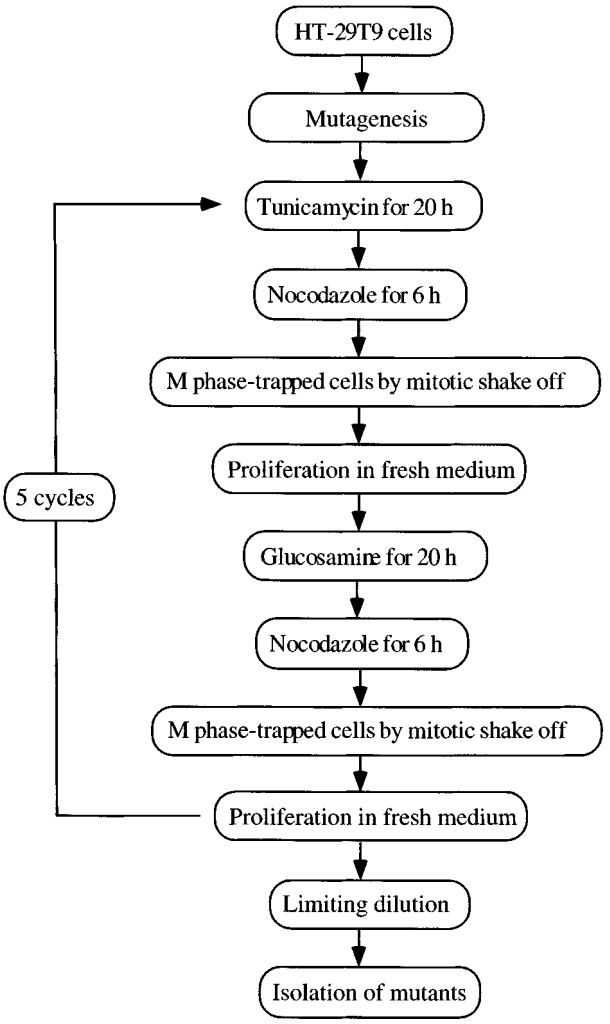

Fig. 1. The scheme of isolation of mutant cells from HT-29T9 cells. HT-29T9 cells mutagenized with EMS were treated with tunicamycin for 20 h. Following removal of tunicamycin, the cells were treated with nocodazole for 6 h. The M phase-trapped cells were collected by gentle pipetting and were allowed to grow in fresh medium. The tunicamycin-selected cells were then treated with glucosamine for 20 h, and the M phase-trapped cells were collected after the nocodazole treatment, as above. After five cycles of sequential tunicamycin and glucosamine selection, mutant clones were isolated by limiting dilution.

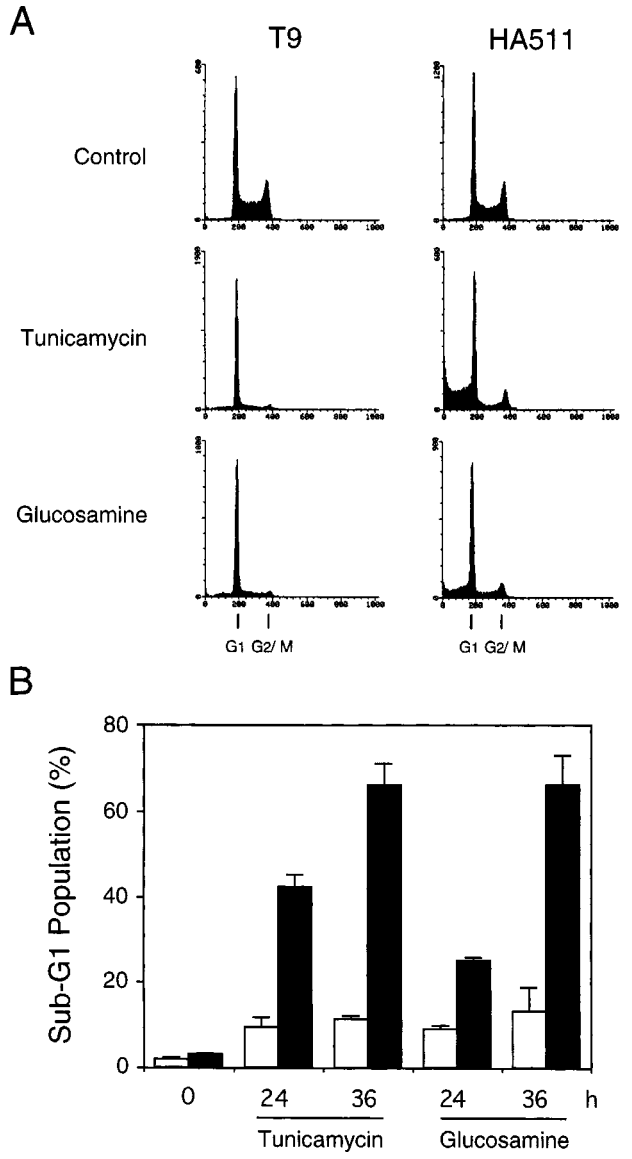

Fig. 2. Flow-cytometric analysis of cellular DNA content. HT-29T9 and HA511 cells were treated with 1 μg/ml of tunicamycin or 10 m*M* glucosamine for 24 h (A) or the indicated periods (B). The DNA contents were determined with a FACScan. The histogram is a representative result of three independent experiments (A). The sub-G1 population was quantified and expressed as a percentage of the total population (B). The data represent mean values, and bars indicate the SD of triplicate determinations. Open columns, HT-29T9; closed columns, HA511.

mycin-selected cells were then treated with 10 m*M* glucosamine for 20 h, and the M phase-trapped cells were collected after nocodazole treatment, as above. The collected cells were allowed to proliferate in fresh medium. The sequential tunicamycin and glucosamine selections were repeated for five cycles, and the cells were cloned by limiting dilution. The mutant clones were further subjected to flow-cytometric analysis (see below), and a clone, designated as HA511, was used in this study.

Flow cytometry and cell staining Flow cytometric analysis of cellular DNA content was performed as described previously.[15] In brief, cells were harvested and fixed in 70% ethanol. The fixed cells were treated with 1 mg/ml of RNase A for 1 h at 37°C and then stained with 50 μg/ml of propidium iodide. Cellular DNA content was analyzed with a FACScan (Becton Dickinson, Braintree, MA). For analysis of BrdU incorporation, cells were pulsed for 30 min with 5 μg/ml of BrdU and fixed in 70% ethanol. The fixed cells were incubated in 4 *N* HCl for 20 min and washed with neutralization solution (0.1 *M* sodium tetraborate). The cells were suspended in PBS (136.9 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4) containing 0.5% Tween 20 and incubated with an FITC-conjugated anti-BrdU antibody for 30 min at room temperature. The cells were washed twice with PBS and further stained with 50 μg/ml of propidium iodide. For determination of cell surface expression of Fas antigen, cells were incubated with 10 μg/ml of an anti-Fas antibody (clone UB-2) for 1 h on ice. Following incubation with the FITC-conjugated second antibody for 30 min on ice, expression of Fas antigen was analyzed with a FACScan. For nuclear morphological analysis, cells were harvested, fixed in PBS containing 4% formaldehyde, and stained with 1 μg/ml of DAPI. The DAPI-stained nuclei were visualized by using a fluorescence microscope.

Peptide cleavage assay Peptide cleavage assay was performed as previously described.[27] Briefly, cells were lysed in 10 m*M* HEPES-KOH (pH 7.4), 2 m*M* EDTA, 5 m*M* dithiothreitol, 0.1% CHAPS, 1 m*M* phenylmethylsulfonyl fluoride, 20 μg/ml of aprotinin. Equal amounts of proteins were incubated with 20 μ*M* DEVD-MCA or YVAD-MCA for 1 h at 37°C. Cleavage of peptide was analyzed with excitation at 380 nm and emission at 460 nm. Activity of control cells was taken as 100%.

Immunoblot analysis For immunoblot analysis, whole cell lysates were prepared as described previously.[15] In brief, cells were rinsed with ice-cold PBS and collected by scraping. For GRP78 analysis, the cell pellets were suspended in 1× SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 m*M* Tris-HCl, pH 6.8) and boiled for 5 min. To examine Bcl-2, Bcl-X_L, CPP32 and PARP, cell pellets were suspended in 50 m*M* Tris-HCl (pH 8), 120 m*M* NaCl, 2 m*M* EDTA, 0.5% NP-40, 1 m*M* phenylmethylsulfonyl fluoride and 20 μg/ml of aprotinin, and rotated for 1 h at 4°C. Insoluble materials were removed by centrifugation. Protein concentrations of the lysates were measured with a BIO-RAD protein assay kit. Equal amounts of proteins were subjected to SDS-poly-

acrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were probed using an enhanced chemiluminescence detection system (Amersham, Tokyo).

Cell survival assay Cells were treated with the stressors, as above, or anti-Fas antibody CH-11 at 100 ng/ml. After the indicated culture time, cells were harvested by trypsinization, and the number of viable cells was determined by trypan blue dye exclusion. The viable cell number at time 0 was taken as 100%.

Cellular sensitivity to anticancer agents was determined by a colony formation assay, as described previously.[8] Briefly, cells were treated with 3 μg/ml of cisplatin, 1 μg/ml of etoposide, 100 n*M* taxol, or 10 ng/ml of camptothecin for 24 h. Immediately after the incubation, cells were replated at appropriate dilution in fresh medium and cultured to form colonies. The colony number of untreated control cells was expressed as 100%.

RESULTS

High apoptotic response of mutant HA511 to chemical stressors The chemical stressors tunicamycin and glucosamine were used for establishment of the mutant HA511 from HT-29T9, a clone of HT-29 cells, by limiting dilution. Treatments of HT-29T9 cells with the chemical stressors for 24 h caused cell cycle arrest at the G1 phase (see Fig. 2A), as previously observed in the parental HT-29 cell line.[15] We initially attempted to isolate a mutant defective in G1 arrest under these chemical stress conditions (Fig. 1). To concentrate non-G1-arrested populations after the 20-h treatment with tunicamycin, we used the reversible mitotic inhibitor nocodazole and harvested M phase-trapped cells after a 6-h treatment. The tunicamycin-selected cells were allowed to grow in fresh medium and were subjected to selection with glucosamine instead of tunicamycin. After five cycles of sequential tunicamycin and glucosamine selection, HA511 was isolated by limiting dilution. Interestingly, the stressor treatments of the mutant HA511 cells led to the emergence of an additional population at less than G1 phase (Fig. 2A). The sub-G1 populations of HA511 cells reached more than 60% after 36 h under the stress conditions (Fig. 2B).

To examine apoptosis, we collected both the adherent and the non-adherent cells after the treatments with the stressors and examined the morphological changes of their nuclei with DAPI staining. The stressor-treated HA511 cells showed nuclear condensation and fragmentation (Fig. 3), characteristics of apoptosis, while most of the parental HT-29T9 cells did not show these changes. The cell size of HA511 cells was somewhat larger than that of HT-29T9 cells, although the reason for this is unclear at present.

Using DEVD-MCA, a specific fluorogenic substrate of the CPP32 (caspase-3)-like death protease, we found that the cleavage activity was induced in HA511 cells by treat-

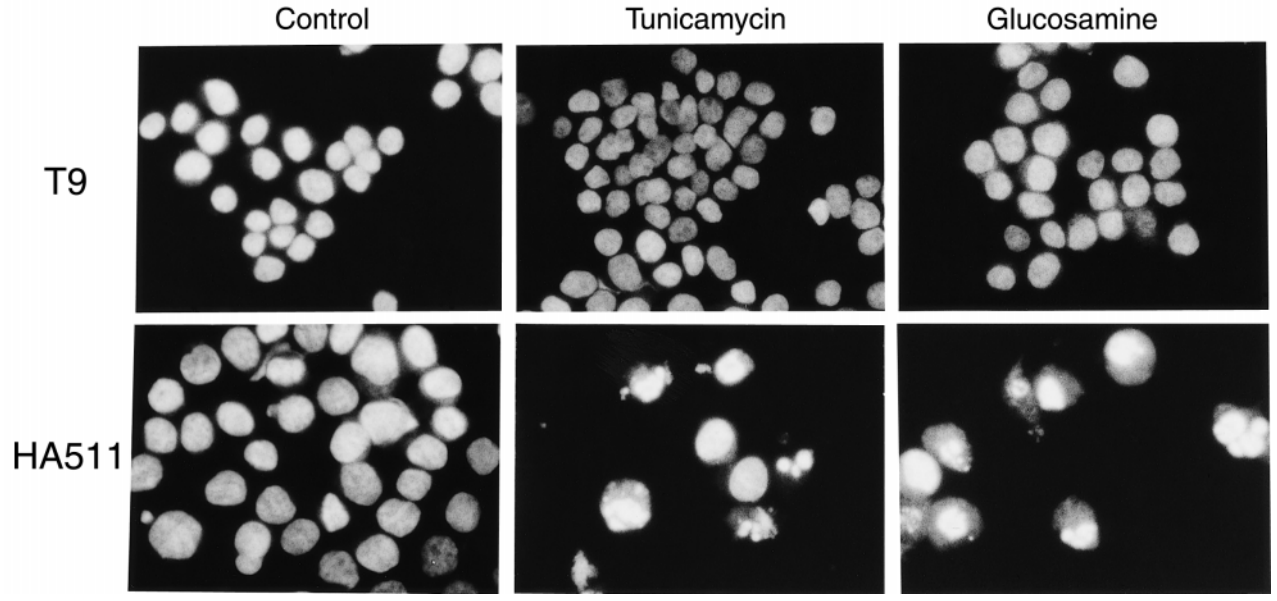

Fig. 3. Apoptotic nuclear condensation and fragmentation induced by chemical stressors. HT-29T9 (upper panels) and HA511 (lower panels) were treated with tunicamycin (1 μg/ml) or glucosamine (10 m*M*) for 48 h. The adherent and nonadherent cells were collected, fixed, and stained with DAPI (1 μg/ml). Photographs were taken at the magnification of ×200.

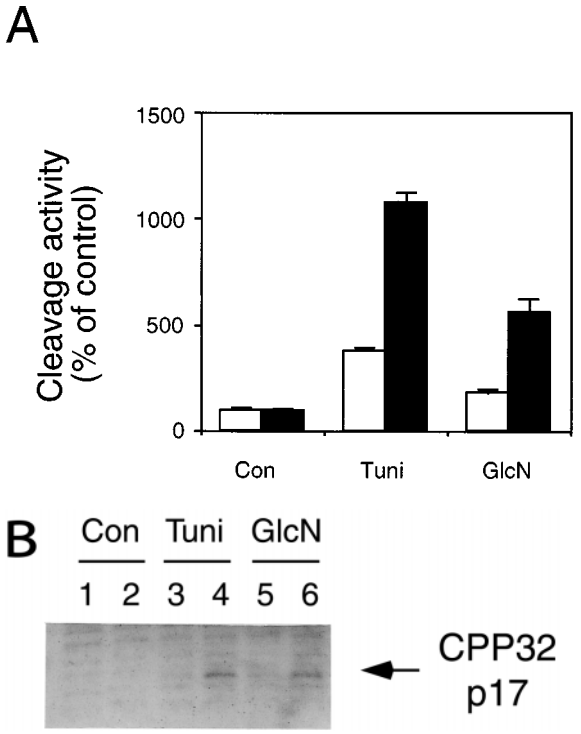

Fig. 4. Activation of CPP32 (caspase-3) in stressor-treated cells. HT-29T9 and HA511 cells were treated with 1 μg/ml of tunicamycin or 10 m*M* glucosamine for 24 h. A, proteolytic activities in the cell lysates were measured using DEVD-MCA as a substrate. The cleavage activities were expressed as a percentage of that in untreated control cell lysates. The data represent mean values, and bars indicate the SD of triplicate determinations. Open columns, HT-29T9; closed columns, HA511. B, immunoblot analysis of CPP32 was carried out for the cell lysates of HT-29T9 (lanes 1, 3, 5) and HA511 cells (lanes 2, 4, 6) treated for 24 h without (lanes 1 and 2), or with 1 μg/ml of tunicamycin (lanes 3 and 4) or 10 m*M* glucosamine (lanes 5 and 6). Con, control; Tuni, tunicamycin; GlcN, glucosamine. p17, active fragment of CPP32.

ments with the stressors for 24 h, compared with the parental HT-29T9 cells (Fig. 4A). Immunoblot analysis of CPP32 showed a 17-kDa fragment, indicating that CPP32 was activated in the stressor-treated HA511 cells (Fig. 4B). In addition, Z-EVD-CH_2DCB, an inhibitor of CPP32,[28] reduced the development of the sub-G1 population resulting from tunicamycin treatment (data not shown). These results collectively indicated that upon exposure to chemical stressors, the mutant HA511 cells underwent apoptosis through the activation of CPP32 more readily than did HT-29T9 cells.

Sequential response of G1 arrest and apoptosis in HA511 cells under stress conditions Fig. 5A shows the survival of HA511 and HT-29T9 cells depending on the exposure time to the stressors, as determined by trypan blue dye exclusion. At 16 h after the addition of stressors, most HA511 cells were viable, although the number of cells showed no increase. Thereafter, HA511 cells began to lose their viability. During the same exposure, HT-29T9 cells showed a high viability and proliferated at a slower rate, compared with untreated cells; the doubling times of the tunicamycin- and the glucosamine-treated cells were 34 and 47 h, respectively, and that of untreated cells was 18 h.

We next performed a double staining with propidium iodide (for DNA content) and BrdU (for DNA synthesis). After a 16-h treatment with tunicamycin, approximately 70% of HT-29T9 cells were in the G1 phase and only 12% of the total cell population incorporated BrdU (Fig. 5B). The BrdU-incoporating population increased to approximately 30% after 24 and 36 h, indicating that cell cycle progression had occurred from G1 to S phase and that the G1 arrest under stress was leaky in HT-29T9 cells. The mutant HA511 cells also showed G1 arrest of the cell cycle at 16 h, but subsequent cell cycle progression to S phase hardly occurred (Fig. 5B). Instead, the apoptotic fraction of sub-G1 (17% at 16 h) increased and reached 66% at 36 h. Similar results were obtained with glucosamine (data not shown). These results indicated that the apoptotic response occurred after the G1 arrest in HA511 cells under stress, while in the parent HT-29T9 cells, G1-S progression occurred in part without substantial cell death.

High apoptotic response of HA511 to hypoxia and glucose deprivation Hypoxia and glucose deprivation are physiological stress conditions that commonly exist in solid tumors and that lead to GRP induction, like treatment of cells with tunicamycin and glucosamine (see Fig. 9A).[1, 3, 4] HA511 cells were hypersensitive to hypoxia, compared with parental HT-29T9 cells (Fig. 6A). The hypoxia-treated HA511 cells showed apoptotic features, including fragmentation of nuclei, emergence of a sub-G1 population (data not shown), formation of an active 17-kDa fragment of CPP32 and formation of a cleaved product of PARP that is a representative substrate protein of CPP32 (Fig. 6B). These results indicated that HA511 cells showed a hyperapoptotic response to hypoxia as well as to the chemical stress conditions. HA511 cells also showed a hyperapoptotic response to glucose deprivation, as determined by the emergence of a sub-G1 population (Fig. 6C) and the DAPI staining of the nuclei (data not shown).

Sensitivity of HA511 cells to other apoptosis-inducing stimuli Solid tumor cells, including HT-29 cells,[29] show resistance to some apoptosis-inducing stimuli; therefore, it was of interest to examine the sensitivity of HA511 to other stimuli. Upon exposure to an agonistic anti-Fas anti-

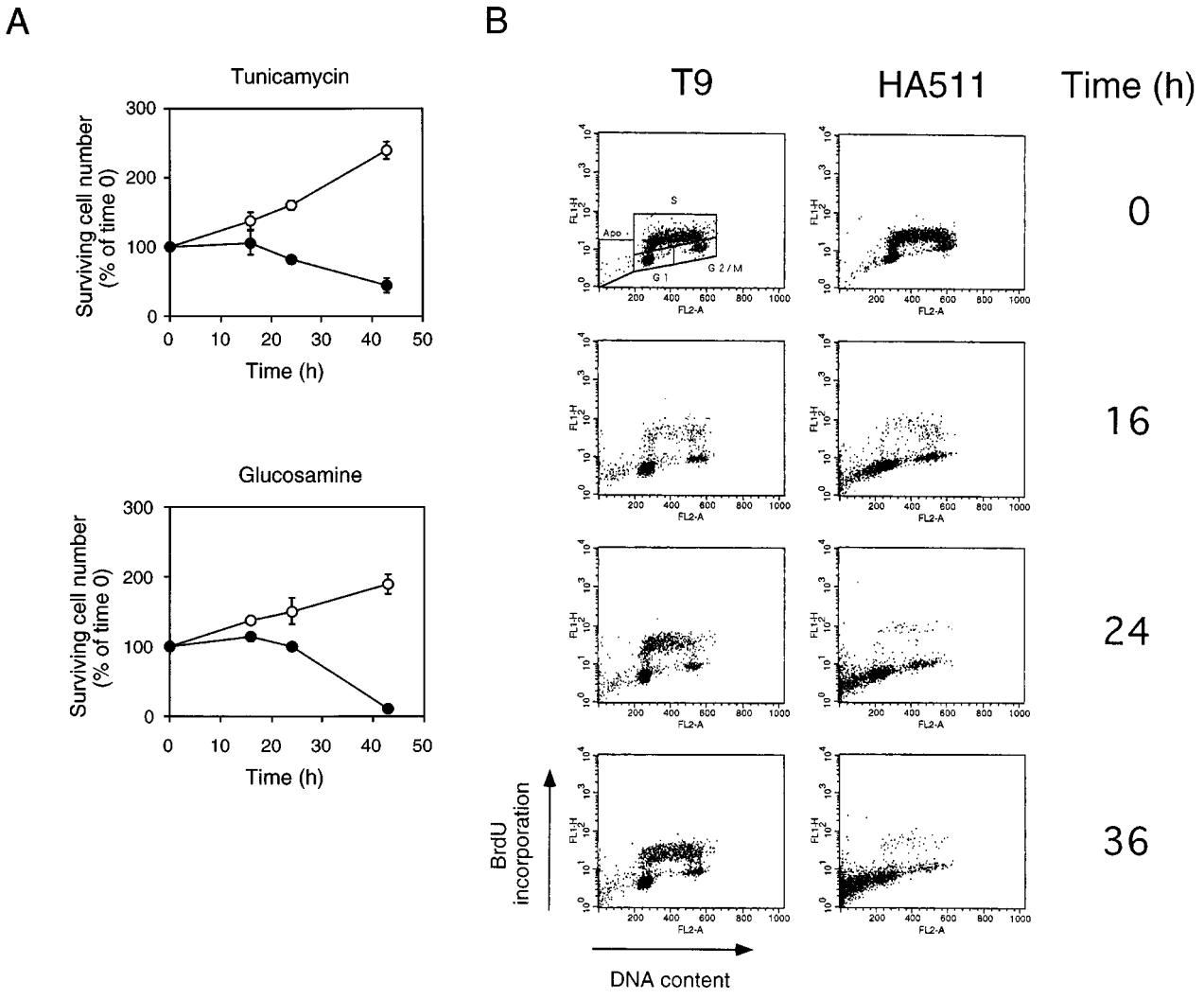

Fig. 5. Induction of apoptosis after G1 phase arrest in the cell cycle. A, HT-29T9 (open circles) and HA511 (closed circles) cells were treated with 1 μg/ml of tunicamycin or 10 m*M* glucosamine for the indicated periods. Surviving cells were determined by trypan blue dye exclusion and expressed as a percentage of the initial cell number. The data represent mean values, and bars indicate the SD of triplicate determinations. B, the tunicamycin-treated cells were pulsed for 30 min with 5 μg/ml of BrdU. The cells were double-stained with an FITC-conjugated anti-BrdU antibody and propidium iodide and analyzed by flow cytometry. FL-1H and FL2-A represent the amounts of BrdU incorporation into DNA and the DNA content in cells, respectively.

body, almost all HA511 cells died within 42 h, while the parental HT-29T9 cells proliferated without substantial cell death (Fig. 7A). The Fas-mediated apoptosis of HA511 cells was further confirmed by the emergence of a sub-G1 population, the nuclear fragmentation and the activation of CPP32 (Fig. 7B and data not shown). The expression of Fas antigen on the cell surface was somewhat increased in HA511 cells, compared to that in HT-29T9 cells (Fig. 7C).

We next examined the sensitivity of HA511 cells to antitumor drugs (Fig. 8). HA511 cells were preferentially hypersensitive to cisplatin. To a lesser extent, HA511 cells showed a hypersensitivity to etoposide. The difference in sensitivity to camptothecin and taxol was marginal between HT-29T9 and HA511 cells.

Expression of stress- and apoptosis-related proteins in HA511 cells GRP78 plays an important role in protecting cells from glucose-regulated stress conditions, and the

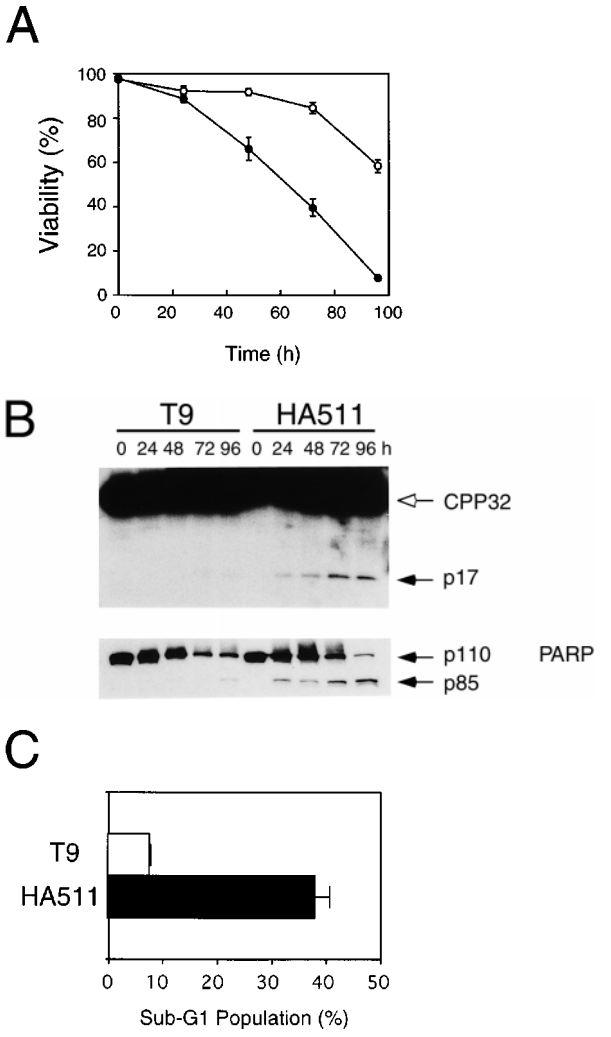

Fig. 6. Induction of apoptosis in HA511 cells under the physiological stress conditions of hypoxia (A and B) and glucose deprivation (C). A, HT-29T9 (open circles) and HA511 (closed circles) cells were exposed to hypoxia for the indicated periods. At least 400 cells were counted and the percentage of viable cells was determined by trypan blue dye exclusion. The data represent mean values, and bars indicate the SD of triplicate determinations. B, immunoblot analyses of CPP32 and PARP were performed for the cell lysates of HT-29T9 and HA511 cells exposed to hypoxia for the indicated periods. p17, active fragment of CPP32; p85, cleaved product of PARP. C, HT-29T9 and HA511 cells were cultured for 24 h under the glucose-free condition, and the sub-G1 population was determined by flow cytometry, as described in Fig. 2.

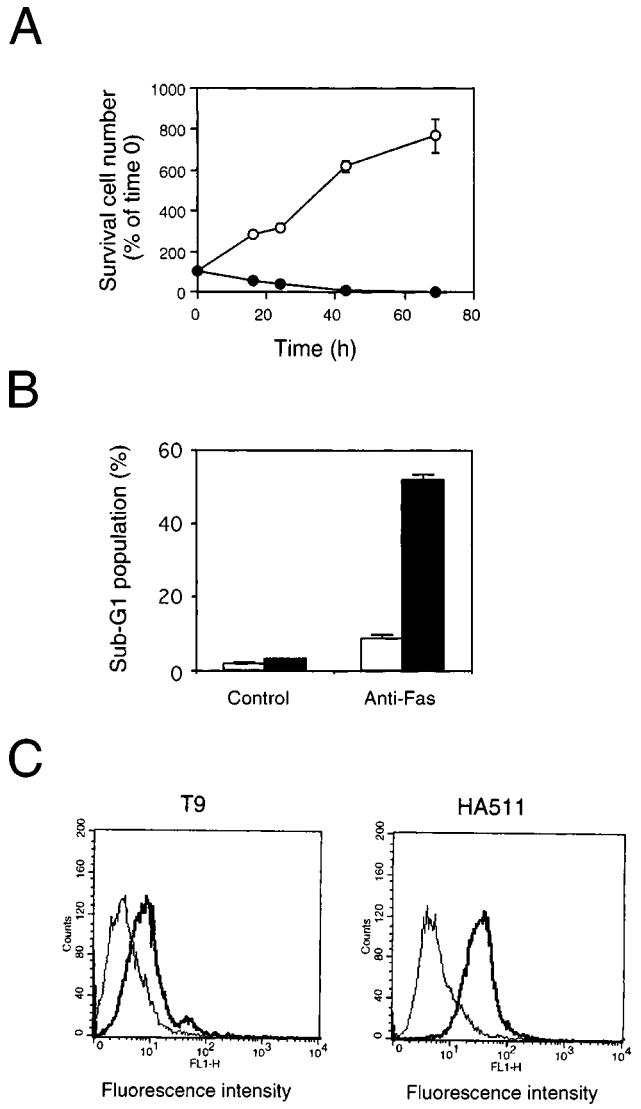

Fig. 7. Induction of Fas-mediated apoptosis in HA511 cells. A, HT-29T9 (open circles) and HA511 (closed circles) cells were treated with 100 ng/ml of death-inducing anti-Fas antibody clone CH-11 (A and B) for the indicated periods. Surviving cells were determined by trypan blue dye exclusion, as described in Fig. 5. B, at 24 h after the anti-Fas antibody treatment, the sub-G1 population was determined by flow cytometry, as described in Fig. 2. Open columns, HT-29T9; closed columns, HA511. C, cell surface expression of Fas antigen was examined with an anti-Fas antibody UB-2. Regular line, control; bold line, Fas expression.

inducibility of GRP78 determines the cellular sensitivity to chemical stressors.[30] However, both the basal and the induced levels of GRP78 expression in HA511 cells were essentially the same as those in HT-29T9 cells (Fig. 9A).

We also examined the expression of apoptosis-regulating proteins. Bcl-X_L was expressed at essentially the same levels in HT-29T9 and HA511 cells (Fig. 9B). The expression of Bcl-2 in HA511 cells was somewhat lower

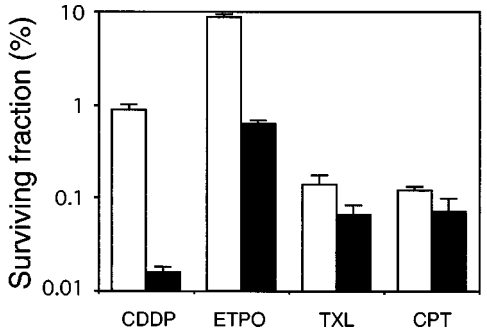

Fig. 8. Hypersensitivity of HA511 cells to cisplatin. HT-29T9 and HA511 cells were treated for 24 h with 3 μg/ml of cisplatin (CDDP), 1 μg/ml of etoposide (ETPO), 100 n*M* taxol (TXL), or 10 ng/ml of camptothecin (CPT). The cellular sensitivities were determined by colony formation assay. The colony number of untreated control cells was taken as 100%. The data represent mean values, and bars indicate the SD of triplicate determinations. Open columns, HT-29T9; closed columns, HA511.

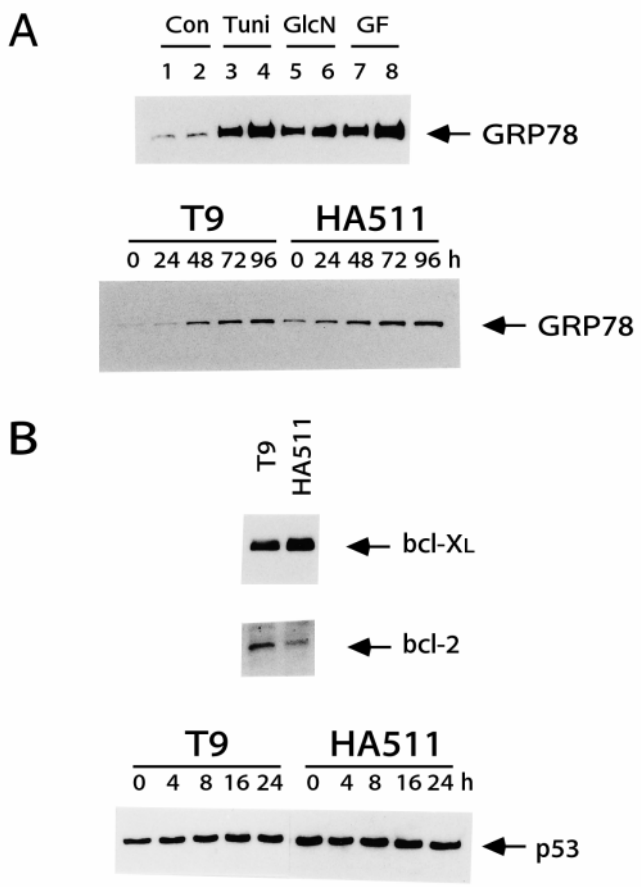

Fig. 9. Immunoblot analysis of GRP78, Bcl-XL, Bcl-2 and p53. A, *upper panel*, HT-29T9 (lanes 1, 3, 5, 7) and HA511 cells (2, 4, 6, 8) were treated for 24 h without (lanes 1 and 2) or with 1 μg/ml of tunicamycin (lanes 3 and 4), 10 m*M* glucosamine (lanes 5 and 6), or under a glucose-free condition (lanes 7 and 8). Con, control; Tuni, tunicamycin; GlcN, glucosamine; GF, glucose-free condition. *Lower panel*, HT-29T9 and HA511 cells were exposed to hypoxia for the indicated periods. The cell lysates were prepared and subjected to immunoblot analysis using a rat polyclonal anti-GRP78 antibody. B, *upper panel*, the lysates of untreated HT-29T9 (lane 1) and HA511 cells (lane 2) were prepared and subjected to immunoblot analysis using anti-Bcl-X_L and anti-Bcl-2 antibodies, respectively. *Lower panel*, HT-29T9 and HA511 cells were treated with 10 m*M* glucosamine for the indicated periods. Immunoblot analysis was performed with an anti-p53 antibody.

than that in HT-29T9 cells (Fig. 9B). However, overexpression of Bcl-2 by cDNA transfection did not confer resistance to tunicamycin, glucosamine and the anti-Fas antibody in HA511 cells (data not shown). HT-29T9 and HA511 cells had similar basal levels of *p53*, the gene that was mutated in HT-29 cells,[31] and the expression levels of *p53* were relatively constant during exposure to glucosamine (Fig. 9B).

DISCUSSION

In this study, we established the mutant HA511, which shows a high apoptotic response to GRP-inducing stress conditions, including hypoxia, glucose deprivation, and treatments with tunicamycin and glucosamine. The success in establishing the HA511 mutant was somewhat surprising because the selection process consisted of intermittent, 20-h exposures to tunicamycin and glucosamine (Fig. 1). However, we have obtained several independent clones that were sensitive to stress-induced apoptosis (data not shown), suggesting that such mutants as HA511 had a selective advantage in our system. Under the stress conditions, HA511 cells underwent apoptosis after a prolonged G1 cell cycle arrest (Fig. 5). This observation raises the possibility that the hypersensitivity of the mutant to stress-induced apoptosis is associated with cell cycle regulation at the G1 phase. In addition, close examination of the data presented in Figs. 2A and 5B revealed that HA511 cells had greater G2/M phase populations than HT-29T9 cells did after 24 h of stressor treatment. Therefore, in our selection system, nocodazole might concentrate such apoptosis-sensitive mutants as HA511 by arresting the cells in the M phase and protecting the cells from apoptosis that occurred after the G1 arrest in the next cell cycle.

HA511 cells were hypersensitive to Fas-mediated apoptosis and expressed somewhat higher levels of Fas antigen at the cell surface than did HT-29T9 cells (Fig. 7). These results suggested that the hypersensitivity of

HA511 cells to Fas-mediated apoptosis can be explained, at least in part, by the increased expression of Fas antigen. However, previous studies have shown that sensitivity to Fas-mediated apoptosis does not always correlate with the expression of Fas antigen on cancer cell lines.[32, 33] In agreement with the previous observations, HT-29T9 cells were essentially insensitive to Fas-mediated apoptosis, although they expressed Fas antigen. Therefore, it is possible that other factors than the increased expression of Fas antigen are involved in the hypersensitivity to Fas-mediated apoptosis in HA511 cells.

Antitumor drug resistance of solid tumors has been thought to be associated with diminished apoptotic response to stress conditions.[12] Thus, it was of interest to examine the drug sensitivity of HA511 cells. We found that HA511 cells were hypersensitive to cisplatin (Fig. 8). This observation suggests that the pathway of the apoptotic response to GRP-inducing stress conditions overlaps with that of cisplatin-induced cell death. In this regard, we have recently found that co-treating with a chemical stressor and cisplatin synergistically induced cell death in HT-29 cells (manuscript in preparation) while the same stressors induced resistance to other antitumor agents.[7, 8] Therefore, even in the apoptosis-resistant HT-29 cells, the pathway of the apoptotic response to stress conditions might be activated when the cells were treated with cisplatin under stress.

The mechanism of increased apoptotic response in HA511 cells remains to be determined. In this study, we found no alteration in the basal expression or the inducibility of GRP78, which is involved in a defense mechanism of cell survival under stress conditions.[34, 35] We also found no alteration in the expression level of Bcl-X_L, a representative anti-apoptotic protein.[22, 36] In addition, overexpression of Bcl-2 in this mutant did not confer resistance to the stressors. Wild-type *p53* has been shown to be involved in hypoxia-induced apoptosis; hypoxia induces the expression of the wild-type *p53*, resulting in *p53*-dependent apoptosis in mouse embryonic fibroblasts transformed oncogenetically.[12] However, both parental HT-29T9 and mutant HA511 cells showed a high level of basal *p53* expression, which is characteristic of the mutant type.[18] Further, *p53* expression was not induced by the stressor glucosamine in either of the cell lines (Fig. 9B). These observations suggest that the hyperapoptotic response of HA511 cells is *p53*-independent.

In conclusion, we have established the mutant HA511, which is hypersensitive to apoptosis induced by hypoxia and glucose deprivation. The stress conditions in solid tumors may represent a selective pressure for tumor cells with diminished or lost apoptotic potential.[12] In agreement with this notion, most solid tumor-derived cell lines show resistance to stress-induced apoptosis. Because of this resistance, it has been difficult to examine the mechanisms of apoptosis in solid tumor cells. The HA511 mutant should be a valuable model to study the mechanism of apoptotic response of solid tumors to physiological stress conditions.

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