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Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME

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ABSTRACT **Stress protein GRP78/BiP is highly induced in progressively growing tumors and has recently been shown to exert a protective role against lysis by cytotoxic T cells and tumor necrosis factor *in vitro*. This raises the question whether the *in vitro* observed protective function of GRP78/BiP translates into the *in vivo* situation in which tumors grow progressively, killing the host. Herein we report that molecular inhibition of GRP78/BiP induction in the fibrosarcoma B/C10ME, while not affecting *in vitro* cell proliferation, causes a dramatic increase in apoptotic cell death upon Ca^{2+} depletion of the endoplasmic reticulum. When B/C10ME cells incapable of inducing GRP78/BiP are injected into mice, tumors are initially formed that, however, regress presumably due to a cytotoxic T-cell response demonstrable by a strong *in vitro* response to the tumor with spleen cells of regressor mice. Since sensitivity to apoptosis is key to tumor rejection, these results may point to new approaches to the therapy of cancer via regulation of stress protein GRP78/BiP.**

The mammalian stress response represents a defense mechanism that allows cells to respond to adverse conditions threatening their survival. These conditions may include systemic viral infections, local tissue inflammation, and deregulated growth of cancer cells. Cells respond to these conditions by inducing synthesis of a small number of evolutionarily conserved proteins. Examples of these stress inducible proteins are the heat shock proteins (1, 2) and the glucose-regulated proteins (GRPs) (3). Of the latter, the most abundant is a 78-kDa protein, GRP78, first detected in rapidly expanding cultures of Rous sarcoma virus-transformed avian fibroblasts (4). GRP78, also known as the immunoglobulin heavy chain binding protein BiP, has been shown to be a molecular chaperone and Ca^{2+} binding protein (5, 6). It is expressed in many cell types and localized in the endoplasmic reticulum (ER). Strikingly, the transcription of GRP78 is highly induced in response to cellular stress. Potent inducers of *grp78* transcription include glucose starvation, oxygen deprivation, and treatment with thapsigargin (Tg), which depletes the ER Ca^{2+} store (7, 8). Therefore, in addition to its housekeeping functions such as protein folding, GRP78 could also serve a protective role in physiological stress conditions, particularly in conditions associated with progressively growing tumors. In support, increased levels of GRP78 expression have been observed in chemically and radiation-transformed murine embryonic cells (9) and in fibrosarcomas in which the level of GRP78 expression correlates with tumor growth (10, 11).

The observation that GRP78 is induced in progressively growing neoplastic cells raises the question as to how this protein could exert its protective function. GRP78 is a Ca^{2+} binding protein and a molecular chaperone that could regulate apoptosis by virtue of its ability to control processing and release of regulatory substances. It could also be involved in the maintenance of Ca^{2+} homeostasis, which is crucial for proper cell function. Inhibition of GRP78 induction by an antisense expression vector in CHO cells has been shown to cause decreased cell survival after treatment with Ca^{2+} ionophore A23187 (12) and elevated cell death during chronic hypoxia (13). Moreover, B/C10ME fibrosarcoma cells treated with GRP inducers express increased resistance to cell-mediated lysis by cytotoxic T lymphocytes (CTLs) and tumor necrosis factor (TNF) (14, 15). Resistance is readily reversible when inducers are removed and cells are recultured in normal medium (15), resulting in GRP78 rephosphorylation, which is nonfunctional (16). Proof that induction of GRP78 causes resistance was sought by establishing a panel of B/C10ME clonal cell lines stably transfected with amplified copies of the *grp78* antisense vector (12, 15). Analysis of this panel of stable transfectants revealed a striking correlation between the ability to induce GRP78 protein and the ability to mount stress-induced resistance to CTL-mediated and TNF-mediated lysis (15). The observation that GRP78 plays a role in protecting tumor cells against CTL-mediated cytotoxicity and the toxic effects of TNF *in vitro* suggests that the induction of GRP78 may protect tumor cells from immune attack *in vivo*. Herein we report that in B/C10ME cells, suppression of GRP78 induction does not affect its *in vitro* growth rate yet causes a dramatic increase in apoptotic cell death upon calcium depletion in the ER. When B/C10ME cells not able to induce GRP78 were injected into mice, they were found to be unable to form tumors. Therefore, suppression of GRP78 induction in tumor cells may provide a new approach to the therapy of cancer.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions. The tumor cell line B/C10ME was grown in Dulbecco's modified Eagle's medium (DMEM) (glucose at 4.5 mg/ml) supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% penicillin–streptomycin–neomycin antibiotics. B/C10ME 78WO clone 9 cells were maintained under the selection of 5 μM methotrexate. In all comparative experiments, both cell lines were grown in non-selective DMEM at 37°C in a humidified 5% CO_2/95% air atmosphere. Cells were stressed by adding 7 μM A23187, 5 mM L-azetidine-2-carboxylic acid, 10 mM 2-deoxyglucose, and tunicamycin (1.5 μg/ml).

Protein Gel Electrophoresis and Immunoblots. Whole cell lysates were made by resuspending and incubating cells in RIPA buffer (10 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/1 mM phenylmethylsulfonyl fluoride/1 mM aprotinin/1 mM benzamidine/1 mM leupeptin/1 mM pepstatin A). Protein concentrations were determined by bicinchoninic acid (BCA) assay. Total cell lysate (100–200 μg) was separated on a 8.5% denaturing SDS/PAGE gel and electrophoretically transblot-

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Abbreviations: TNF, tumor necrosis factor; ER, endoplasmic reticulum; Tg, thapsigargin; CTL, cytotoxic T lymphocyte.

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ted onto a Hyperbond membrane (Amersham). Immunoblot analysis was performed by blocking the membrane in 2.5% nonfat dry milk and subsequently incubating with rabbit polyclonal anti-GRP78 (a gift of Robert Pedersen, State University of New York, Buffalo), rat monoclonal anti-GRP94, or mouse monoclonal anti-HSP70 (StressGen Biotechnologies, Sidney, Canada) at dilutions of 1:10,000, 1:1000, and 1:1000, respectively. The secondary antibodies used were goat anti-rabbit, rabbit anti-rat, and goat anti-mouse (all from Cappel), respectively, conjugated to horseradish peroxidase and diluted 1:3000.

Growth Rate Measurement. The measurement of the growth rate of B/C10ME and clone 9 cells was accomplished by seeding 10^4 viable cells into 10-cm dishes in duplicates. Every 24 hr for 6 days, cells were trypsinized and cell numbers were determined by microscopic counting with trypan blue.

Cell Survival Analysis. The cell survival assay was performed as described (17). Briefly, 2×10^3 viable cells were seeded on 100-mm diameter dishes. After 24 hr, the cells were treated with the indicated stress inducer for 16 hr. The cells were then washed twice with phosphate-buffered saline (PBS), and fresh medium was added. Cells were allowed to grow 7–14 days until colonies formed. Colonies were counted after fixing in 100% methanol and staining with the Giemsa stain solution (Fisher Scientific). Two independent experiments were performed, each in duplicate.

DNA Fragmentation Measurements. Fragmentation of cellular DNA was measured essentially as described (18). Approximately 4×10^4 viable B/C10ME or clone 9 cells were seeded in duplicate in flat-bottom 24-well cell culture plates (Corning). After a 20-hr incubation with [^{3}H]thymidine at 20 μCi/ml (1 mCi/ml; 1 Ci = 37 GBq; Dupont/NEN), cells were washed four times with PBS and subjected to various concentrations of Tg added to 500 μl of medium per well for 16 hr. Cells were then washed with PBS and incubated with 1 ml of hypotonic lysing buffer (10 mM Tris/1 mM EDTA/0.5% Triton X-100) for 30 min at 37°C. After lysis, 400 μl of the solution was centrifuged at 14,000 rpm for 20 min to separate intact from fragmented DNA. Supernatant (200 μl) was emulsified in scintillation fluid (Readysolv, Beckman) and counted on a Packard liquid scintillation analyzer. Maximum cpm was determined by counting 100 μl of the cell lysate without prior centrifugation. Fragmented DNA was calculated by using the formula 100% × [cpm (experimental) − cpm (spontaneous)]/[cpm (maximum) − cpm (spontaneous)].

Tumor Formation. Confluent cultures of B/C10ME were harvested with trypsin/EDTA (GIBCO/BRL) and washed with PBS. From 2 to 3.5×10^7 viable cells were resuspended in 200 μl of PBS. Six- to 8-week-old BALB/c mice obtained from The Jackson Laboratory were subcutaneously injected with an 18-gauge needle in their right flank. The tumors that formed were palpable within a week of inoculation and biperpendicular measurements were taken of the progressively growing tumor two to three times weekly until it became approximately 4 cm in size. Tumors were determined to have regressed after losing both measurability and palpability.

Cell-Mediated Cytotoxicity Assay. In 20 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids, 2×10^7 BALB/c spleen cells from mice were stimulated with 10^5 10,000-rad-irradiated B/C10ME cells (1 rad = 0.01 Gy). Cells were harvested for use in cytotoxicity assays after 5 days. Exponentially growing target cells were labeled with $Na_2{}^{51}CrO_4$ for 60 min at 37°C in RPMI then washed three times in PBS (15). Labeled targets (10^4 cells per well) were incubated in a volume of 200 μl with effector cells in RPMI 1640 medium in 96-well round-bottom microtiter plates. After 4 hr of incubation, and 5 min of centrifugation, 100 μl of the supernatant was harvested and radioactivity was measured on a Wallac 1272 CliniGamma (Gaithersburg, MD). The percentage of cytotoxicity was calculated as the percentage of releasable counts after subtraction of spontaneous release.

RESULTS AND DISCUSSION

Specific Suppression of GRP78 Protein During Stress Induction in B/C10ME Clone 9 Cells. We have established (15) a panel of B/C10ME clonal cell lines stably transfected with amplified copies of the *grp78* antisense vector pRSV-78WO. These clones can be grouped into sets based on their ability to induce GRP78 and we observed a correlation between GRP78 stress induction and cell-mediated cytotoxicity. Clone 9 is a representative of those clones with reduced GRP78 stress induction and increased sensitivity to CTL and TNF killing. The protein synthesis profile of this clone had been analyzed by pulse labeling with [^{35}S]methionine and found to be similar to that of control cells with the exception that stress-induced synthesis of nascent GRP78 was impaired (15). To further elucidate the steady-state levels of GRPs in clone 9, protein extracts were prepared from parental B/C10ME and clone 9 cells grown under normal culture conditions, and after treatment with A23187, tunicamycin, and 2-deoxyglucose. The levels of GRP78 and GRP94, a 94-kDa GRP that tends to be coordinately regulated with GRP78 (7), were probed by Western blot with an antisera specific for the respective stress proteins. Compared with B/C10ME, clone 9 cells expressed a slightly lower basal level of GRP78 and the induction of GRP78 by all three stress inducers tested was impaired (Fig. 1). In contrast, the basal level of GRP94 was moderately elevated when compared with the parental cell and maintained at high levels under stress conditions, suggesting that the cells may accumulate more GRP94 to compensate for the antisense to GRP78. Because previously induction of heat shock protein HSP70 had been reported to correlate with protection against CTL-induced target cell lysis (19, 20) and that HSP70 and GRP78 show partial amino acid identity (5), we determined whether the *grp78* antisense vector affects basal and/or induced levels of HSP70 in clone 9 cells. Fig. 1 shows that HSP70 is readily induced in parental as well as clone 9 cells upon treatment with azetidine. Thus, the *grp78* antisense vector suppresses GRP78 but not HSP70 induction.

***In Vitro* Growth of B/C10ME Clone 9 Cells Is Unchanged but Calcium Depletion Causes Increased Cell Death by Apoptosis.** Growth rates of clone 9 cells when compared with those of parental B/C10ME cells were indistinguishable. Seeding efficiencies of B/C10ME and clone 9 cells were found to be

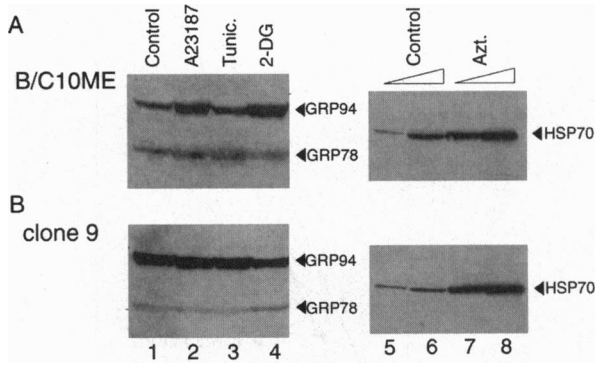

FIG. 1. Suppression of GRP78 stress induction in clone 9 cells. Western blots of protein extracts prepared from parental B/C10ME (*A*) and clone 9 (*B*) cells cultured under normal growth conditions (control) or stressed with the various inducers (A23187, tunicamycin, 2-deoxyglucose, and azetidine) as indicated. For lanes 1–4, 200 μg of protein extract was applied. For lanes 5 and 6 and lanes 7 and 8, 100 and 200 μg of protein from control and azetidine-treated cells were applied, respectively. The protein blots were probed with antisera against GRP94, GRP78, and HSP70.

close to 100% and 85%, respectively. During the first 2 days of culture, the cell number of clone 9 cells lags somewhat behind that of B/C10ME but in subsequent days growth rates are essentially the same (Fig. 2). Virtually identical results were obtained with clone 9 cells cultured in the presence or absence of methotrexate (data not shown).

Because Ca^{2+} homeostasis plays an important role in cell function and viability, clone 9 cells were tested for ability to cope with Ca^{2+} depletion from the ER. To deplete ER Ca^{2+}, the sesquiterpene lactone Tg was used. In contrast to A23187 whose effect is pleiotropic, Tg specifically inhibits Ca^{2+}-ATPase in the ER, causing a loss of Ca^{2+} from the ER but an increase of Ca^{2+} in the cytoplasm (21). Among the known inducers of GRP78, Tg is comparable in its potency to A23187 but much less toxic (8). Because the induction of GRP78 by Tg is suppressed in clone 9 cells (data not shown), we examined cell survival after a brief exposure to this drug. Results in Fig. 3 show that clone 9 cells are about 5 times more sensitive to Tg-induced cell death than parental B/C10ME cells.

To determine whether Tg-induced cell death in clone 9 is due to programmed cell death, the extent DNA fragmentation was assayed. Cells were collected, and genomic DNA was extracted and analyzed by agarose gel electrophoresis. Higher levels of DNA fragmentation were observed in clone 9 compared with parental B/C10ME cells (data not shown). To quantitate this difference, cells were metabolically labeled with [^{3}H]thymidine prior to Tg treatment and the release of DNA fragments from cells into the culture medium was assayed. Fig. 4 shows that clone 9 cells release 7–10 times higher amounts of labeled DNA fragments than B/C10ME cells under identical drug concentrations. These results demonstrate that the parental cells are much more resistant to Tg-induced apoptosis than clone 9 cells.

Tumor Progression *in Vivo* Is Impaired in B/C10ME Clone 9 Cells. Given the above observation that the rate of cell growth is not changed in clone 9 cells yet the sensitivity to apoptosis induction by Tg is significantly increased, the question arises whether these *in vitro* effects have *in vivo* consequences on tumor growth. It is now well established that target cell lysis by CTL progresses through apoptosis and is associated with irreversible Ca^{2+} increase in targets (22). Consequently, maintenance of Ca^{2+} homeostasis could amount to a protective effect in cells attacked by the cell-mediated immune system. Thus, B/C10ME and clone 9 cells were injected subcutaneously into syngeneic BALB/c mice to assess tumor establishment and progression. Results from four experiments are summarized in Table 1 and examples of tumors growing in individual mice are shown in Fig. 5. At a dose of 2 to 3.5 $\times$ 10^7 B/C10ME cells per mouse, tumors grew within 2 weeks in all

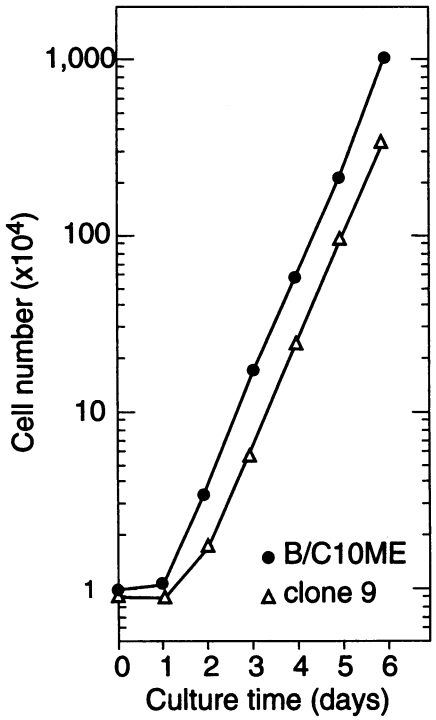

FIG. 2. *In vitro* growth rates of B/C10ME and clone 9 cells. Equal numbers of viable cells from each line were seeded and their growth rates were followed for 6 days in culture by microscopic counting.

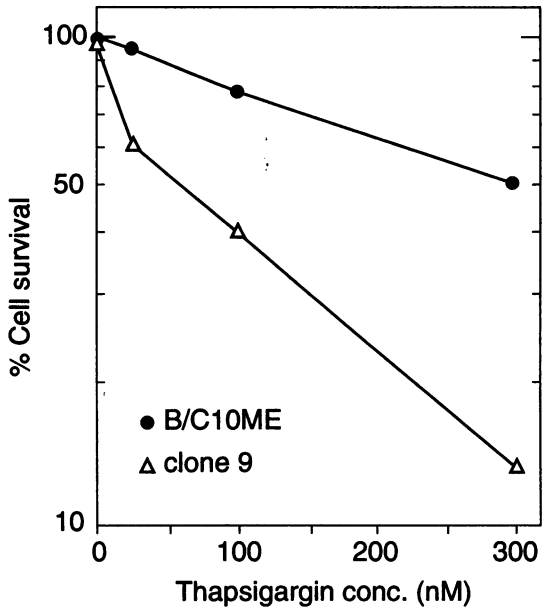

FIG. 3. Cell survival after ER calcium depletion with Tg. B/C10ME and clone 9 cells were treated with various concentrations of Tg for 16 hr. Cell survival was determined by a colony-forming assay 7–14 days after the stress treatment.

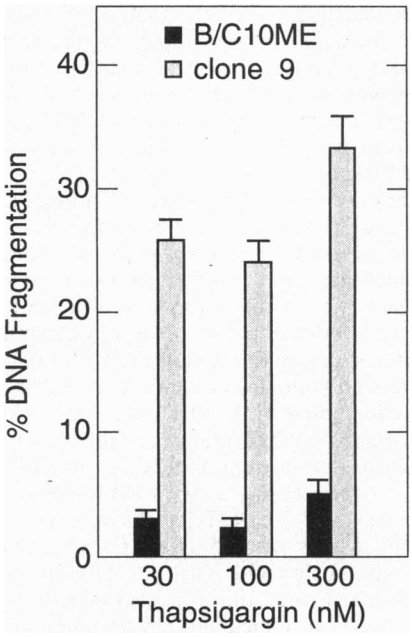

FIG. 4. Increased apoptosis is induced by calcium depletion in the ER in clone 9 cells. Equal numbers of B/C10ME and clone 9 cells were seeded into 96-well plates and labeled with [^{3}H]thymidine prior to treatment with the indicated concentration of Tg for 16 hr. Cells were then lysed and the percent of small DNA fragments was assayed.

Table 1. *In vivo* tumor incidence and regression

Exp.	Cells injected: No. ($\times 10^{-7}$)	Cells injected: Name	Tumor growth, no. grew/ total no.	Tumor regression, no. regressed/ total no.	Tumor progression, no. progressed/ total no.
1	2	B/C10ME	5/5	0/5	5/5
2	2	B/C10ME	5/5	0/5	5/5
3	3.5	B/C10ME	5/5	0/5	5/5
4	3.5	B/C10ME	2/2	0/2	2/2
			17/17	0/17	17/17
1	2	Clone 9	1/5	0/5	1/5
2	2	Clone 9	1/5	1/5	0/5
3	3.5	Clone 9	3/5	3/5	0/5
4	3.5	Clone 9	4/4	4/4	0/4
			9/19	8/19	1/19

mice. Tumors grew progressively and ultimately lead to death of the animal, requiring termination of the experiment by about 30 days. Quite a different result was seen in animals injected with equivalent doses of clone 9 cells. Only a proportion of animals (i.e., 9 out of 19 mice) developed tumors, demonstrating that tumor establishment is significantly impaired with clone 9 cells. Moreover, and most significantly, only a minority of clone 9 tumors that initially grew continue to do so. In fact, 8 out of the 9 tumors that grew during the first 2 weeks regressed within 2–3 days of reaching a diameter of 0.5–1.5 cm (Fig. 5), and in only one animal did the tumor continue to grow progressively. These results indicate that clone 9 cells are impaired in their ability to establish initial growth and, even after their tumor has reached a palpable size, clone 9 cells continue to be subject to regression processes.

B/C10ME Clone 9 Tumors Induce Strong Memory of Cytotoxic T Cells *in Vivo*. The above result that clone 9 cells tend to regress after initial growth is of particular interest because it may point to a rejection mechanism operative specifically against tumor cells in which induction of GRP78 is inhibited. To examine this possibility, *ex vivo* spleen cells from regressor and progressor mice were tested for cytolytic activity. Results from these tests did not reveal, however, any cytolytic activity on either B/C10ME or clone 9 targets. Therefore, assays for CTL memory cells were initiated. Spleen cells from naive as well as regressor and progressor mice were cultured for 5 days with irradiated B/C10ME stimulator cells then assayed for cytolytic activity on B/C10ME targets. Results in Fig. 6 show that spleen cells from naive mice mount a small but significant cytotoxic response to B/C10ME stimulator cells, consistent with previous results showing that this tumor is weakly immunogenic *in vivo* (unpublished results). In contrast, spleen cells from progressor mice fail to mount a cytotoxic response, suggesting absence of memory CTLs in the spleen of these mice or presence of a potent suppressor mechanism. In contrast, spleen cells from regressor mice show consistently very high CTL responses, suggesting that tumor regression had been caused by a cytotoxic response against the growing tumor. Presumably, regression could occur because clone 9 cells are unable to resist elimination because of their failure to induce protective GRP78 stress protein.

A large number of correlative studies have accumulated linking heat shock and Ca^{2+} binding chaperone proteins to resistance against chemotherapy, photodynamic therapy, radiation therapy, and apoptosis (13, 19, 20, 23–27). To our knowledge, our results provide the first demonstration in an

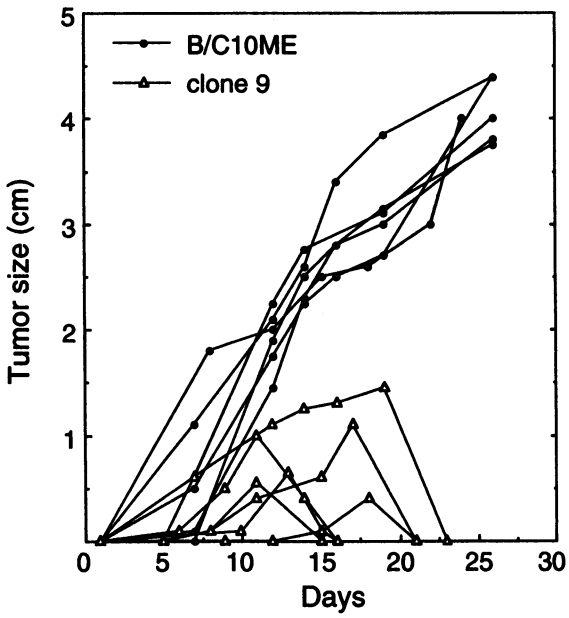

FIG. 5. Tumor growth curves for B/C10ME and clone 9 cells. Equivalent numbers of viable cells ranging from 2 to 3.5 × 10^7 cells were injected subcutaneously into BALB/c mice. Biperpendicular measurements of tumors were taken over a period of 26 days.

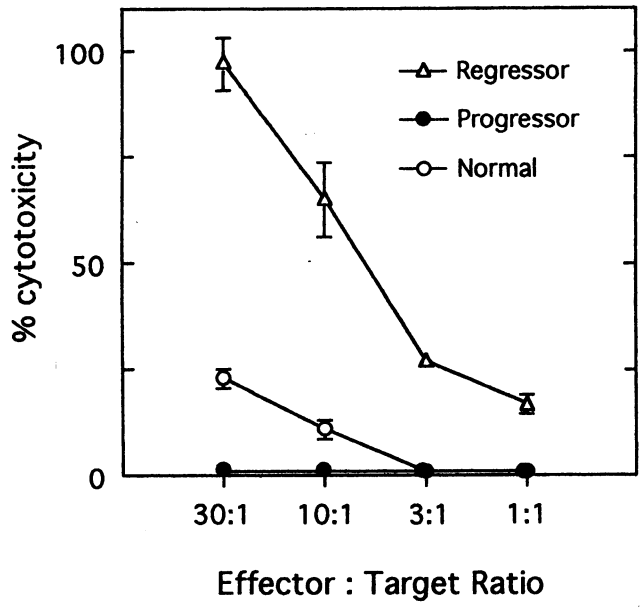

FIG. 6. Memory CTLs are present in the spleen of tumor regressor but not progressor mice. Spleen cells from regressor and progressor normal mice were harvested between 16 and 20 days after tumor inoculation. Spleen cells were cultured for 5 days with irradiated B/C10ME cells and tested at the indicated effector/target ratios in a 4-hr Cr^{51} release assay on B/C10ME targets.

animal model that suppression of a stress protein may lead to tumor regression and/or inhibition of tumor progression. Thus, GRP78/BiP appears to be a new candidate protein responsible for the control of apoptotic cell death in tumor cells whose suppression could offer a novel approach to cancer therapy.

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1. Hightower, L. E. (1991) *Cell* **66,** 191–197.
2. Morimoto, R. I. (1993) *Science* **259,** 1409–1410.
3. Lee, A. S. (1992) *Curr. Opin. Cell Biol.* **4,** 267–273.
4. Shiu, R. P. C., Pouysségur, J. & Pastan, I. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3840–3844.
5. Munro, S. & Pelham, H. R. B. (1986) *Cell* **46,** 291–300.
6. Little, E., Ramakrishnan, M., Roy, B., Gazit, G. & Lee, A. S. (1994) *Crit. Rev. Eukaryotic Gene Expression* **4,** 1–18.
7. Lee, A. S. (1987) *Trends Biochem. Sci.* **12,** 20–23.
8. Li, W. W., Alexandre, S., Cao, C. & Lee, A. S. (1993) *J. Biol. Chem.* **268,** 12003–12009.
9. Patierno, S. R., Tuscano, J. M., Landolph, J. R. & Lee, A. S. (1987) *Cancer Res.* **47,** 6620–6624.
10. Cai, J. W., Henderson, B. W., Shen, J. W. & Subjeck, J. R. (1993) *J. Cell. Physiol.* **154,** 229–237.
11. Gazit, G., Kane, S. E., Nichols, P. & Lee, A. S. (1995) *Cancer Res.* **55,** 1660–1663.
12. Li, L.-J., Li, X., Ferrario, A., Rucker, N., Liu, E. S., Wong, S., Gomer, C. & Lee, A. S. (1992) *J. Cell. Physiol.* **153,** 575–582.
13. Koong, A. C., Chen, E. Y., Lee, A. S., Brown, J. M. & Giaccia, A. J. (1994) *Int. J. Radiat. Oncol.* **28,** 661–666.
14. Sugawara, S., Nowicki, M., Xie, S., Song, H. W. & Dennert, G. (1990) *J. Immunol.* **145,** 1991–1998.
15. Sugawara, S., Takeda, K., Lee, A. & Dennert, G. (1993) *Cancer Res.* **53,** 6001–6005.
16. Hendershot, L. M., Ting, J. & Lee, A. S. (1988) *Mol. Cell. Biol.* **8,** 4250–4256.
17. Li, X. & Lee, A. S. (1991) *Mol. Cell. Biol.* **11,** 3446–3453.
18. Flieger, D., Riethmuller, G. & Ziegler-Heitbrock, H. W. L. (1989) *Int. J. Cancer* **44,** 315–319.
19. Jäättelä, M. & Wissing, D. (1993) *J. Exp. Med.* **177,** 231–236.
20. Wei, Y.-Q., Zhao, X., Kariya, Y., Teshigawara, K. & Uchida, A. (1995) *Cancer Immunol. Immunother.* **40,** 73–78.
21. Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R. & Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2466–2470.
22. Poenie, M., Tsien, R. Y. & Schmitt-Verhulst, A. M. (1987) *EMBO J.* **6,** 2223–2232.
23. Shen, J., Hughes, C., Chao, C., Cai, J., Bartels, C., Gessner, T. & Subjeck, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3278–3282.
24. Gomer, J., Ferrario, A., Rucker, N., Wong, S. & Lee, A. S. (1991) *Cancer Res.* **51,** 6574–6579.
25. Dowd, D. R., MacDonald, P. N., Komm, B. S., Haussler, M. R. &. Miesfeld, R. L. (1992) *Mol. Endocrinol.* **6,** 1843–1848.
26. Ulatowski, L. M., Lam, M., Vanderburg, G., Stallcup, M. R. & Distelhorst, C. W. (1993) *J. Biol. Chem.* **268,** 7482–7488.
27. Ramsamooj, R., Notario, V. & Dritschilo, A. (1995) *Cancer Res.* **55,** 3016–3021.