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A model system for investigating the fundamental mechanisms of programmed cell death, apoptosis, in breast cancer cells has been developed. This model is based on the induction of apoptosis by the calcium pump inhibitor, thapsigargin. Our results indicate that thapsigargin-induced apoptosis in breast cancer cells is mediate through caspase activation and is inhibited by Bcl-2. Also, we have found that lymphoma cells are much for susceptible to thapsigargin-induced apoptosis than breast cancer cells. Our results indicate that this difference in susceptibility to apoptosis is due to the ability of breast cancer cells to mount a protective stress response that is absent in lymphoma cells. Understanding the mechanism of apoptosis through use of this model system should provide a basis for developing new therapies for breast cancer, based on abrogating endogenous cellular stress responses that reduce susceptibility to apoptosis induction.

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Clark W. Distelhorst 10/21/97

PI - Signature Date

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(5) INTRODUCTION

The overall purpose of the proposal is to investigate the role of calcium in the signal transduction cascade that mediates programmed cell death (PCD), or apoptosis, in breast cancer cells. Apoptosis is a fundamentally important process in all cells that regulates development and tissue homeostasis (1). Furthermore, abnormalities of apoptosis underlie many diseases, including cancer. In breast cancer, apoptosis is important for many reasons, but perhaps the one most relevant to this proposal is that many chemotherapeutic agents work by inducing apoptosis.

The emphasis of this proposal is on basic mechanisms that mediate apoptosis and regulate the susceptibility of breast cancer cells to apoptosis. It is anticipated that an improved understanding of the mechanism of apoptosis and how it is controlled in breast cancer will facilitate the development of novel therapeutic strategies based on inducing apoptosis and/or regulating the susceptibility of breast cancer cells to apoptosis.

One hypothesis addressed in this proposal is that mobilization of calcium from the endoplasmic reticulum (ER) may be a critical step in the apoptotic pathway of breast cancer cells induced by a variety of death signals, including growth factor withdrawal, treatment with calcium mobilizing agents, and treatment with certain chemotherapeutic agents. As a major component of the research funded through this grant, we have developed direct support for this hypothesis by demonstrating that the calcium-ATPase inhibitor, thapsigargin (TG), induces apoptosis in breast cancer cells (2). In parallel with these studies, we have also demonstrated that TG induces apoptosis in lymphoma cells. However, an important distinction exists: the lymphoma cells are much more susceptible to TG-induced apoptosis than breast cancer cells. This observation has led to the discovery in our laboratory that breast cancer cells and lymphoma cells differ in a fundamental way: the breast cancer cells mount a strong stress response to TG-mediated calcium mobilization, whereas the lymphoma cells do not (3). The potential importance of this observation becomes obvious when one recalls the marked difference between breast cancer cells and lymphoma cells with regard to chemotherapy sensitivity in general, a factor that underlies the slower progress made in the treatment of breast cancer compared to lymphomas.

The continuation of our work, described in more detail in the sections that follow, will further investigate the mechanism and significance of this observation. We anticipate that the continuing work on this project well produce information that will lead to a greater understanding of the mechanisms of apoptosis in breast cancer cells, and how the

susceptibility to apoptosis is regulated. This understanding should provide a basis for novel approaches to therapy, based on regulating the cancer cell's own endogenous stress responses that determine susceptibility to apoptosis induction.

(6) BODY OF PROGRESS REPORT

This progress report is organized according to the Statement of Work submitted with the original application and specifically covers the third year of funding. For clarity, progress made in the first two years, and consequently described in preceding progress reports, is summarized briefly at the start of each section.

Task 1, Develop model systems for studying PCD in breast cancer, Months 1-8:

a. MCF-7 and T47D breast cancer cell lines will be acquired and optimal culture conditions will be established.

During the first two years of work, described in detail in earlier progress reports, a major goal was to identify a good model system for investigating apoptosis in breast cancer cells. To this end, we extensively characterized two breast cancer cells lines, MCF-7 and MDA-MB-468, with regard to Bcl-2 expression, susceptibility to apoptosis induction by estrogen withdrawal, and the effect of estrogens on Bcl-2 expression. T47D was less extensively characterized. In summary, we found that MCF-7 cells express Bcl-2 at a sufficiently high level that the cells have a relatively low susceptibility to apoptosis induction. Furthermore, we found that the level of Bcl-2 expression was regulated by estrogen, with a repression of Bcl-2 following estrogen withdrawal, and an increase in Bcl-2 following estrogen supplementation. However, we found that the magnitude of change in Bcl-2 level was insufficient to affect the susceptibility of cells to apoptosis.

Therefore, we turned most of our attention to the MDA-MB-468 cell line. This line has a low level of endogenous estrogen receptor, and hence displays estrogen-independent cell growth. Furthermore, this line has a very low level of Bcl-2, in contrast to the much higher constitutive level in MCF-7 cells. We did find, and have described in earlier progress reports, that the level of Bcl-2 in the MDA-MB-468 line varies according to cell density (i.e., the level of Bcl-2 increases when cell density is high). Hence, this is a factor that we take into account in all experiments dealing with apoptosis induction in

this cell line. Understanding the mechanism of Bcl-2 regulation in this situation may be important, but is presently considered beyond the scope of this research grant. Finally, during the course of our studies, we found that other laboratories well versed in breast cancer research employ the MDA-MB-468 line as a model, and that apoptosis can be induced in this line by several mechanisms, including treatment with epidermal growth factor (for example, see ref. 4).

Although we have settled on the MDA-MB-468 cell line as our primary model system, we will confirm and extend our findings using other breast cancer cell lines, including T47D. We will also employ an MDA-MB-468 derivative in which we have stably overexpressed Bcl-2. Hence, we will be able to use this model system to investigate the mechanism of apoptosis inhibition by Bcl-2.

b. Methods of inducing PCD will be established, using both hormonal and non-hormonal manipulations.

During the first two years of funding, we investigated the possibility of inducing PCD by estrogen withdrawal, using primarily the MCF-7 cell line. This work has been described in detail in preceding progress reports. Briefly, we found little evidence of apoptosis when estrogen was withdrawn from MCF-7 cells. This led us to the conclusion that the endogenous level of Bcl-2 in MCF-7 cells was sufficient to preserve cell viability when estrogen was withdrawn, and that the repression of Bcl-2 expression observed during estrogen withdrawal was insufficient to induce cell death. Therefore, we sought a better way of inducing apoptosis in our studies, which led directly into Task 3, where we have found that TG treatment is an effective way of inducing apoptosis in breast cancer cells (see below). Please note that at this point we decided to begin work on task 3 before task 2 was completed, so as to test the strength of our model system and to make more efficient progress. Thus, task 3 was given higher priority than task 2.

c. PCD assays, including DNA fragmentation assays, will be applied to breast cancer cells undergoing PCD.

As described in preceding progress reports, our laboratory has become proficient in two apoptosis assays: DNA fragmentation by agarose gel electrophoresis and morphological assessment by fluorescence microscopy of ethidium bromide and acridine orange stained cells. We have successfully applied both of these techniques without difficulty to the breast cancer cell systems described in this progress report. These assays are sufficient for the studies currently underway in Tasks 1-3 of this proposal. These assays are efficient, reliable and inexpensive.

Thus, they will be utilized in our work, unless need for other types of assays develops.

Task 2, Adapt established calcium assays to breast cancer cell system, Months 4-12:

For reasons discussed above, we elected to move directly into Task 3, before completing Task 2. This was possible, because Task 3 is not dependent upon the findings in Task 3. In fact, in retrospect, it would have been more logical to place Task 3 ahead of Task 2 in our original application. Under Task 2a, we have begun to work out methods for loading breast cancer cells with Fura-2 AM, and to assess intracellular calcium by fluorometry. Also, we have initiated work on a new method of directly measuring calcium concentration within organelles, based on expression of aequorin, a protein that fluoresces when it binds calcium ions (5). To this end, we have obtained the aequorin cDNA and placed in the pSFFVneo and pcDNA3 expression vectors. We have begun initial experiments to transfect cells with these expression vectors, in order to achieve stable expression of aequorin in the endoplasmic reticulum. This experimental approach will complement and extend our original proposal using Fura-2 AM to monitor calcium fluxes, and will have the advantage of directly measuring calcium within intracellular pools.

Task 3, Determine effect of thapsigargin on growth and viability of breast cancer cells, Months 12-18:

a. Measure effects of thapsigargin treatment on growth and viability of breast cancer cells over a broad dose-response range.

Thapsigargin (TG) is a selective inhibitor of the endoplasmic reticulum-associated calcium-ATPase that induces apoptosis by mobilizing calcium from the ER lumen into the cytoplasm. In our laboratory, TG has been used as an effective reagent for investigating the role of calcium in mediating apoptosis. For example, in studies using lymphoma cells, we have found that TG induces apoptosis, accompanied by nuclear chromatin condensation and extensive DNA fragmentation, and that apoptosis induction by TG is inhibited by Bcl-2 overexpression (6). Furthermore, TG has been useful for investigating the mechanism of action of Bcl-2; for example, our findings suggested that Bcl-2 inhibits calcium efflux from the ER following TG-mediated calcium-ATPase inhibition (6) and thereby maintains the ER calcium pool (7). Also, we have found that ER

calcium pool depletion may be an important trigger of apoptosis (7).

Therefore, in the first two years of progress already summarized in previous reports, we determined the effect of TG on cell growth and viability in breast cancer cells. To briefly summarize, we found that 100 nM TG was sufficient to inhibit cell proliferation. In both cell lines, treatment with 100 nM TG impaired cell growth and induced loss of adherence. In MDA-MB-468 cells, treatment with 100 nM TG caused cell death, measured by increased incorporation of trypan blue dye, whereas TG treatment did not appear to induce cell death in MCF-7 cells. In more recent experiments, not described previously, we have found that 50 nM TG was sufficient to induce loss of adherence of MDA-MB-468 cells and that loss of adherence preceded morphological signs of cell death by 24 - 48 h. Based on these initial observations, we proceeded directly to the next planned experiments, designed to measure morphological and biochemical parameters to determine whether TG-induced loss of viability was due to induction of apoptotic cell death.

b. Measure morphological and biochemical parameters to determine whether thapsigargin-induced loss of viability is due to induction of apoptotic cell death.

In work accomplished during the first two years of funding, and summarized in previous progress reports, we investigated whether or not TG induces apoptosis in MCF-7 and MDA-MB-468 cells. Briefly, using nuclear chromatin condensation as a hallmark of apoptosis, we detected evidence of apoptosis in TG-treated MDA-MB-468 cells, but not in TG-treated MCF-7 cells. Moreover, the failure of MCF-7 cells to undergo apoptosis in response to TG treatment was not overcome by estrogen withdrawal, indicting that constitutive levels of Bcl-2 in MCF-7 cells were sufficient to inhibit TG-induced apoptosis. Based on this information, and for reasons summarized above, we have focused our attention on the MDA-MB-468 cell line as a model for investigation of apoptosis in breast cancer cells.

Therefore, in work carried our during the third year of funding, we characterized apoptosis induction in MDA-MB-468 cells in more detail. This work has been published in Oncogene (2). Our approach was twofold: first, to characterize apoptosis induction by TG in MDA-MB-468 cells; second, to investigate the role of caspases in apoptosis induction. With regard to the first aim, we found that TG treatment does induce apoptosis in MDA-MB-468 cells, based on morphological evidence and DNA fragmentation. However, the onset of apoptosis evolved over a 48 to 72 hr period following TG addition, in contrast to lymphoma cells in which apoptosis

evolved rapidly after TG addition. The decreased susceptibility of MDA-MB-468 cells to apoptosis induction by TG was not overcome by increasing TG concentration, and hence is unlikely to be secondary to resistance to TG at the level of the ER calcium-ATPase. Hence, in subsequent work described in the next section, we investigated the role of the ER stress response (i.e., grp78, grp94 induction) in regulating the susceptibility of MDA-MB-468 cells to apoptosis induction.

In the second approach, we focused on the role of caspases in mediating apoptosis induction in MDA-MB-468 cells. Caspases (ICE-like proteases) compose a family of cysteine proteases that characteristically cleave proteins at aspartic acid residues (8,9). Recent findings suggest that at least some of these proteases operate within an amplifiable protease cascade, culminating in activation of caspase-3 (CPP32) (8,10). Cleavage of selected target proteins by caspase-3 (CPP32) and related proteases appears to be directly responsible for the stereotypic morphological changes characteristic of apoptosis (10). To test the role of caspases in apoptosis induction in breast cancer cells, we used two experimental approaches: the first was to examine the effect of the baculovirus p35 protein on apoptosis induction; the second was to examine the effect of cell permeable caspase inhibitors on apoptosis induction. Baculovirus p35 is a poor substrate of most caspases, and hence is an effective inhibitor of their activity. We obtained the cDNA for baculovirus p35 from Paul Friesen, University of Wisconsin, and cloned it into two different expression vectors, pSFFVneo and pcDNA3, in the sense orientation. MDA-MB-468 cells were stably transfected with the p35 expression vectors and selected for resistance to G418. Multiple clones were characterized with regard to p35 expression by both polymerase chain reaction and Northern hybridization. The results indicated that p35 inhibits induction of apoptosis by TG in MDA-MB-468 cells.

In the second approach, we found that TG-induced apoptosis was inhibited by Z-VAD-fmk, a cell permeable, broad spectrum inhibitor of caspases. The tripeptide sequence in Z-VAD-fmk corresponds to the P1 to P3 residues of the pro-IL-1ß cleavage site ($Tyr_4ValAlaAsp_1Gly$), where caspase-1 (ICE) cleaves between the Asp and Gly residue (11). Deletion of the Tyr broadens the inhibitory spectrum to include not only caspase-1, but other closely related caspases. An effective treatment regimen was empirically derived in which cells were treated with 200 µM doses of Z-VAD-fmk added 1 h prior to TG, and every 12 h thereafter over a period of 48 h. In addition to Z-VAD-fmk, Z-FA-fmk, a cathepsin inhibitor, was employed as a negative control. Apoptosis induction by TG, measured by fluorescence microscopy, was inhibited by Z-VAD-fmk, but not

the control inhibitor, Z-FA-fmk, thus confirming that caspase activity is necessary for mediating apoptosis in response to TG treatment.

Details of these findings are found in the paper published in Oncogene, included with this progress report.

c. Determine if cells adapt to growth in thapsigargin, and if cells so adapted become resistant to induction of PCD by hormonal manipulations.

This task is not as high a priority as other tasks, and therefore its initiation has been delayed.

d. Measure other parameters of thapsigargin-induced endoplasmic calcium pool depletion, including transcriptional induction of grp78 and grp94 genes, alterations of protein processing, and capacitative calcium entry.

The ER is the major intracellular reservoir of calcium in non-muscle cells. The ER calcium pool is essential for a number of vital cellular functions which include protein processing within the ER, maintaining high translation rates of newly transcribed messages, preserving the structural integrity of the ER, and regulating cell proliferation and cell cycle progression. Under physiological conditions, the ER calcium pool is maintained by an associated calcium-ATPase that pumps calcium into the ER lumen from the cytoplasm. The ER calcium pool can be depleted by treating cells with the calcium ionophore A23187 or the selective ER calcium-ATPase inhibitor thapsigargin (TG). Recent findings in our laboratory have indicated that ER calcium pool depletion, in response to TG treatment, triggers apoptosis (7).

ER function is mediated, in part, by intraluminal calcium binding proteins which include the glucose regulated proteins (GRP's) GRP78 and GRP94 (12). GRP78 and GRP94 are found constitutively within the ER, and transcription of the genes for these proteins is elevated in response to malfolded proteins, inhibition of glycosylation and ER calcium pool depletion. GRP78 is a highly conserved 78 kDa protein that shares 60% amino acid homology with the 70 kDa heat shock protein (HSP 70). GRP78 (also known as BiP) associates transiently with nascent proteins as they traverse the ER and aids in their folding and transport. The binding of immature proteins by GRP78 requires ATP, and GRP78 has both ATP binding and ATPase activities. GRP94 is a 94 kDa glycoprotein that shares 50% amino acid homology with HSP90. GRP94 acts in concert with GRP78 to fold nascent proteins, and

also exhibits ATPase activity.

In epithelial cells and fibroblasts, grp78 and grp94 are coordinately regulated through common calcium-responsive promoter elements that respond to ER calcium pool depletion (12). Thus, ER calcium pool depletion, induced by either A23187 or TG, signals an increase in grp78/grp94 transcription, producing a 5- to 20 fold elevation of grp78/grp94 mRNA levels. In these cells, the loss of ER calcium induced by TG or A23187 does not result in a loss of viability, unless the grp78/grp94 stress response is repressed by antisense, promoter competition or ribozyme techniques. Moreover, grp78/grp94 induction restores protein synthesis under conditions where intracellular calcium is depleted. This indicates that grp78/grp94 gene induction is a protective response mechanism by which cells accommodate to potentially lethal stress caused by the disruption of intracellular calcium homeostasis.

Based on this background information, we reasoned that the decreased susceptibility of breast cancer cells to apoptosis induction by TG, compared to lymphoma cells (described above), might be secondary to differences in grp78/grp94 stress response induction. To test this hypothesis, we measured the level of grp78 mRNA following TG treatment in WEHI7.2 lymphoma cells and several breast cancer cell lines, Mm5MT, MCF-7, and MDA-MB-468. This work has been published in The Journal of Biological Chemistry (3). Briefly, we found that TG induced calcium loss from the ER of WEHI7.2 cells does not induce grp78 transcription, even if cells are protected from undergoing apoptosis by overexpressing Bcl-2 (W.Hb12 is a subclone of WEHI7.2 cells stably transfected with cDNA encoding Bcl-2). In contrast, grp78 transcription was strongly induced in each of the breast cancer cell lines. In summary, these findings have two important implications. First, the grp78 stress response may be differentially regulated among different types of cells, with a much greater response observed in non-lymphoid cells than in lymphoid cells. Second, regulation of the grp78 stress response may be a major factor in deciding whether a cell lives or dies in response to disruption of intracellular calcium homeostasis. Indeed, the absence of a calcium-mediated grp78 stress response may be the basis for the marked susceptibility of certain lymphoma cells to TG-induced apoptosis, and the decreased susceptibility of breast cancer cells to TG-induced apoptosis.

(7) CONCLUSIONS

Task 1. The MCF-7 and MB-MDA-468 human breast cancer cell lines provide excellent model systems for carrying out the proposed research, although MDA-MB-468 cells are more immediately applicable to the research goals of this proposal

because of their lower endogenous level of Bcl-2.

a. MCF-7 represents a hormone-responsive line that expresses significant levels of Bcl-2.

b. In MCF-7 cells, the level of Bcl-2 can be down-regulated by estrogen withdrawal, and induced by estrogen supplementation.

c. In MCF-7 cells, the downregulation of Bcl-2 following estrogen withdrawal is not associated with apoptosis.

d. MB-MDA-468 cells do not express Bcl-2, unless they have been maintained at confluence for several days.

e. In breast cancer cells (both MCF-7 and MB-MDA-468), the level of Bcl-2 expression correlates with the phase of cell growth.

f. Morphological assessment, by fluorescence microscopy of acridine orange and ethidium bromide stained cells, is a feasible and efficient method for detecting apoptosis in the breast cancer cell lines we are using.

Task 2. While this aim has not been given as high priority in our work as the other aims, we are beginning to make progress toward using Fura-2 AM and/or aequorin to measure calcium fluxes and intracellular calcium concentrations as they relate to apoptosis induction in MDA-MB-468 cells. It is expected that further progress will be made under this aim in the next year of work.

Task 3. The intracellular calcium pump inhibitor, thapsigargin, induces apoptosis in breast cancer cells, and thapsigargin-induced apoptosis will be an excellent model system for studying the role of calcium in mediating programmed cell death in breast cancer.

a. Thapsigargin (TG) induces growth arrest of MCF-7 cells, without significant loss of viability, due to the significant level of Bcl-2 in MCF-7 cells.

b. Estrogen withdrawal does not increase the susceptibility of MCF-7 cells to TG-induced apoptosis, suggesting that the reduction in the level of Bcl-2 following estrogen withdrawal is insufficient to render cells susceptible to apoptosis.

c. TG-induces marked loss of viability in MDA-MB-468 cells.

d. TG-induced cell death in MDA-MB-468 cells is accompanied by typical morphological features of apoptosis and DNA fragmentation.

e. TG-induced apoptosis of MDA-MB-468 cells is mediated through activation of caspases and is inhibited by overexpression of the baculovirus p35 protein caspase inhibitor or by treatment with Z-VAD-fmk, both of which inhibit ICE-like protease activities.

f. Although TG induces apoptosis in MDA-MB-468 breast

cancer cells, the induction of cell death is much slower than in lymphoma cells.

g. MDA-MB-468 and Mm5MT breast cancer cells induce transcription of the stress response gene, grp78, in response to TG-induced ER calcium release, whereas the lymphoma cells do not. Thus, induction of grp78 stress response may be a factor that regulates whether or not a cell undergoes apoptosis in response to TG treatment. The decreased susceptibility of breast cancer cells to apoptosis induction following TG treatment, compared to lymphoma cells, may be due to the inherent capacity of breast cancer cells to mount a grp78 stress response.

Implications of the research

During the first three years of support, this laboratory has made significant progress toward the long term goal of the research, which is to understand the programmed cell death process in breast cancer cells so that new therapeutic strategies can be developed. In partial fulfillment of Technical Objective C2 of the original proposal, we have discovered that the intracellular calcium pump inhibitor, thapsigargin, induces apoptosis in a human breast cancer cell line. This finding is significant because it indicates that calcium mediated signal transduction pathways that mediate apoptosis are active in human breast cancer cells, indicating that it may be feasible to develop novel forms of therapy based on triggering programmed cell death through a calcium-mediated signal. In partial fulfillment of Technical Objective C3, we have found that TG-induced cell death is inhibited by Bcl-2. This will allow us, in future years of support, the opportunity to test hypotheses, outlined in the original proposal, regarding the mechanism of Bcl-2 action in breast cancer cells. Moreover, the observation that TG induces apoptosis, and that TG-induced apoptosis is inhibited by Bcl-2, sets the stage for pursuit of Technical Objective 4, in which we will investigate how endoplasmic reticulum calcium release signals programmed cell death in breast cancer cells. Most importantly, we have validated our TG-induced model of apoptosis by showing that TG-induced apoptosis is mediated by caspases. We are among the first laboratories to stably express the baculovirus p35 apoptosis inhibitory protein in breast cancer cells to prove that apoptosis is indeed mediated through caspase activation.

Perhaps the most exciting, and therapeutically most relevant, finding is that breast cancer cells mount a strong grp78 stress response when the ER calcium pool is disrupted. This is in stark contrast to lymphoma cells, which fail to mount a similar stress response. The implications are that the differential susceptibility of breast cancer cells and lymphoma cells to apoptosis induction, which underlies the slower progress made in treating breast cancer compared to lymphoma,

may be explained by differential expression of the grp78 stress response. In our future work, we intend to pursue this concept with the overall objective of determining how the grp78 stress response is mediated and regulated in breast cancer cells. From this, we hope to develop novel therapeutic approaches with the intent of abrogating the grp78 stress response and thereby increase the susceptibility of breast cancer cells to apoptosis induction.

Finally, there will be no significant changes from the original plans of research and the specific aims of the original research proposal will be followed during the fourth year of support.

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(9) APPENDICES

The appendix contains two reprints references in the Body of the Progress Report. These reprints represent work funded in total or in part by this grant.

McCormick, Thomas. S., McColl, Karen, S. and Distelhorst, Clark W. Mouse lymphoma cells destined to undergo apoptosis in response to thapsigargin treatment fail to generate a calcium-mediated grp78/grp94 stress response. J. Biol. Chem. 272:6087-6092, 1997

Qi, Xiao-Mei, He, Huiling, Zhong, Hongying, and Distelhorst, Clark W. Baculovirus p35 and Z-VAD-fmk inhibit thapsigargin-induced apoptosis of breast cancer cells. Oncogene 15:1207-1212, 1997.

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Mouse Lymphoma Cells Destined to Undergo Apoptosis in Response to Thapsigargin Treatment Fail to Generate a Calcium-mediated *grp78/grp94* Stress Response*

(Received for publication, May 31, 1996, and in revised form, December 6, 1996)

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***grp78/grp94* induction is critical for maintaining the viability of epithelial cells and fibroblasts following treatment with thapsigargin (TG), an inhibitor of Ca^{2+} uptake into the endoplasmic reticulum. In contrast to these cell types, WEHI7.2 mouse lymphoma cells undergo apoptosis when treated with TG, prompting us to examine the *grp78/grp94* stress response in WEHI7.2 cells. TG treatment failed to induce *grp78/grp94* transcription in WEHI7.2 cells, measured by Northern hybridization and nuclear run-on assays, even if the cells were protected from apoptosis by overexpressing *bcl-2*. However, *grp78/grp94* transcription was induced by the glycosylation inhibitor tunicamycin, suggesting that there are at least two *grp78/grp94* signaling pathways, one in response to TG-induced endoplasmic reticulum Ca^{2+} pool depletion, which is inoperable in WEHI7.2 cells, and one in response to glycosylation inhibition, which is operable in WEHI7.2 cells. Studies of additional lymphoid lines, as well as several nonlymphoid lines, suggested a correlation between *grp78/grp94* induction and resistance to apoptosis following TG treatment. In conclusion, the vulnerability of TG-treated WEHI7.2 cells to apoptosis may be due to failure to signal a *grp78/grp94* stress response.**

The endoplasmic reticulum (ER)[1] is the major intracellular reservoir of Ca^{2+} in nonmuscle cells (1). The ER Ca^{2+} pool is essential for a number of vital cellular functions, which include protein processing within the ER (2, 3), maintaining high translation rates of newly transcribed messages (4), preserving the structural integrity of the ER (5, 6), and regulating cell proliferation and cell cycle progression (7). Under physiological conditions, the ER Ca^{2+} pool is maintained by an associated Ca^{2+}-ATPase that pumps Ca^{2+} into the ER lumen from the cytoplasm (8). The ER Ca^{2+} pool can be depleted by treating cells with the Ca^{2+} ionophore A23187 or the selective ER Ca^{2+}-ATPase inhibitor thapsigargin (TG) (9).

ER function is mediated, in part, by intraluminal Ca^{2+}-binding proteins, which include the glucose-regulated proteins GRP78 and GRP94 (5, 10, 11). GRP78 and GRP94 are found constitutively within the ER, and transcription of the genes for these proteins is elevated in response to malfolded proteins, inhibition of glycosylation, and ER Ca^{2+} pool depletion (12–14). GRP78 is a highly conserved 78-kDa protein that shares 60% amino acid homology with the 70-kDa heat shock protein (HSP70). GRP78 (also known as BiP) associates transiently with nascent proteins as they traverse the ER and aids in their folding and transport (15–20). The binding of immature proteins by GRP78 requires ATP, and GRP78 has both ATP binding and ATPase activities (21). GRP94 is a 94-kDa glycoprotein that shares 50% amino acid homology with HSP90 (11, 22). GRP94 acts in concert with GRP78 to fold nascent proteins and also exhibits ATPase activity (22–24).

In epithelial cells and fibroblasts, *grp78* and *grp94* are coordinately regulated through common Ca^{2+}-responsive promoter elements that respond to ER Ca^{2+} pool depletion (10, 25). Thus, ER Ca^{2+} pool depletion, induced by either A23187 or TG, signals an increase in *grp78/grp94* transcription, producing a 5–20-fold elevation of *grp78/grp94* mRNA levels (25). In these cells, the loss of ER Ca^{2+} induced by TG or A23187 does not result in a loss of viability, unless the *grp78/grp94* stress response is repressed by antisense, promoter competition, or ribozyme techniques (26–28). Moreover, *grp78/grp94* induction restores protein synthesis under conditions where intracellular Ca^{2+} is depleted (29). This indicates that *grp78/grp94* gene induction is a protective response mechanism by which cells accommodate to potentially lethal stress caused by the disruption of intracellular Ca^{2+} homeostasis.

In contrast to epithelial cells and fibroblasts, we have found that WEHI7.2 mouse lymphoma cells undergo apoptosis in response to TG-induced ER Ca^{2+} loss, unless protected by overexpression of the anti-apoptotic oncogene *bcl-2* (30). Given this observation, we chose to examine the *grp78/grp94* stress response in WEHI7.2 mouse lymphoma cells. We report for the first time that TG-induced Ca^{2+} loss from the ER of WEHI7.2 cells does not induce *grp78/grp94* transcription, even if cells are protected from undergoing apoptosis by *bcl-2*. Interestingly, treatment with tunicamycin (TN), an inhibitor of *N*-linked glycosylation, does induce *grp78/grp94* transcription, suggesting that ER Ca^{2+} pool depletion and accumulation of underglycosylated proteins signal an increase in *grp78/grp94* transcription through independent pathways, the former pathway being inoperative in WEHI7.2 cells. Moreover, in three breast cancer cell lines and two additional lymphoma lines, the induction of *grp78* correlated with resistance to TG-induced apoptosis. These findings suggest that inherent differences in the susceptibility of cells to apoptosis induction by TG can be determined, at least in part, by the cell's capacity to mount a *grp78/grp94* stress response.

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[1] The abbreviations used are: ER, endoplasmic reticulum; TG, thapsigargin; TN, tunicamycin.

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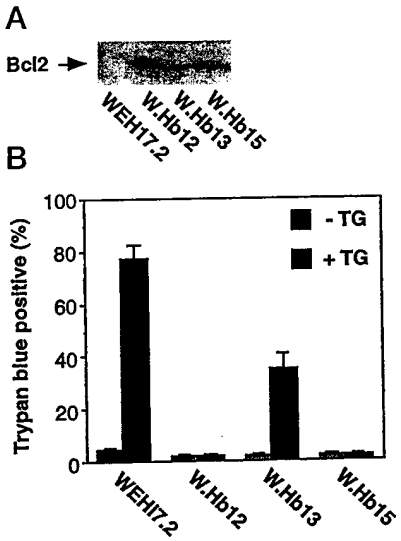

FIG. 1. **Effect of Bcl-2 on WEHI7.2 viability after TG treatment.** *A*, using an antibody specific for human Bcl-2, the levels of Bcl-2 protein expressed by WEHI7.2 cells and stable transfectants are shown by Western blot analysis. *B*, exponentially growing cells were diluted to a concentration of 0.3×10^6/ml with fresh culture medium 24 h before adding 100 nM TG at the zero time point. The percentage of trypan blue-positive cells was measured 24 h after TG addition. *Error bars* represent the mean of duplicate determinations in multiple experiments.

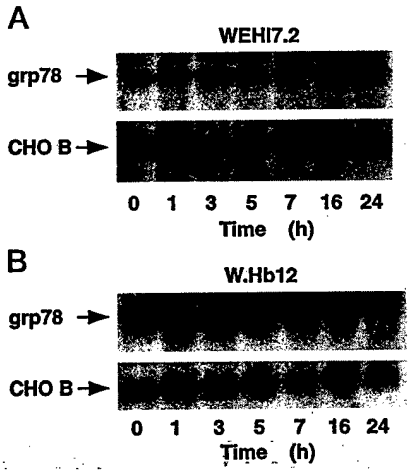

FIG. 2. **Effect of TG treatment on *grp78* mRNA levels in WEHI7.2 and W.Hb12 cells.** Exponentially growing cells were diluted to a concentration of 1×10^6/ml with fresh culture medium before adding 100 nM TG. RNA was isolated at the indicated times following TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. Representative blots for WEHI7.2 cells (*A*) and W.Hb12 cells (*B*) are shown.

at each time point by densitometry with normalization to the CHO-B standard. The maximum ratio was 1.7 ± 0.2, which did not represent a reproducible elevation above pretreatment levels ($p \geq 0.05$). The failure of TG treatment to induce an elevation of the *grp78* mRNA level was confirmed at several other concentrations of TG (10, 50, and 300 nM) (data not shown).

To determine whether or not the failure of TG treatment to increase *grp78* mRNA levels in WEHI7.2 cells was secondary to early changes accompanying cell death, we examined the *grp78* stress response in W.Hb12 cells, which are protected from apoptosis by *bcl-2*. As shown by the Northern blot in Fig. 2*B*, the *grp78* mRNA level did not appear to increase following treatment of W.Hb12 cells with 100 nM TG. In multiple experiments, the maximum post-treatment to pretreatment *grp78*

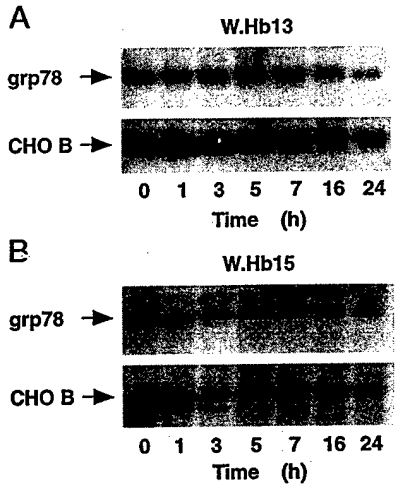

FIG. 3. **Effect of TG treatment on *grp78* mRNA levels in W.Hb13 and W.Hb15 cells.** Exponentially growing cells were diluted to a concentration of 1×10^6/ml with fresh culture medium before adding 100 nM TG. RNA was isolated at the indicated times following TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. Representative blots for W.Hb13 cells (*A*) and W.Hb15 cells (*B*) are shown.

mRNA ratio was 2.1 ± 0.4, which did not represent a significant elevation above pretreatment levels ($p \geq 0.05$). Northern blot analysis of two other Bcl-2-expressing clones, W.Hb13 and W.Hb15, confirmed that *grp78* mRNA levels did not increase following treatment with 100 nM TG (Fig. 3, *A* and *B*). Note that in Fig. 3, *grp78* mRNA levels actually decreased relative to CHO-B levels at 16 and 24 h after TG addition. This observation was variable among experiments, including those with WEHI7.2 and W.Hb12 cells. Note that we have previously shown, in WEHI7.2 cells and derivatives expressing Bcl-2, that TG treatment inhibits the ER Ca^{2+}-ATPase, producing cytosolic Ca^{2+} elevation and ER Ca^{2+} pool depletion (30, 36). Hence, the failure to significantly elevate *grp78/grp94* transcription following TG treatment is not due to a failure of TG to disrupt Ca^{2+} homeostasis.

Levels of GRP78 and GRP94 proteins, assessed by Western blotting, were the same in untreated WEHI7.2 and W.Hb12 cells, indicating that *bcl-2* does not affect basal levels of GRP78/GRP94 expression at the protein level (Fig. 4, *A* and *B*). Furthermore, levels of GRP78 protein did not increase following TG treatment in either WEHI7.2 or W.Hb12 cells (Fig. 4*C*).

Both WEHI7.2 and W.Hb12 cells up-regulated *grp78* mRNA levels by 6–7-fold when treated with 0.75 μM TN (Fig. 5). Thus, although ER Ca^{2+} pool depletion failed to induce an up-regulation of *grp78* mRNA, accumulation of unglycosylated proteins in the ER induced a strong up-regulation of *grp78* mRNA levels. These findings suggest that there is more than one signal transduction pathway for *grp78* induction (see "Discussion").

To assess if *grp94* is regulated in the same manner as *grp78* in WEHI7.2 and W.Hb12 cells, we examined the steady-state level of *grp94* mRNA after treatment with 100 nM TG (Fig. 6). A modest elevation of *grp94* mRNA levels appeared to occur at 5 h after TG addition in both WEHI7.2 and W.Hb12 cells. In multiple experiments, however, the maximum ratio of post-treatment to pretreatment *grp94* mRNA levels in WEHI7.2 cells was only 2.0 ± 0.5, which did not represent a reproducible elevation above base-line levels ($p \geq 0.05$). In W.Hb12 cells, the maximum ratio was only 1.5 ± 0.2, which also did not represent a significant elevation above base-line levels ($p \geq 0.05$).

The preceding findings suggest that TG treatment does not signal an increase in *grp78/grp94* transcription in the WEHI7.2

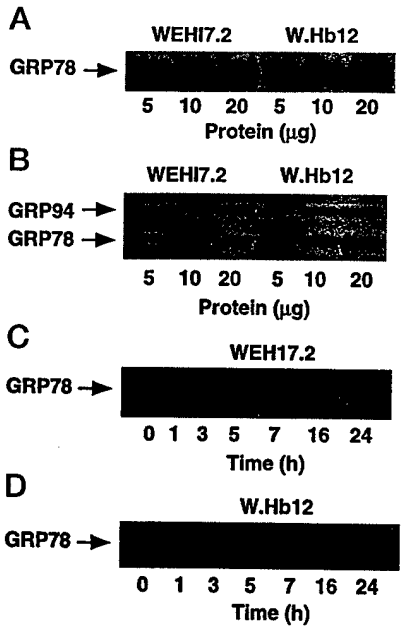

FIG. 4. **GRP78 and GRP94 protein levels in WEHI7.2 and W.Hb12 cells.** *A* and *B*, protein was isolated from exponentially growing cells and separated by SDS-polyacrylamide gel electrophoresis in the amounts shown. Western blots were probed with a monoclonal antibody to GRP78 (*A*) or with a polyclonal antibody that recognizes both GRP94 and GRP78 (*B*). *C* and *D*, protein, isolated from WEHI7.2 and W.Hb12 cells, respectively, at various times after adding 100 nM TG, was separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using a monoclonal antibody to GRP78.

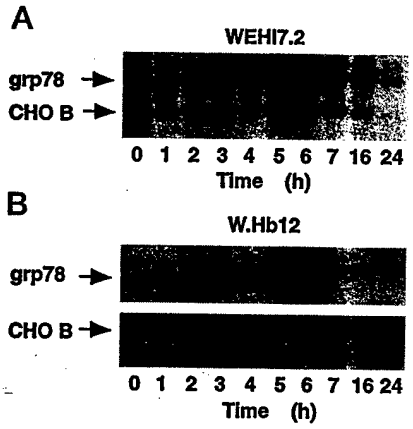

FIG. 5. **Effect of TN treatment on *grp78* mRNA levels in WEHI7.2 and W.Hb12 cells.** Exponentially growing cells were diluted to a concentration of 1×10^6/ml with fresh culture medium before adding 0.75 μM TN. RNA was isolated at the indicated times following TN addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. Representative blots for WEHI7.2 cells (*A*) and W.Hb12 cells (*B*) are shown.

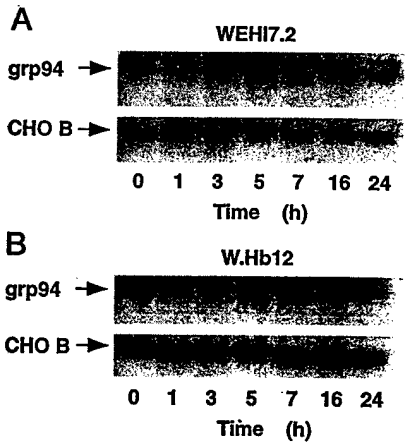

FIG. 6. **Effect of TG treatment on *grp94* mRNA levels in WEHI7.2 and W.Hb12 cells.** Exponentially growing cells were diluted to a concentration of 1×10^6/ml with fresh culture medium before adding 100 nM TG. RNA was isolated at the indicated times following TG addition and analyzed by Northern hybridization using radiolabeled *grp94* and CHO-B cDNA probes. Representative blots for WEHI7.2 cells (*A*) and W.Hb12 cells (*B*) are shown.

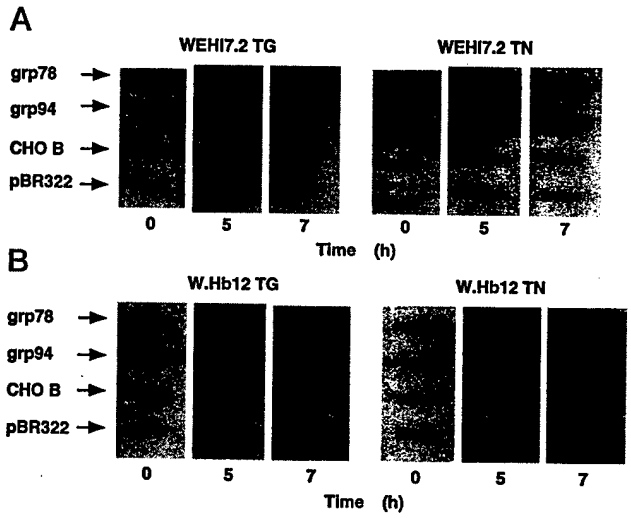

FIG. 7. **Effect of TG and TN treatment on *grp78* and *grp94* transcription.** Nuclei were isolated from WEHI7.2 cells (*A*) and W.Hb12 cells (*B*) after treatment with 100 nM TG or 0.75 μM TN for the indicated times. Equal amounts (cpm) of ^{32}P-labeled nuclear run-off RNA were hybridized to slot blots containing 5 μg of immobilized *grp78* or *grp94* plasmids. Hybridization of run-off RNA to slot blots containing the CHO-B plasmid and the empty plasmid vector pBR322 was used as a control. The time samples taken following TG or TN addition are indicated. Results are representative of three experiments.

lymphoma cell line or its derivatives that express Bcl-2. To confirm that this is the case, we measured the effect of TG treatment on the transcription rate of *grp78* and *grp94* genes by nuclear run-off assays using isolated nuclei from WEHI7.2 and W.Hb12 cells. An increase in newly expressed *grp78/grp94* message after TG treatment was not detected in WEHI7.2 cells (Fig. 7*A*) or W.Hb12 cells (Fig. 7*B*). TN treatment, however, did induce a significant increase in *grp78* and *grp94* transcription, which was detected by 5 and 7 h, respectively. This indicates that *grp78/grp94* transcription is not induced by TG in WEHI7.2 cells or derivatives that express Bcl-2, but is induced by TN.

Because earlier studies of *grp78* regulation have emphasized epithelial cells and fibroblasts (see the Introduction), as a positive control, we examined the effect of TG treatment on *grp78* mRNA levels in three epithelial breast cancer lines, Mm5MT, MDA-MB-468, and MCF-7. Treatment of Mm5MT cells with 100 nM TG did not induce cell death (Fig. 8*D*), but did induce a 5-fold elevation of *grp78* mRNA levels detectable within 7 h of adding TG (Fig. 8*B*). MDA-MB-468 and MCF-7 cells were also much less sensitive than WEHI7.2 cells to TG-induced cell death (Fig. 8*D*) and displayed marked induction of *grp78* mRNA levels in response to TG treatment (Fig. 8, *B* and *C*).

To determine if the defect in TG-mediated *grp78* signaling is observed in other lymphoid cells, we measured the effect of TG treatment on *grp78* mRNA levels in two additional Bcl-2-negative mouse lymphoma lines, W7.MG1 and S49.1. *grp78* transcription is induced by TN treatment in both of these lines (31). The level of *grp78* mRNA failed to increase following TG treatment in W7.MG1 cells, which rapidly lost viability following TG

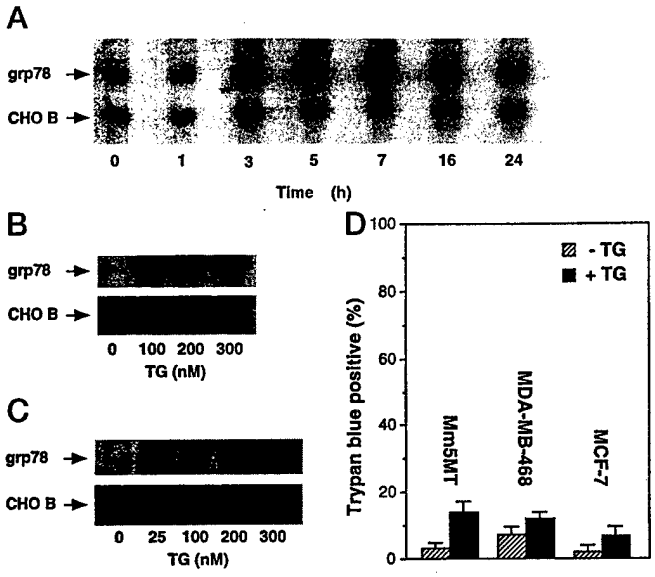

FIG. 8. **Effect of TG on *grp78* mRNA levels and viability in breast cancer cells.** *A*, exponentially growing Mm5MT cells were treated with 100 nM TG. RNA was isolated at the indicated times following TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. *B*, exponentially growing MDA-MB-468 cells were treated with multiple concentrations of TG. RNA was isolated 7 h after TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. *C*, exponentially growing MCF-7 cells were treated with multiple concentrations of TG. RNA was isolated 7 h after TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. *D*, exponentially growing cells were incubated for 24 h in the presence or absence of 100 nM TG. The percentage of trypan blue-positive cells was measured 24 h after TG addition. *Error bars* represent the mean of duplicate determinations in multiple experiments.

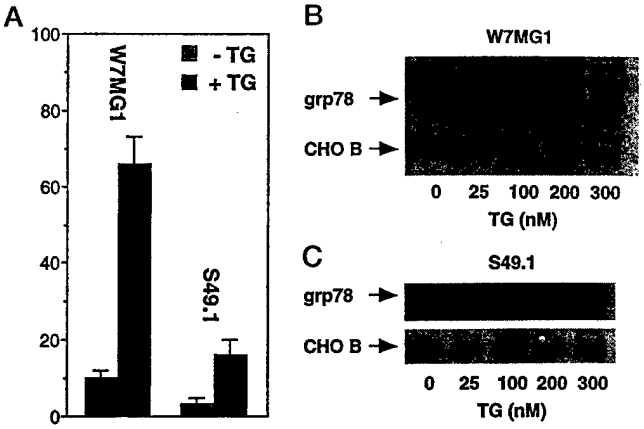

FIG. 9. **Effect of TG on *grp78* mRNA levels and viability in lymphoma cells.** *A*, exponentially growing cells were incubated for 24 h in the presence or absence of 100 nM TG. The percentage of trypan blue-positive cells was measured 24 h after TG addition. *Error bars* represent the mean of duplicate determinations in multiple experiments. *B*, exponentially growing W7.MG1 cells were diluted to a concentration of 0.5×10^6/ml in fresh culture medium 7 h before adding 100 nM TG. RNA was isolated 7 h after TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes. *C*, exponentially growing S49.1 cells were diluted to a concentration of 0.5×10^6/ml in fresh culture medium 7 h before adding 100 nM TG. RNA was isolated 7 h after TG addition and analyzed by Northern hybridization using radiolabeled *grp78* and CHO-B cDNA probes.

treatment, whereas the level of *grp78* mRNA did increase 3–4-fold following TG treatment in S49.1 cells, which were relatively resistant to TG-induced cell death (Fig. 9). These data are consistent with the concept that a deficiency of *grp78* induction increases susceptibility to TG-induced cell death.

DISCUSSION

We have discovered that the transcription of *grp78* and *grp94* is not significantly increased in WEHI7.2 cells in response to treatment with the ER Ca^{2+}-ATPase inhibitor TG, even when apoptosis is inhibited by overexpressing grp78. Examination of two additional lymphoma lines revealed an absence of *grp78* induction in W7.MG1 cells and 3–4-fold induction of *grp78* in S49.1 cells following TG treatment. By comparison, TG treatment induced a marked elevation of *grp78* mRNA levels in all three nonlymphoid lines tested (Mm5MT, MDA-MB-468, and MCF-7), consistent with studies indicating that TG treatment substantially induces *grp78/grp94* transcription in epithelial cells and fibroblasts (13).

We have previously shown, in WEHI7.2 cells and derivatives expressing Bcl-2, that TG treatment inhibits the ER Ca^{2+}-ATPase, producing cytosolic Ca^{2+} elevation and ER Ca^{2+} pool depletion (30, 36). Hence, the failure to significantly elevate *grp78/grp94* transcription following TG treatment is not due to a failure of TG to disrupt Ca^{2+} homeostasis. Moreover, in the present study, we show that TN treatment induces a substantial *grp78/grp94* transcriptional response. This observation is important for two reasons. First, it provides evidence that the *grp78/grp94* stress response is not already maximally induced in WEHI7.2 cells. Second, it suggests that the *grp78/grp94* stress response induced by Ca^{2+} mobilization may be regulated differently than that induced by TN. Ca^{2+} mobilization and inhibition of glycosylation have been shown to induce *grp78/grp94* transcription through common promoter elements (12). Therefore, the deficiency in the TG-induced *grp78/grp94* transcriptional response observed in WEHI7.2 cells is unlikely to reside at the promoter level. One possible explanation for our findings is that two independent ER-to-nucleus *grp78/grp94* signaling pathways may exist: one Ca^{2+}-mediated and the other mediated by glycosylation inhibition. Both pathways are operative in fibroblasts and epithelial cells, which induce *grp78/grp94* in response to both TG and TN, but only the glycosylation inhibition signaling pathway appears to be operative in WEHI7.2 cells.

Little is known about the ER-to-nucleus signaling pathway that activates *grp78/grp94* transcription. ER-to-nucleus signaling may be Ca^{2+}/calmodulin-regulated (37) or may be mediated through tyrosine kinases and/or serine/threonine kinases (38, 39). Recently, it has been shown that IRE1p (Ern1), a yeast transmembrane serine/threonine kinase required for the induction of *KAR2*, the yeast homologue of *grp78*, may play a role in the ER-to-nucleus signaling pathway mediating *KAR2/grp78* up-regulation in response to malfolded proteins (40, 41). Overexpression of IRE1p in fibroblasts produced a modest increase in the ability of transfectants to up-regulate *grp78* in response to TG treatment (39). The WEHI7.2 cell line described in this report may be a useful model for the delineation of ER-to-nucleus signaling pathways. For example, it will be interesting to determine whether or not expression of IRE1p/Ern1 restores Ca^{2+}-mediated *grp78/grp94* transcriptional induction in these cells, thus further elucidating the role of IRE1p/Ern1 proteins in the pathway of *grp78/grp94* induction.

Understanding ER-to-nucleus signaling pathways should provide insight into mechanisms that regulate apoptosis induction during ER Ca^{2+} pool depletion. Indeed, our findings suggest that cells deficient in *grp78* stress response signaling are more susceptible to TG-induced apoptosis than cells that mount a *grp78* stress response. These findings are consistent with those of earlier work by Lee and co-workers (26–28) in fibroblasts and epithelial cells, indicating that up-regulation of *grp78* and coordinately regulated *grp94*, in response to ER Ca^{2+} pool depletion, prevents cell death. Hence, when the

grp78/grp94 response was inhibited, fibroblasts died in response to treatment with agents that mobilize Ca^{2+} from the ER, including TG and the Ca^{2+} ionophore A23187. Using a *grp78* antisense plasmid, they demonstrated that the inability to up-regulate *grp78* resulted in increased cell death following A23187 treatment (26). Similarly, when *grp78* induction was inhibited by amplification of the *grp78* core promoter region, an increased sensitivity to A23187 was observed (27). Furthermore, when induction of *grp78/grp94* was inhibited by ribozyme cleavage of newly transcribed *grp94* mRNA, increased sensitivity to A23187 and TG was observed (28). Interestingly, abrogation of the *grp78/grp94* stress response did not enhance the cytotoxicity of TN, suggesting that the increase in *grp78/grp94* transcription provides specific protection against ER Ca^{2+} pool depletion (28). In agreement with their findings, we have found that WEHI7.2 and W.Hb12 cells were killed by TN (data not shown), even though TN increased *grp78/grp94* transcription.

Understanding the role of *grp78/grp94* in regulating cell death may have important implications for cancer therapy. In this regard, there is evidence that prior induction of *grp78* can make cells less susceptible to death following treatment with photodynamic therapy (42), the superoxide-generating anticancer agent doxorubicin (43), and the topoisomerase inhibitor etoposide (44).

In summary, the findings reported here have three important implications. First, the *grp78/grp94* stress response may be differentially regulated among different types of cells, with a much greater response observed in nonlymphoid cells than in lymphoid cells. Second, there may be at least two signal transduction pathways that mediate the *grp78/grp94* stress response, one in response to ER Ca^{2+} mobilization and the other in response to protein glycosylation inhibition. Third, regulation of the *grp78/grp94* stress response may be a major factor in deciding whether a cell lives or dies in response to disruption of intracellular Ca^{2+} homeostasis. Indeed, the absence of a Ca^{2+}-mediated *grp78/grp94* stress response may be the basis for the marked susceptibility of WEHI7.2 cells to TG-induced apoptosis.

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Baculovirus p35 and Z-VAD-fmk inhibit thapsigargin-induced apoptosis of breast cancer cells

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Programmed cell death, or apoptosis, is inhibited by the antiapoptotic oncogene, Bcl-2, and is mediated by a cascade of aspartate-specific cysteine proteases, or caspases, related to interleukin 1-β converting enzyme. Depending on cell type, apoptosis can be induced by treatment with thapsigargin (TG); a selective inhibitor of the endoplasmic reticulum-associated calcium-ATPase. The role of caspases in mediating TG-induced apoptosis was investigated in the Bcl-2-negative human breast cancer cell line, MDA-MB-468. Apoptosis developed in MDA-MB-468 cells over a period of 24–72 h following treatment with 100 nM TG, and was prevented by Bcl-2 overexpression. TG-induced apoptosis was associated with activation of caspase-3 and was inhibited by stable expression of the baculovirus p35 protein, an inhibitor of caspase activity. Also, TG-induced apoptosis was inhibited by treating cells with Z-VAD-fmk, a cell-permeable fluoromethylketone inhibitor of caspases. These findings indicate that TG-induced apoptosis of MDA-MB-468 breast cancer cells is subject to inhibition by Bcl-2 and is mediated by caspase activity. This model system should be useful for further investigation directed toward understanding the role of calcium in signaling apoptosis, and its relationship to Bcl-2 and the caspase proteolytic cascade.

Keywords: thapsigargin; apoptosis; Bcl-1; ICE-like protease

Introduction

Programmed cell death (PCD), or apoptosis, is important for proper development and homeostasis of tissues; thus, insight into PCD mechanisms will contribute to improved understanding and treatment of diseases in which the normal balance between cell proliferation and cell death is disrupted (Thompson, 1995). For example, the PCD process mediates both the physiologic involution of normal breast epithelium following lactation and the regression of breast cancer following hormonal manipulation or chemotherapy administration (McCloskey *et al.*, 1996).

Studies in the primitive nematode, *C. elegans*, have contributed considerably to our understanding of fundamental PCD mechanisms by proving that apoptosis involves a systematic, stepwise process of cellular destruction regulated by genes encoding either death inhibitors or effectors (Steller, 1995). For example, the *C. elegans* gene ced-9 encodes an inhibitor of cell death, whereas the ced-3 and ced-4 genes encode death effectors. Mammalian cells express genes homologous to ced-9 and ced-3, indicating that the apoptotic pathway is highly conserved. The mammalian homolog of ced-9 is bcl-2, a potent inhibitor of apoptosis that prevents PCD triggered by diverse death signals (Cory, 1995). The mammalian homolog of ced-3 is the cysteine protease, interleukin 1-β converting enzyme (ICE) (Yuan *et al.*, 1993). ICE, along with a number of ICE-like proteases, or caspases, compose a family of cysteine proteases that cleave proteins at aspartic acid residues (Henkart, 1996). Recent findings suggest that a caspase proteolytic cascade mediates cell death by cleaving selected target proteins (Martin and Green, 1995; Fraser and Evan, 1996). Bcl-2 appears to inhibit apoptosis by preventing caspase activation Chinnaiyan *et al.*, 1996; Jacobson *et al.*, 1996; Shimizu *et al.*, 1996). ICE-like proteases have been implicated as mediators of apoptosis in response to a variety of death signals, including trophic factor deprivation, protein kinase inhibition by staurosporine, and cytotoxic lymphocyte killing (Milligan *et al.*, 1995; Jacobson *et al.*, 1996; Martin *et al.*, 1996). Thus, activation of a caspase cascade may be a final common pathway where diverse cell death signals converge.

We have been investigating the mechanism of cell death signaling following inhibition of calcium ion uptake into the endoplasmic reticulum (ER) by the sesqueterpene lactone tumor promoter thapsigargin (TG). In lymphoma cells, TG treatment induces apoptotic cell death, which can be prevented by Bcl-2 overexpression (Lam *et al.*, 1993, 1994). In the present report, we show that TG also induces apoptosis in the human breast cancer cell line, MDA-MB-468, and that TG-induced apoptosis involves caspase-3 activation and is prevented by expressing the baculovirus anti-apoptotic protein p35, an inhibitor of caspases (Bump *et al.*, 1995). Also, we show that TG-induced apoptosis can be inhibited by treating cells with a cell-permeable, irreversible, tripeptide inhibitor of caspases, Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) (Jacobson *et al.*, 1996). These findings indicate that TG-induced apoptosis is mediated through a highly conserved PCD process regulated by Bcl-2 and mediated by caspases.

Results

When treated with 100 nM TG, MDA-MB-468 cells underwent apoptotic cell death. The distinction between viable cells and apoptotic cells was based on nuclear chromatin pattern, assessed by fluorescence microscopy of cells stained with acridine orange (Figure 1). The nuclear chromatin of viable cells was

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dispersed, whereas that of apoptotic cells was condensed into densely fluorescent apoptotic bodies. To test the effect of Bcl-2 on TG-induced apoptosis, MDA-MB-468 cells were stably transfected with pSFFV-neo-Bcl-2 or pSFFV-neo, producing 468-Bcl-2 and 468-neo cells, respectively. Bcl-2 was present at very low levels in 468-neo cells, but was readily detected by Western blotting in 468-Bcl-2 cells (Figure 2a). Following exposure to 100 nM TG, 468-neo cells underwent apoptosis, which evolved over a period of 24–72 h (Figure 2b). Thus, the kinetics of apoptosis induction were much slower than reported previously in lymphoma cells, where a peak in the percentage of apoptotic cells was detected within 12 h of adding 100 nM TG (McCormick *et al.*, 1997). TG-induced apoptosis was markedly repressed in 468-Bcl-2 cells, representative of multiple Bcl-2-expressing clones, indicating that TG-induced apoptosis is mediated through a Bcl-2-regulated pathway (Figure 2b).

To determine if TG-induced apoptosis is associated with caspase activation, the level of pro-caspase-3 (CPP32, Yama, or apopain) was assessed in MDA-MB-468 cells by Western blotting using a monoclonal antibody to human pro-caspase-3. During activation, pro-caspase-3 undergoes cleavage to two subunits that form the active heterotetramer (Henkart, 1996). The level of pro-caspase-3 declined within 48 h of TG addition, indicating conversion of pro-caspase-3 to an active form (Figure 3). Two methods were employed to determine whether or not ICE-like protease activation is a necessary step in TG-induced apoptosis. In the first, MDA-MB-468 cells were stably transfected with the pSFFV-neo expression vector into which the cDNA encoding p35, a baculovirus inhibitor of ICE-like proteases, had been cloned. Cells stably transfected with this vector are referred to as 468-p35 cells, while cells stably transfected with empty vector alone are referred to as 468-neo cells. p35 mRNA was detected by Northern hybridization in two 468-p35 subclones (1 and 4), but not in 468-neo cells (Figure 4). On light microscopy, healthy 468-neo cells have a somewhat elongated appearance and are adherent to plastic tissue culture wells, whereas apoptotic cells have a small rounded appearance and are non-adherent (Figure 5). Following treatment of 468-neo cells with TG, there was a marked increase in the proportion of apoptotic cells. Untreated 468-p35 cells have an appearance similar to that of 468-neo cells, except that fewer spontaneously apoptotic cells were observed in culture. In addition, fewer apoptotic cells were detected in cultures of 468-p35 cells following TG treatment compared to TG-treated cultures of 468-neo cells. To corroborate these findings, the percentage of apoptotic cells following TG treatment was quantitated by fluorescence microscopy (Figure 6a). The data confirm the inhibition of TG-induced apoptosis by p35.

In a second approach, cells were treated with TG in the presence or absence of the peptide fluoromethylketone inhibitor Z-VAD-fmk. The tripeptide sequence in Z-VAD-fmk corresponds to the P_1 to P_3 residues of the pro-IL-1β cleavage site (Tyr^{4}ValAlaAsp^{1}Gly), where ICE cleaves between the Asp and Gly residue (Yuan *et al.*, 1993). Deletion of the Tyr residue broadens the inhibitory spectrum to include not only ICE, but other proteases closely related to ICE. An effective treatment regimen was empirically derived in which cells were treated with 200 μM doses of Z-VAD-fmk added 1 h prior to TG, and every 12 h thereafter over a period of 48 h. Z-VAD-fmk had a marked inhibitory effect on

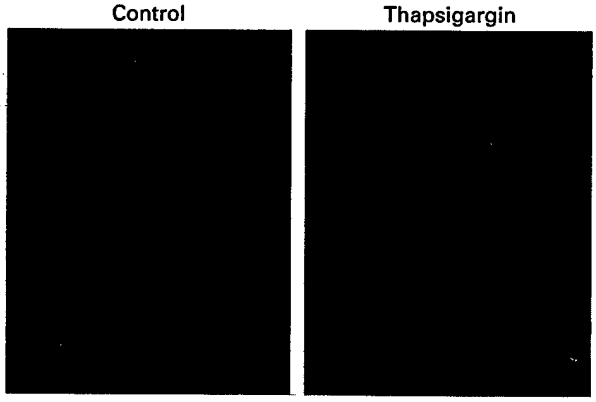

Figure 1 TG induces apoptosis of MDA-MB-468 cells. Cells in exponential phase of cell growth were treated with 100 nM TG dissolved in DMSO. A replicate cell culture, treated with an equivalent amount of DMSO, served as a control. Nuclear morphology of cells stained with ethidium bromide and acridine orange was visualized by fluorescence microscopy 48 h after adding TG or DMSO. Small dense fluorescent bodies seen in TG-treated cells represent nuclear chromatin condensation characteristic of apoptosis

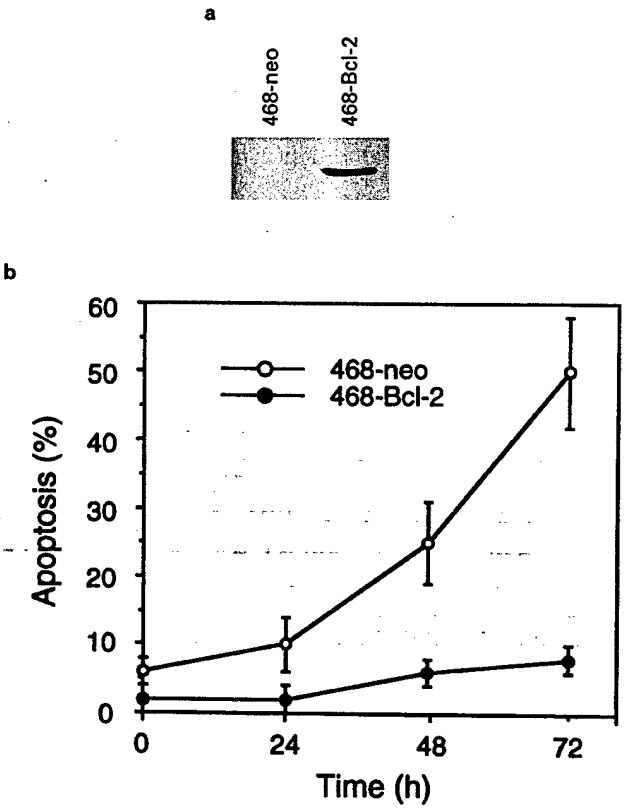

Figure 2 Inhibition of TG-induced apoptosis by Bcl-2. (**a**) Protein was isolated from MDA-MB-468 cells that had been stably transfected with pSFFV-neo (468-neo) or pSFFV-neo-Bcl-2 (468-Bcl-2). Protein (50 μg) was resolved by SDS–PAGE and analysed by Western blotting using a Bcl-2 monoclonal primary antibody. (**b**) The percentage of 468-neo and 468-Bcl-2 cells with an apoptotic nuclear morphology at various times after adding 100 nM TG was measured by fluorescence microscopy as described in Figure 1. Symbols represent the mean ± s.e. of four experiments

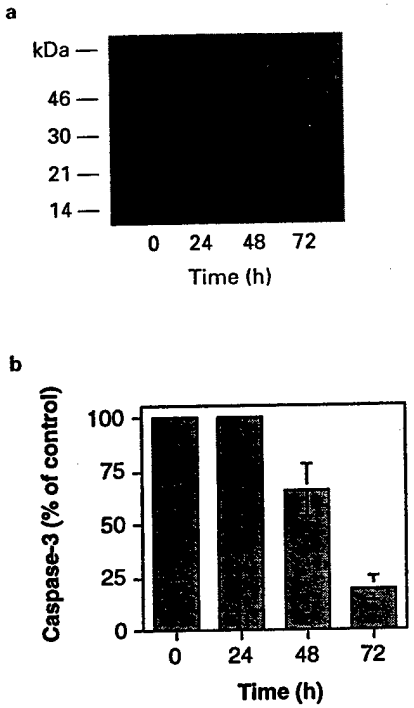

Figure 3 Caspase-3 activation by TG. (**a**) Protein was isolated from MDA-MB-468 cells at time intervals following addition of 100 nM TG and subjected to Western blotting to detect the level of caspase-3 (CPP32/Yama/Apopain). (**b**) The level of caspase-3 on Western blots was measured by densitometry. Symbols represent the mean ± s.e. of three experiments

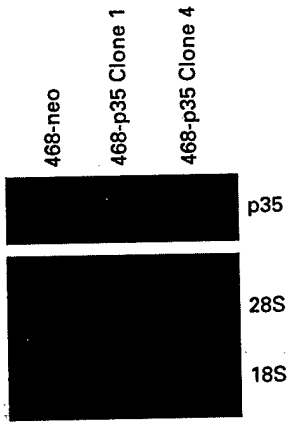

Figure 4 Northern blot analysis of p35 mRNA in MDA-MB-468 cells. RNA was isolated from MDA-MB-468 cells that had been transfected with pSFFV-neo (468-neo) and pSFFV-neo-p35 (468-p35, clones 1 and 4), and analysed by Northern hybridization using a cDNA probe for p35 (top panel). The bottom panel shows ethidium bromide-stained 28S and 18S rRNA from each sample, indicating equivalent RNA loading

TG-induced apoptosis (Figure 6b), whereas Z-FA-fmk, a cathepsin inhibitor, actually induced cell death (data not shown).

Discussion

In experiments reported here, we found that TG treatment induces apoptosis of MDA-MB-468 cells, an estrogen receptor negative human breast cancer cell line that has been employed by others to investigate

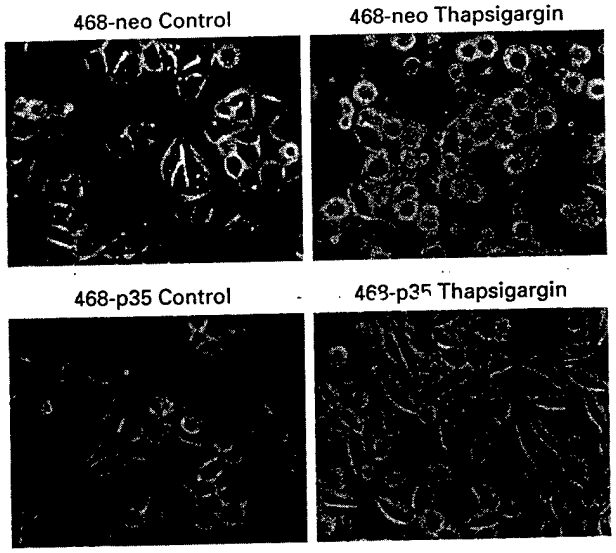

Figure 5 Effect of baculovirus p35 on TG-induced cell death. MDA-MB-468 transfectants, 468-neo and 468-p35 (clone 1), were treated with either 100 nM TG dissolved in DMSO or an equivalent volume of DMSO (control). Shown are photographs of the cells 48 h after treatment was initiated

PCD in breast cancer (Armstrong *et al.*, 1992, 1994). Our findings add to the number of recognized cell types in which TG treatment induces apoptosis, including normal thymocytes (Jiang *et al.*, 1994), lymphoma cells (Lam *et al.*, 1993, 1994) and prostate carcinoma cells (Furuya *et al.*, 1994). However, not all cell types readily undergo apoptosis following TG treatment. For example, smooth muscle cells, fibroblasts and epithelial cells have been reported to undergo growth arrest, without loss of viability, following TG treatment (Ghosh *et al.*, 1989; Li *et al.*, 1993). In the case of smooth muscle cells, survival in the presence of TG is mediated through induction of TG-insensitive calcium pumps that restore calcium homeostasis within the ER lumen (Waldron *et al.*, 1995); whereas in the case of fibroblasts and epithelial cells, maintenance of cell viability following TG treatment is dependent upon the induction of the grp78 and grp94 genes encoding the resident ER calcium-binding proteins, GRP78 and GRP94 respectively (Li *et al.*, 1993). Moreover, the rate of cell death induction following TG treatment also varies among different types of cells. For example, in lymphoma cells, TG treatment induces rapid onset of apoptosis within less than 12 h; whereas, as shown in the present study, induction of apoptosis in breast cancer cells evolves more slowly following TG treatment. In part, the kinetics of cell death induction in TG-treated cells may depend upon the magnitude of grp78/grp94 induction, a concept based on the observation that grp78/grp94 induction is detected in TG-treated breast cancer cells, but not in TG-treated lymphoma cells (McCormick *et al.*, 1997).

The mechanism of apoptosis induction by TG is not fully understood, but appears to be directly related to inhibition of ER calcium-ATPase activity, which produces sustained elevation of cytosolic calcium and sustained depletion of the ER calcium pool (Thastrup *et al.*, 1990). Apoptosis induction may be signaled in response to cytosolic calcium elevation, consistent with evidence that factors interfering with cytosolic calcium elevation inhibit TG-induced apoptosis (Jiang *et al.*,

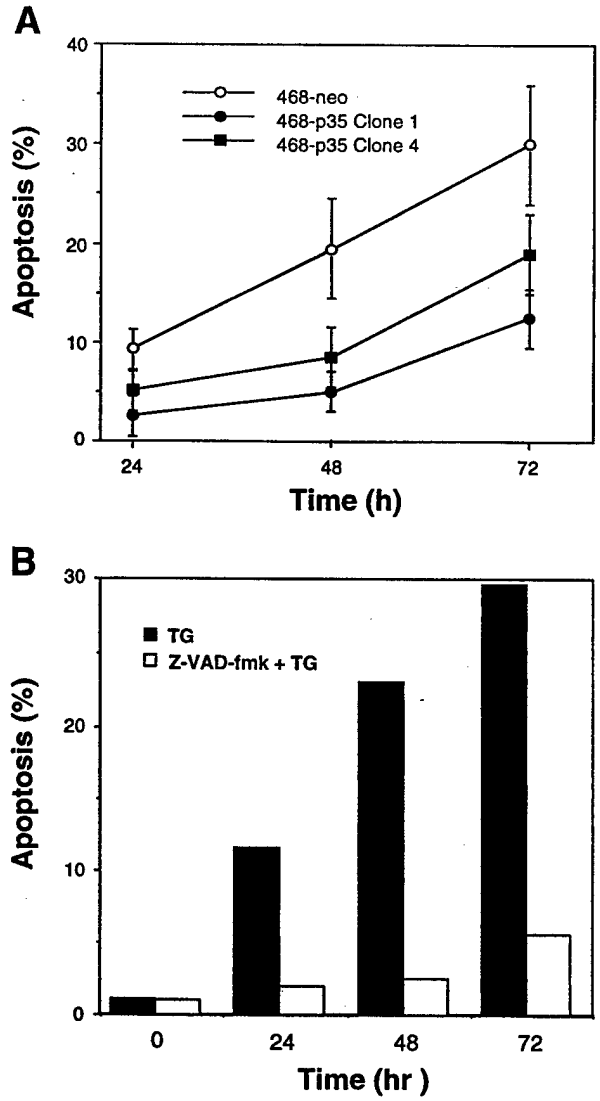

Figure 6 Effect of baculovirus p35 and Z-VAD-fmk on TG-induced apoptosis. (a) MDA-MB-468 transfectants, 468-neo and 468-p35 (clones 1 and 4), were treated with 100 nM TG. The percentage of cells with an apoptotic nuclear morphology was determined by fluorescence microscopy at the indicated times after adding TG. Symbols represent the mean±s.e. of multiple cell counts in two separate experiments. (b) MDA-MB-468 cells were treated with 100 nM TG in the presence or absence of Z-VAD-fmk. TG (100 nM) was added to cells at time 0. Z-VAD-fmk (200 μM) was added to one set of cells 1 h before adding TG and at 12, 24, and 36 h thereafter. The percentage of cells with an apoptotic nuclear morphology was determined by fluorescence microscopy at the indicated time points after adding TG. Symbols represent the mean of multiple determinations in a single experiment that was repeated once with the same result

1994; Furuya *et al.*, 1994). Also, it is possible that ER calcium pool depletion may trigger apoptosis, a concept that is both supported by experimental evidence (Reynolds and Eastman, 1996) and consistent with the fact that the ER calcium pool is essential for a number of vital cellular functions, including protein processing (Lodish *et al.*, 1992), signal transduction (Schonthal *et al.*, 1991; Little and Lee, 1995), and cell division (Short *et al.*, 1993). Because Bcl-2 is associated with the ER/perinuclear membrane, in addition to the outer mitochondrial membrane, we suggested the possibility that Bcl-2 might inhibit apoptosis by regulating calcium fluxes across intracellular membranes (Lam *et al.*, 1994), a concept that is intriguing in view of recent evidence that Bcl-XL, a Bcl-2 homolog, has a structure consistent with that of an ion channel (Muchmore *et al.*, 1996).

The present report extends our understanding of TG-induced apoptosis by providing evidence of involvement of ICE-like proteases. One type of evidence is the inhibition of TG-induced apoptosis by baculovirus p35, an inhibitor of a number of ICE-family proteases (Bump *et al.*, 1995). Expression of p35 has been used by others to document the involvement of ICE-like proteases in mediating PCD in insect cells (Cartier *et al.*, 1994), *C. elegans* (Sugimoto *et al.*, 1994), *Drosophila* (Hay *et al.*, 1994), and mammalian cells (Rabizadeh *et al.*, 1993; Beidler *et al.*, 1995). Therefore, inhibition of TG-induced cell death in MDA-MB-468 cells expressing p35 suggests that ICE-like protease activity is essential for apoptosis induction by TG. Although p35 mRNA expression in transfected cells was detected by RT–PCR, p35 protein was not detected (data not shown). This is consistent with earlier data indicating that low level p35 expression is sufficient to inhibit apoptosis (Hershberger *et al.*, 1994). Moreover, inhibition of ICE-like proteases by p35 involves p35 cleavage, reducing the level of intact p35 protein available for detection by Western blotting (Bump *et al.*, 1995; Xue and Horvitz, 1995).

A second type of evidence for involvement of ICE-like proteases in TG-induced apoptosis is the inhibition observed in cells exposed to Z-VAD-fmk. The use of this reagent to confirm the role of ICE-like proteases in mediating apoptosis in response to several different signals has been reported previously (Zhu *et al.*, 1995; Jacobson *et al.*, 1996). It is important to note that the concentration of Z-VAD-fmk employed in our experiments, although in the micromolar range, did not repress cell growth or decrease cell viability, whereas a control peptide fluoromethylketone, Z-FA-fmk, was toxic to cells.

In summary, the findings of the present study indicate that TG induces apoptosis of MDA-MB-468 cells, mediated through a highly conserved, Bcl-2-regulated process that involves ICE-like proteases. This model system should be useful for further investigation directed toward understanding the role of calcium in apoptosis signaling, and its relationship to Bcl-2 and the ICE-like proteolytic cascade.

Materials and methods

Materials

TG was purchased from LC Laboratories and serum from Hyclone. Antibiotics were from Gibco BRL. Benzyloxycarbonyl-Val-Ala-Asp (O-methyl)-fluoromethylketone (Z-Val-Ala-Asp (O-Me)-CH_2F; Z-VAD-fmk) and benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-Phe-Ala-fmk; Z-FA-fmk) were from Enzyme Systems Products, Inc. All other chemicals, unless noted otherwise, were obtained from Sigma. Monoclonal antibody to human pro-caspase-3 was from Transduction Laboratories.

Cell Culture and Treatment Conditions

MDA-MB-468 human breast cancer cells (from M Lippman, Georgetown University) were cultured in Improved Minimal

Essential Medium (Biofluids) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C in a 7% CO_2 atmosphere.

A 1 mg/ml stock of TG was made in DMSO and stored in aliquots at −20°C. A working stock was prepared by diluting TG in fresh culture medium to a final concentration of 1 μM. TG was then added to the cell cultures to achieve the desired final concentrations as noted in the text. Untreated cultures received the same volumes of DMSO without TG. Z-FA-fmk and Z-VAD-fmk were dissolved in DMSO to give a final concentration of 50 mM and added to 1 ml culture wells at a final concentration of 100 or 200 μM respectively 1 h before adding TG and every 12 h thereafter for a total of four additions.

Cell viability and apoptosis assays

Cells were counted on a hemocytometer after suspension in trypan blue dye. Viability was defined by the ability of cells to exclude trypan blue dye. Apoptotic cells were detected by fluorescence microscopy, using a previously published method (McCormick *et al.*, 1997). Briefly, five million cells were gently pelleted and resuspended in 0.2 ml tissue culture medium and both ethidium bromide and acridine orange were added from 100 μg/ml stock solutions to achieve 4 μg/ml final concentrations of each. Cells were examined under a glass coverslip with u.v. illumination using a Nikon Optiphot microscope and cells were scored as apoptotic according to nuclear morphological changes, including chromatin condensation and apoptotic body formation.

Western blotting

Levels of Bcl-2 in cell lysates were measured by Western blotting as described previously using the 15131A monoclonal antibody (PharMingen) (Lam *et al.*, 1994). To assess the level of caspase-3 at various time points after treatment with 100 nM TG, cells were washed twice with ice cold phosphate buffered saline and then lysed for 40 min in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% triton-X-100, 0.1% SDS, 1 mM PMSF, 1 μg/ml leupeptin). The lysates were centrifuged in a microfuge at 13 200 r.p.m. for 10 min at 4°C, the supernatants collected and the protein concentrations determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, California). Thirty μg of each protein lysate was resolved by electrophoresis on a 14% SDS-polyacrylamide gel under reducing conditions, and transferred to PVDF membrane (Millepore Co., Bedford, Massachusetts). The membranes were incubated in 5% non-fat dry milk in T-TBS (18 mM Tris-HCl, pH 7.6, 122 mM NaCl, 0.1% Tween-20) at room temperature for 2 h and then incubated with monoclonal antibody to human caspase-3.

(Transduction Laboratories, Lexington, Kentucky) at 1:1000 dilution at 4°C for 18 h, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (GIBCO BRL, Gaithersburg, Maryland). The immune complexes were detected with the ECL Western blotting detection reagents (Amersham Corporation, Arlington Heights, Illinois) according to the manufacturer's protocol, followed by exposure to X-ray film (Sigma, St Louis, Missouri).

Vector construction and transfection

MDA-MB-468 cells were transfected by the calcium phosphate method (GIBCO/BRL Calcium Phosphate Transfection System) with the pSFFV-Bcl-2 expression vector, described previously (Cuende *et al.*, 1993). Stable transfectants were isolated by resistance to G418. The full length cDNA encoding baculovirus p35 (P Friesen) was cloned in the sense direction into the pSFFV-neo vector (G Nunez), producing pSFFV-p35. MDA-MB-468 cells were transfected with either pSFFV-neo or pSFFV-p35 by the calcium phosphate method and stably transfected, G418-resistant, clones were isolated.

Northern blotting

Total cellular RNA was prepared using TRIZOL Reagent (GIBCO BRL). Thirty μg RNA per sample was separated by electrophoresis in a 1.2% agarose gel and blotted onto a nitrocellulose membrane. The blot was prehybridized at 65°C for 30 min and hybridized with α-^{32}P-dCTP-labeled (Stratagene Prime-It II Kit) plasmid pPRM-35K-ORF containing the full length cDNA for baculovirus p35 (Hershberger *et al.*, 1994).

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