Effects of Alterations in Calcium Homeostasis on Apoptosis during Neoplastic Progression

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ABSTRACT

Our previous studies showed that early, stage I preneoplastic cells (sup"I) are highly susceptible to apoptosis, whereas the later, stage II preneoplastic cells (sup"II) are relatively resistant. To examine possible mechanisms that might explain these differences in the regulation of apoptosis, Ca²⁺ homeostasis was analyzed and comparisons were made between these two Syrian hamster embryonic cell lines. The Ca²⁺ indicator, fura-2, and fluorescent microscopy were used to measure intracellular free calcium concentrations, [Ca²⁺]ᵢ. The results indicated that the [Ca²⁺]ᵢ level in logarithmically growing sup"I cells (~100 nM) was considerably lower than that observed in sup"II cells (~260 nM). Serum removal resulted in a reduction of [Ca²⁺]ᵢ in the sup"I cells (~82 nM), whereas the [Ca²⁺]ᵢ level in sup"II cells did not change. Endoplasmic reticulum (ER) calcium levels were determined by measuring thapsigargin-releasable Ca²⁺. Reduced ER calcium was consistently observed in cells induced to undergo apoptosis. Specifically, thapsigargin-releasable Ca²⁺ was greatly reduced in sup"I cells (45 nM) as compared to sup"II cells (190 nM) after 4 h in low serum. When sup"II cells were placed under conditions that resulted in apoptosis (thapsigargin or okadaic acid), decreased ER calcium was observed. To determine whether reduced ER calcium had a causative effect in apoptosis, ER calcium levels were exogenously increased in sup"I cells by raising extracellular Ca²⁺ to 3 mM; ER calcium levels were maintained, and apoptosis was blocked. Studies were performed to determine whether the decrease in ER calcium could be attributed to reduced Ca²⁺ influx at the plasma membrane. To measure directly whether Ca²⁺ entry was decreased in sup"I cells in 0.2% serum, Mn²⁺ uptake was used to monitor Ca²⁺ influx. The data show that in low serum, the rate of thapsigargin-induced Mn²⁺ entry in sup"I cells was approximately 50% lower than that of sup"II cells, demonstrating that capacitative entry is reduced in sup"I cells. In further support of this hypothesis, thapsigargin-treated sup"I cells (0.2% serum) showed decreased Ca²⁺ entry upon raising extracellular Ca²⁺ from 0 to 2 mM. We report the novel finding that early preneoplastic cells, which exhibit a high propensity to undergo apoptosis, have decreased calcium entry at the plasma membrane, resulting in decreased ER calcium pools.

INTRODUCTION

Tumor development can occur either by disruption of cell cycle arrest, decreased rates of cell death, or both (1–3). Although tumor cells generally retain the intrinsic ability to undergo apoptosis, specific induction signals can be defective (1). A focus of our investigations has been to study the dysregulation of apoptosis during the neoplastic process. Our earlier work had shown that alterations in the regulation of apoptosis can be an early event in tumorigenesis (2). Specifically, we showed that cells at an early, preneoplastic stage of progression have a sharp increase in the activation of apoptosis in low serum conditions when compared to normal cells. Furthermore, cells at a later stage of progression, but prior to becoming tumorigenic, show a dramatic decrease in apoptotic cell death. Although these data provide insight into when changes in the regulation of apoptosis homeostatic processes might occur during progression, it is necessary to extend these studies to determine how a cell might modulate signals to block apoptosis. There is some evidence that Ca²⁺ signaling is involved in apoptosis, although the role of Ca²⁺ is still unclear (3–7). It was originally proposed that an increase in cytosolic free Ca²⁺ concentration, [Ca²⁺]ᵢ, was of primary importance during apoptosis (8), and that Ca²⁺ was required for the activation of an endonuclease responsible for DNA nucleosomal cleavage (9). However, in some systems, apoptosis has been observed in the absence of any detectable rise in [Ca²⁺]ᵢ (5, 10, 11). In fact, apoptosis induced by growth factor withdrawal can be suppressed by increasing [Ca²⁺]ᵢ (12). Studies investigating Ca²⁺ compartmentation during apoptosis in lymphocytes have shown an association between apoptosis and decreased ER calcium pools (13, 14). Furthermore, a recent publication proposes that maintenance of high nuclear calcium levels can dictate whether a cell will survive or die (15). Interestingly, studies suggesting that transformation is associated with modulations of Ca²⁺-sensitive signaling pathways have shown that lowering of extracellular Ca²⁺ resulted in a reversible block in the cell cycle of normal cells, whereas SV40-transformed cells continued to proliferate in low Ca²⁺ levels (16).

Calcium oscillations responsible, in part, for Ca²⁺ signaling are produced by the generation of InsP₃ and the subsequent release of ER calcium. Most of the Ca²⁺ in the cell is sequestered and resides in the ER (8). The steep concentration gradient from the cytosol to the ER lumen is maintained by an endoplasmic reticulum-Ca²⁺-ATPase pump. Transitory release of ER calcium can be stimulated by many hormones, which generate InsP₃, InsP₄ elevation results in the release of ER calcium, and this release is coupled with an influx of external Ca²⁺ (17). This coupling, termed capacitative entry, appears to be regulated by the calcium content of the ER (10). The mechanism of capacitative entry is debated, but many studies suggest a role for phosphorylation/dephosphorylation (11–13), small G-proteins (18), and diffusible messengers (19).

Two preneoplastic Syrian hamster embryo (SHE) cell lines were used for the studies presented here. One cell line, isolated as an immortal clone, is representative of early stage, preneoplastic cells and has been termed sup"I (tumor suppressor gene plus), because these cells suppress tumorigenicity when hybridized with tumor cells. The other cell line, termed sup"II (tumor suppressor gene minus), is representative of a later stage of preneoplastic cells; these cells no longer have the capability to suppress tumorigenicity in cell hybrids (20). The tumor suppressor gene altered in sup"II cells has not been conclusively identified, although the cells differ in the expression of putative suppressor genes, H19 and tropomyosin I (21). The RB and p53 genes are wild type in both cell types (22). sup"I cells are highly
susceptible to activation of apoptosis under antiproliferative conditions, whereas sup II cells, although not tumorigenic, display a decreased susceptibility to apoptotic death.

\[ [Ca^{2+}]_i = K_d \times \frac{(R - R_{min})(R_{max} - R)}{S_F/S_b} \]

\( R_{min} \) and \( R_{max} \) are the excitation wavelength ratios at 340:380 nm for uncomplexed (excess EGTA) and \( Ca^{2+} \)-saturated fura-2, respectively. \( S_F/S_b \) are the fluorescence intensities measured at 380 nm excitation for fura-2 with excess EGTA and with excess \( Ca^{2+} \), respectively. \( R \) is the 340:380 excitation ratio of the sample to be measured. The previously determined \( K_d \) of 224 nM (24) was used. \( R_{min} \), \( R_{max} \), and \( S_F/S_b \) were measured in fura-2-loaded cells following the addition of ionomycin in the presence of excess \( Ca^{2+} \) and excess EGTA. The values obtained agree well with those determined in a solution containing 120 mM KCl, 20 mM NaCl, and 2 \( \mu \)M fura-2, buffered with 4-morpholinepropanesulfonic acid to pH 7.05.

Thapsigargin inhibits the ER \( Ca^{2+} \)-ATPase, and due to continued release of \( Ca^{2+} \) through the ER calcium release channel, this leads to a rapid release of ER calcium into the cytosol that can be measured by fura-2 (25). To reduce capacitative \( Ca^{2+} \) entry (26), cells were placed in \( Ca^{2+} \)-free PBS. Thapsigargin (Calbiochem) was added to a final concentration of 2 \( \mu \)M from a 100X stock in DMSO. The addition of vehicle (DMSO) showed no effect on fura-2 fluorescence.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture Conditions.** Normal, diploid SHE cell lines were established as described by Koi and Barrett (23). The preneoplastic stage I immortalized cell lines were derived after treatment of normal SHE cells with carcinogen (23). Early passage cells (sup+) retained the ability to suppress tumorigenicity when hybridized with tumor cells. Later passage cell populations were subcloned, and cell variants were isolated that no longer displayed tumorigenicity when hybridized with tumor cells. Concomitant with the loss of suppressor gene function was the loss of the dependency for attachment for cell growth demonstrated by growth in soft agar (20). Cells were maintained in Dulbecco’s modified IBM medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Rehydratin), 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin and were incubated at 37°C in 10% CO2 in air.

**Fluorescence Measurements of \([Ca^{2+}]_i\).** Cells were grown on 22-mm diameter (0 thickness) round glass coverslips (Carolina Biological Supply Co.). Fura-2 was introduced into the cells by the addition of 2 \( \mu \)M fura-2-AM to each coverslip and incubation at 37°C for 20 min. Coverslips were washed twice with the appropriate medium (10 or 0.2% serum) and then incubated for an additional 15 min to allow recovery from the loading. The coverslips, containing the fura-2-loaded cells, were placed in a custom-built, temperature-controlled holder that was placed on the stage of a Nikon inverted epifluorescence microscope, coupled to a PFI Deltacin dual wavelength excitation spectrophotometer. Measurements were taken over an area containing two to three cells. Excitation spectra were run from 300 to 420 nm, with emission measured at 510 nm. Time-dependent changes in emission fluorescence were measured in response to simultaneous excitation of the preparation with 340 and 380 nm wavelengths. Autofluorescence, measured at the same wavelengths on cells from the same culture that were not loaded with fura-2, was subtracted from the respective excitation fluorescence spectra of fura-2-loaded cells. \([Ca^{2+}]_i\) was calculated using the following equation, as described by Grynkiewicz et al. (24):

**RESULTS**

**Measurement of \([Ca^{2+}]_i\).** \( Ca^{2+} \) homeostasis was assessed in cells that undergo apoptosis in low serum and in those that do not. \([Ca^{2+}]_i\) was measured at various times after incubation of cells in either 10 or 0.2% serum. Cells were loaded with the fluorescent \( Ca^{2+} \) indicator, fura-2, 30 min prior to the measurement. To assure that we were studying changes in \([Ca^{2+}]_i\) that occurred prior to and not as a result of cell death, changes in \([Ca^{2+}]_i\) were measured prior to DNA fragmentation, which can be observed at 8 h. Initially, \([Ca^{2+}]_i\) was measured after 1, 4, and 8 h in 0.2% serum. At no time was an increase in \([Ca^{2+}]_i\), observed; however, a slight decrease in \([Ca^{2+}]_i\), was consistently observed at 4—5 h. Therefore, subsequent studies were performed at 4 h after removal of serum. Fig. 1 shows data collected after 4 h in either 10 or 0.2% serum. sup+I cells in 10% serum had a mean \([Ca^{2+}]_i\) of 100 ± 5 nm (n = 61 from 11 cultures); this value decreased significantly to 82 ± 4 nm (n = 96 from 12 cultures) after 4 h in 0.2% serum. sup-II cells in 10% serum had a mean \([Ca^{2+}]_i\) of 260 ± 28 nm (n = 14 from six cultures) and after 4 h in 0.2% serum, \([Ca^{2+}]_i\), averaged 282 ± 33 nm (n = 13 from six cultures). It is worth noting that sup-I cells had a significantly lower \([Ca^{2+}]_i\) (100 ± 5 nm) than the sup-II cells (260 ± 28 nm) in 10% serum.

**Depletion of ER Calcium Results in Apoptosis.** To compare differences in ER calcium sequestration between cell types, sup+I and sup-II cells were treated with thapsigargin, a specific inhibitor of the sarcoplasmic reticulum/ER \( Ca^{2+} \)-ATPase. The addition of thapsigarin-

![Fig. 1. Cytosolic free \( Ca^{2+} \) measured in cells incubated in 10 or 0.2% serum (4 h). Measurements were made on fura-2-loaded cells bathed in medium containing either 10 or 0.2% serum. Values are expressed as means; bars, SE.](image-url)
REduced calCium influx during apoptosis

serum were treated with OKA (1 nM), an inducer of apoptosis in these cells. As predicted, not only did the cells die by apoptosis (Fig. 4), but they showed reduced ER calcium (Fig. 5B). Also, the addition of diamide, an oxidizing agent, to sup"I cells in 10% serum caused DNA fragmentation and reduced ER calcium from 113 to 47 nm (Fig. 5A).

Mechanism for the Decrease in ER Calcium. If the decrease in ER calcium is causally related to apoptosis, then blocking the decrease in ER calcium should block apoptosis. To accomplish this, sup"I cells were placed in high extracellular Ca\(^{2+}\) (3 mM). Raising extracellular Ca\(^{2+}\) increases the driving force for net Ca\(^{2+}\) entry into the cell and also increases the energy requirement for Ca\(^{2+}\) efflux. The decline in ER calcium in sup"I cells (0.2% serum) was blocked by 3 mM Ca\(^{2+}\) (Fig. 5A), and DNA fragmentation was blocked also (Fig. 4). These data suggest that the sup"I cells in low serum have an alteration in the Ca\(^{2+}\) transport across the plasma membrane. Depletion of ER calcium normally leads to the opening of a plasma membrane channel (\(I_{\text{CRAC}}\)), which results in an elevation of [Ca\(^{2+}\)^{cytosol}], and refilling of ER calcium. If there was defective capacitative Ca\(^{2+}\) entry in sup"I cells in low serum, then a signal to release ER calcium, such as by InsP\(_3\), would lead to a prolonged decrease in ER calcium. To directly measure whether capacitative Ca\(^{2+}\) entry was decreased in sup"I cells in 0.2% serum, we used Mn\(^{2+}\) uptake to monitor Ca\(^{2+}\) influx (29). Depletion

gin resulted in a release of ER calcium into the cytosol, where it was measured by fura-2 as an increase in [Ca\(^{2+}\)^{cytosol}]. (25, 27, 28). To block capacitative Ca\(^{2+}\) entry (26), these studies were done in the absence of extracellular Ca\(^{2+}\). Fig. 2 shows a typical fluorescence trace of sup"I and sup"II cells treated with thapsigargin (2 \(\mu\)M). Cells were incubated for 4 h in either 10% (Fig. 2A) or 0.2% serum (Fig. 2B), washed, and placed in Ca\(^{2+}\)-free PBS for measurement of thapsigargin-releasable Ca\(^{2+}\). A summary of multiple experiments (Fig. 3) measuring thapsigargin-releasable ER calcium has been plotted as a net increase in [Ca\(^{2+}\)^{cytosol}]. Thapsigargin-releasable Ca\(^{2+}\) in sup"I cells was 113 ± 26 nm and in sup"II cells was 178 ± 45 nm in 10% serum controls. However, in the sup"I cells in 0.2% serum, thapsigargin-releasable Ca\(^{2+}\) was much lower (56 ± 9 nm) than in sup"II cells in 0.2% serum (194 ± 31 nm).

If ER calcium depletion is involved in initiating apoptosis, then agents that deplete ER calcium, such as thapsigargin, should induce apoptosis in cells that do not normally undergo apoptosis (e.g., sup"I cells in 10% serum and sup"II cells in 0.2 and 10% serum). This supposition was supported by the fact that when sup"I and sup"II cells were treated with 0.2 \(\mu\)M thapsigargin in 10% serum, apoptosis was induced (Fig. 4). Other agents that induce apoptosis should also deplete ER calcium. To test this hypothesis, sup"II cells in 0.2%
REDUCED CALCIUM INFLUX DURING APOPTOSIS

Table I Ca\textsuperscript{2+} influx measured as the rate of thapsigargin-induced Mn\textsuperscript{2+} quenching of fura-2 fluorescence

<table>
<thead>
<tr>
<th>Cells</th>
<th>Serum concentration (%)</th>
<th>Initial rate of quenching (photon decay/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sup\textsuperscript{1}I</td>
<td>10</td>
<td>13,350 ± 4,700</td>
</tr>
<tr>
<td>sup\textsuperscript{1}I</td>
<td>0.2</td>
<td>7,450 ± 1,890</td>
</tr>
<tr>
<td>sup\textsuperscript{II}</td>
<td>10</td>
<td>11,570 ± 3,570</td>
</tr>
<tr>
<td>sup\textsuperscript{II}</td>
<td>0.2</td>
<td>16,050 ± 5,710</td>
</tr>
</tbody>
</table>

*a Values are means ± SE. Initial rate of quenching was measured by a linear fit of the initial 15 s.


density of ER calcium that occurs with the addition of thapsigargin has been shown to induce a Ca\textsuperscript{2+} influx across the plasma membrane (26). Mn\textsuperscript{2+} has been shown to enter the cell via this plasma membrane Ca\textsuperscript{2+} channel. Once inside the cell, Mn\textsuperscript{2+} can bind to the Ca\textsuperscript{2+} indicator fura-2 and quench the fluorescence (29). Thus, the rate of Mn\textsuperscript{2+} quenching of fura-2 fluorescence can be used as a measure of Ca\textsuperscript{2+} entry. Cells incubated in 0.2% serum for 4 h were placed in Ca\textsuperscript{2+}-free PBS, and thapsigargin was added to deplete ER calcium. The rate of quenching of fura-2 fluorescence (measured during the first 15 s) is a measure of thapsigargin-stimulated Mn\textsuperscript{2+} entry. The data in Table 1 show similar rates of thapsigargin-induced Mn\textsuperscript{2+} entry in both sup\textsuperscript{1} and sup\textsuperscript{II} cells in 10% serum. However, incubation in 0.2% serum resulted in a significant decrease in the rate of thapsigargin-induced Mn\textsuperscript{2+} entry in sup\textsuperscript{1} cells. The data indicate that capacitative entry is reduced in sup\textsuperscript{1} cells in low serum, which would account for the observed decrease in ER calcium. To test this further, capacitative Ca\textsuperscript{2+} entry was measured. Fig. 6A illustrates a typical fluorescence trace, and Fig. 6B gives a summary of a series of experiments. Thapsigargin was added to fura-2-loaded cells in Ca\textsuperscript{2+}-free PBS, and ER calcium release was measured by fura-2 in the cytosol (Fig. 6A, first arrow). After the ER calcium had been removed from the cytosol, most likely by extrusion via the plasma membrane Ca\textsubscript{2+}-ATPase, Ca\textsuperscript{2+} was added to raise the extracellular Ca\textsuperscript{2+} to 2 mM (Fig. 6B, second arrow). Calcium entry across the plasma membrane, induced by the addition of extracellular Ca\textsuperscript{2+} compared to

**DISCUSSION**

We report the novel finding that preneoplastic sup\textsuperscript{1} cells, which have a high propensity to undergo apoptosis, have decreased capacitative Ca\textsuperscript{2+} entry and decreased ER calcium, thus providing new insight into a mechanism responsible for increased apoptosis. We found that elevation of extracellular Ca\textsuperscript{2+} not only rescued cells in low serum from apoptosis but also blocked the loss of ER calcium, thus demonstrating that alterations in calcium homeostasis can be causally involved in apoptosis. Also, we found that induction of apoptosis by a wide variety of agents (low serum, okadaic acid, diamide, and thapsigargin) results in a decrease in ER calcium.

The concept has been proposed that an increase in [Ca\textsuperscript{2+}]\textsubscript{i} is involved in activating apoptosis (8, 30—33). We did not find a rise in [Ca\textsuperscript{2+}]\textsubscript{i} to be associated with apoptosis. Indeed, we observed a slight

![Graph A](image1.png)

**Fig. 4.** ER calcium measured as an increase in [Ca\textsuperscript{2+}]\textsubscript{i} after the addition of thapsigargin. A, sup\textsuperscript{1} cells incubated (4 h) in 10% serum, 0.2% serum, 10% serum plus diamide, or 0.2% serum plus extracellular Ca\textsuperscript{2+} (3 mM). B, sup\textsuperscript{II} cells incubated (4 h) in 10% serum, 0.2% serum, or 0.2% serum plus OKA (1 nM). Values are expressed as means; bars, SE.

![Graph B](image2.png)

**Fig. 5.** ER calcium measured as an increase in [Ca\textsuperscript{2+}]\textsubscript{i} after the addition of thapsigargin. A, sup\textsuperscript{1} cells incubated (4 h) in 10% serum, 0.2% serum, 10% serum plus diamide, or 0.2% serum plus extracellular Ca\textsuperscript{2+} (3 mM). B, sup\textsuperscript{II} cells incubated (4 h) in 10% serum, 0.2% serum, or 0.2% serum plus OKA (1 nM). Values are expressed as means; bars, SE.

![Graph C](image3.png)

**Fig. 6.** Calcium release-activated calcium channel entry. A, cells were treated with thapsigargin in Ca\textsuperscript{2+}-free PBS to cause release of ER calcium (first arrow). Extracellular Ca\textsuperscript{2+} was added (2 mM, second arrow), and capacitative Ca\textsuperscript{2+} was measured as changes in [Ca\textsuperscript{2+}]\textsubscript{i}. B, a summary of a series of experiments measuring capacitative Ca\textsuperscript{2+} entry, expressed as net increase in [Ca\textsuperscript{2+}]\textsubscript{i}. Bars, SE.
These findings are consistent with a growing body of data in which present in serum facilitate capacitative entry. The supI cells, which used to induce apoptosis, a decrease in ER calcium always precedes provide additional support to the proposal that alterations in calcium dependence on exogenous growth factors (20), which could account for decreased ER calcium in apoptosis is demonstrated by the fact that increasing extracellular Ca²⁺ to block the decrease in ER calcium reduces ER calcium. Thapsigargin, which inhibits ER calcium refill, induces apoptosis in both cell types. Furthermore, a causal role for decreased ER calcium in apoptosis is demonstrated by the fact that defects in capacitative Ca²⁺ entry have been reported in immunodeficient lymphocytes in which oxidizing agent, induces apoptosis in supI cells in 10% serum and from Ca²⁺ influx at the plasma membrane (36—38). Diamide, an oxidizing agent, induces apoptosis in supI cells in 10% serum and reduces ER calcium. OKA induces apoptosis in supII cells and reduces ER calcium. In some cell types, OKA uncouples ER calcium depletion from Ca²⁺ influx at the plasma membrane (36—38). Diamide, an oxidizing agent, induces apoptosis in supI cells in 10% serum and reduces ER calcium. Because the ER calcium ATPase operates near thermodynamic equilibrium (41), a decrease in [Ca²⁺], would ultimately lead to a decrease in ER free Ca²⁺ provided ER calcium release continues and that there is no change in available free energy from ATP (42). Furthermore, a steady-state decrease in [Ca²⁺], is most likely due to altered plasma membrane transport (42). Although an increased uptake or release of Ca²⁺ by an intracellular organelle can transiently alter [Ca²⁺], the intracellular organelles will quickly be saturated or emptied of Ca²⁺ and, therefore, cannot contribute to the long-term regulation of [Ca²⁺]. A plausible hypothesis to account for the decrease in [Ca²⁺], and the decrease in ER calcium in the supI cells is that in 0.2% serum, a signal is generated that leads to release of ER calcium. Normally, the depletion of ER calcium would stimulate a plasma membrane Ca²⁺ entry pathway that elevates [Ca²⁺], and also lead to refilling of ER calcium stores. This process has been termed capacitative Ca²⁺ entry (26). A release of ER calcium with impaired capacitative entry might be expected to result in decreased ER calcium and a decreased [Ca²⁺], and our data show this to be the case in the supI cells in low serum. Capacitative Ca²⁺ entry, as probed by Mn²⁺ quenching of fura-2 fluorescence and by Ca²⁺ influx upon addition of extracellular Ca²⁺ to thapsigargin-treated cells, was slower in supI cells in low serum compared to supII cells. In further support of the importance of Ca²⁺ entry, an elevation of extracellular Ca²⁺ blocked low serum-induced apoptosis and the decrease in ER calcium. Interestingly, defects in capacitative Ca²⁺ entry have been reported in immunodeficient lymphocytes in which cell proliferation is deficient (43). It has been reported also that capacitative Ca²⁺ entry is uncoupled from ER calcium depletion during mitosis (44). The supI cells, which undergo apoptosis, are deficient in capacitative Ca²⁺ entry but only when placed in low serum conditions. One explanation for this is that growth factors present in serum facilitate capacitative entry. The supII cells, which represent a later stage of neoplastic progression, show a decreased dependence on exogenous growth factors (20), which could account for normal capacitative Ca²⁺ entry, even in low serum. In support of this, the supI cells have higher basal [Ca²⁺], than the supII cells. Consistent with this concept, transformed Madin-Darby canine kidney cells exhibit spontaneous Ca²⁺ oscillations that are not present in normal Madin-Darby cells (34).

The present study has focused on establishing a role for ER calcium in apoptosis in nonlymphoid cells. The mechanism by which a decrease in ER calcium leads to apoptosis is beyond the scope of this study; however, it should be noted that there are several plausible hypotheses that would link a decrease in ER calcium and apoptosis. Calcium signals arising from the ER have been shown to be important in cell cycle progression (35). Thus, the lack of a crucial calcium signal due to reduced ER calcium stores might be involved in a signal for apoptosis. Alternatively, ER calcium depletion results in the repression of the initiation of transcription, and maintenance of ER calcium is necessary for maintaining high rates of protein synthesis (45). By blocking translation, the fate of the cell could be determined by the protein half-life of key apoptosis-regulating proteins. Interestingly, there are some data that suggest that Bcl-2 can influence Ca²⁺ partitioning that would normally result in ER depletion, and again, the data suggest that terminal depletion of the ER calcium occurs prior to apoptotic cell death (13). The relative expression of Bcl-2 family members in the cell lines used in our studies has not been examined; however, gene transfer-mediated overexpression of Bcl-2 protein suppresses apoptosis in the supI cells (46).

The tumor suppressor gene(s) responsible for the supI/supII phenotypes has not been determined; however, it has been shown that expression of the actin-binding protein tropomyosin is reduced in the supII cells (47). Reduced expression of tropomyosin has been shown to be directly associated with changes in cytoskeletal organization and anchorage-independent growth in these cells. The function of tropomyosin in skeletal muscle has been shown to be associated with the troponin complex in mediating the effects of calcium on the actin-myosin interaction in muscle contraction (48). Although tropomyosin is not a tumor suppressor gene per se, it is feasible that reduced tropomyosin could result in changes in Ca²⁺ homeostasis.

The level of apoptosis is related to tumor development and understanding the factors that control the level of apoptosis are important for better understanding the overall process of oncogenesis. Discerning the contribution of decreased ER calcium to apoptosis and the consequences of the converse is an important step in this understanding.

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