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Programmed Cell Death Pathways in Tumor Initiation and Progression

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The effects of cellular apoptotic regulators on tumor initiation and progression are difficult to predict, as oncogenes may have either anti-apoptotic (bcl-2) and pro-apoptotic (c-myc) effects. Anti-apoptotic genes may promote tumor growth by rescuing cells susceptible to apoptosis, due to DNA damage, for example. Pro-apoptotic genes, on the other hand, might increase DNA damage as a substrate for genetic instability. We tested the ability of transgenic bcl-2 to interact with DMBA in the formation of murine mammary tumors. A modest increase in tumor incidence at early time points (7-8 weeks post exposure) was observed in DMBA-treated transgenic vs control female mice. We have examined mammary tumors from transgenic mice for apoptosis using the in situ TUNEL assay. Unexpectedly, apoptotic cells were present at relatively high levels, compared to normal breast tissue (20.5 +/- 12.7/hpf vs 14.2 +/- 4.8/hpf).

Apatosis is often associated with increased oxidative stress, a potential cause of DNA damage. We have examined Rat-1 cells undergoing c-myc-regulated cell death for early changes in mitochondrial structure and function. Induction of apoptosis led to initial mitochondrial proliferation followed by collapse of mitochondrial transmembrane potential. Increased numbers of mitochondria with a disorganized cristae and ultracondensed morphology were observed in myc-induced apoptosis. Constitutive c-myc expression has been demonstrated to lead to genetic instability. An association between mitochondrial dysfunction, ROS, and genetic instability may explain the apparent retention of apoptotic programs in experimental and human tumors.

apoptosis, bcl-2, mitochondria, oxidative stress

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[Signature]

Principal Investigator's Signature

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Date
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Introduction

The bcl-2 gene is one of the best studied regulators of apoptosis, capable of inhibiting apoptotic cell deaths in a wide variety of experimental models (Hockenbery 1995). Several lines of evidence point to the importance of programmed cell death pathways as brakes against tumor development. The bcl-2 gene was first identified as an oncogene in follicular lymphomas bearing the t(14;18) chromosomal translocation. High bcl-2 expression in acute myelocytic leukemia correlates with poor chemotherapy responses and shortened survival (Campos and others 1993). In addition, many studies implicate p53 function in apoptotic death following gamma irradiation and exposure to certain chemotherapy drugs. Using a model of choroid plexus tumors in SV40 T antigen-expressing transgenic mice, Symonds et al demonstrated that suppression of apoptosis during tumor growth was tightly linked to inhibition of p53 function and tumor progression, suggesting the role of p53 as a tumor suppressor is regulation of apoptosis. (Symonds and others 1994).

Apoptotic mechanisms appear to be closely intertwined with other cellular processes, including cell division and differentiation (Rubin and others 1993; Howard and others 1993). In support of this cross talk between pathways, overexpression of Bcl-2 has been shown to inhibit cell growth, promote G1-S cell cycle transition, or promote cellular differentiation in separate models (Pietenpol and others 1994; Miyazaki and
others 1995; Linette and others 1994). Survival mediated by Bcl-2 in factor-deprived FDCPmix cells occurs with complete progression to differentiated myeloid or erythroid phenotypes, while Bcl-xL maintains comparable survival of multipotent precursors (Haughn and Hockenbery, unpublished data). The net effect of Bcl-2 overexpression in tumor cells is difficult to predict, in view of these multiple, sometimes opposing, effects. Although many tumors express high levels of Bcl-2, in breast cancers and an older age population with non-small cell lung cancers, Bcl-2 expression correlates with a favorable prognosis (Joensuu and others 1994; Leek and others 1994; Bhargava and others 1994; Pezzella and others 1993).

Paradoxically, several known oncogenes (c-fos, c-myc) are pro-apoptotic. Intermediate steps in transformation may be associated with lowered apoptotic thresholds (Preston and others 1994). One possible explanation for these observations is that "apoptosis" contributes to full tumorigenicity in some way. While the phenotype of apoptotic death includes internucleosomal cleavage of DNA, whether a sublethal number of DNA breaks due to incomplete activation of apoptotic pathways can occur is unknown. Several reports have recently shown that mitochondrial damage with cytochrome c release occurs in viable cells apoptotically stimulated in the presence of inhibitors of the cell death proteases, caspases (Deshmukh and Johnson 1998). Infrequent double-stranded breaks may occur in this setting, which may increase the frequency of chromosomal rearrangements and amplifications, one form of genetic instability associated with tumors. The observation that Bcl-2 may inhibit apoptosis by
increasing cellular resistance to oxidative stress is consistent with this hypothesis, as oxidative stress can be mutagenic and activate apoptotic cell death at different dose levels.

Based on published experiments conducted in different systems, the anti-apoptotic effect of Bcl-2 could increase tumorigenesis, by sustaining cells that would be vulnerable to apoptosis at several steps in the tumorigenic process, or Bcl-2 could have a tumor suppressor effect, by inhibiting cell growth, promoting differentiation, or possibly by counteracting a mechanism involved in genetic instability. While transgenic mice that express Bcl-2 in both B and T lymphocyte lineages are predisposed to cancer, the overall incidence of cancer is low and delayed when compared to other oncogenes. Other lineages that have been targeted for Bcl-2 overexpression in transgenic mice, including breast, intestine and myeloid, have not been noted to have an increased incidence of tumors (Lagasse and Weissman 1993; Hockenbery, unpublished observations). Our goal is to determine the effect of bcl-2 overexpression in several tumor models and develop an understanding of how apoptosis interacts with the process of carcinogenesis, using breast cancer as our focus.
Task 1 - Analysis of genetic instability in cell lines.

Cells triggered to undergo apoptosis have increased generation of reactive oxygen species. This change in redox state may be important in activation of caspase proteases, the executioners of apoptosis, as well as contribute to single-stranded DNA breaks observed in apoptosis (Peitsch and others 1993). The source of endogenous oxidative stress in apoptosis has been determined to be mitochondria, and the mechanisms of mitochondrial dysfunction in apoptosis remains an important problem in apoptosis research. Bcl-2 and other anti-apoptotic proteins are localized to mitochondrial membranes and prevent ROS generation as well as altered mitochondrial function during apoptosis. Bax, a pro-apoptotic factor also localized to mitochondria, results in rapid mitochondrial dysfunction characterized by initial hyperpolarization followed by eventual depolarization of mitochondria (Minn and others 1999).

Rat-1 fibroblasts expressing the inducible mycER construct undergo myc-dependent apoptosis under low serum conditions. This model of apoptosis is also accompanied by increased ROS generation, as shown by the ROS-sensitive dye, dichlorofluorescein (DCFH) (Figure 1).
Figure 1. Peroxide production by Rat-1 mycER cells grown in 10% FCS (solid line) or with serum deprivation and 1 nM tamoxifen (dashed line) for 18 hrs. Cells were stained with DCFH and analyzed by flow cytometry.

We characterized mitochondrial structure and function in Rat-1 cells according to myc expression and serum conditions. 2 x 10^5 Rat-1 myc-ER cellswere plated in 10 cm dishes initially in phenol red-free DMEM with 10% FCS. After 1 day, cells were washed and switched to 0.1% FCS or recultured in 10% FCS. After an additional 2 days, myc induction was achieved by adding 1 nM tamoxifen. Cells were tested for viability by trypan blue staining at successive time points. Mitochondrial staining with the fluorescent dye JC-1 was analyzed by flow cytometric measurement of FL-1 (green) and FL-2 (red) emissions. Specific staining of mitochondria by JC-1 includes a ΔΨm-independent component (green/monomers) and a delta Ψm-sensitive component (red/aggregates). Cells are harvested by trypsinization, incubated at 37° C with 1 μM JC-1 in culture media for 15 min, and analyzed using a Becton-Dickinson FACscan. At several time points, cell
pellets were fixed in 1/2 strength Karnovsky’s solution and processed for transmission electron microscopy.

Figure 2. JC-1 fluorescence in Rat-1-mycER cells grown in 0.1% serum with and without 1 nM tamoxifen. Two color dot-plot representations of the data for JC-1 monomers (correlating with mitochondrial mass) and JC-1 aggregates (correlating with ΔΨᵢ). Significant loss of viability only occurred in tamoxifen-treated cells under low serum conditions. Cell death was first apparent at 48 hours after tamoxifen addition. Morphology of dead cells was consistent with apoptotic death. Rat-1 cells transfected with vector controls (pBabe-neo) retained high viability for all conditions. JC-1 staining was altered in tamoxifen-treated cells, but only in combination with low serum. The initial pattern of JC-1 staining consisted of an increase in green fluorescence seen at 24 hours (Figure 2). Subsequently, green^{HI} cells accumulated with low red staining. These
results suggested that mitochondria in myc-dependent apoptosis were initially triggered to proliferate and subsequently became depolarized.

These changes are correlated with the development of a disorganized, complex pattern of cristal membranes and dense matrix spaces that ultimately appear to fuse with autophagosomes (see Figure 3 in appendix). Electron micrographs demonstrated structural alterations corresponding to the JC-1 patterns. Serum-deprived, myc-induced NIH-3T3 fibroblasts contained greater numbers of mitochondria than under basal conditions. Mitochondrial structure was also altered, with elongated mitochondrial shapes and numerous, non-aligned cristae. The matrix space appeared dense, in some instances obscuring the pattern of cristae. The ultrastructural appearance of mitochondria in myc-induced, serum-starved cells suggested increased biogenesis of mitochondria and a pattern of mitochondrial injury previously described as ultra condensation. Bcl-2-protected cells appeared to have milder structural alterations, with an increase in cristae density.

To investigate this unexpected process of mitochondrial proliferation in apoptosis, we examined the regulation of mitochondrial respiratory chain subunits during apoptosis. For these studies, we used a panel of monoclonal antibodies raised against individual subunits of electron transport or ATP synthase complexes (Marusich and others, 1997). We found that expression of the nuclearly coded mitochondrial proteins COx IV, Va, Vb and SDH2 increased from 1.5-5-fold within 24 h after induction of c-myc activity in serum-starved NIH 3T3 cells (Figure 4). In contrast, none of the proteins
coded by mitochondrial DNA (Cox I and ND4) were found expressed at higher levels.

![Graph showing relative expression of mitochondrial proteins]

Figure 4. Expression levels of mitochondrial proteins in cell lysates prepared 24 h after tamoxifen addition. Densitometry readings from immunoblots are normalized to values for untreated cells. Values are the means of three experiments.

The lack of coordinated production of mitochondrial proteins at a time of mitochondria proliferation may lead to an accumulation of unassembled subunits within mitochondria. To address the nature of the dense matrix material that forms in mitochondria during apoptosis, we examined mitochondrial morphology after a gentle protease treatment. As shown in Figure 5 (appendix), the matrix material is protease-sensitive. This result appears to indicate that, concurrent with the induction of a subset of mitochondrial proteins, mitochondria fill up with protein, which is followed by mitochondrial swelling and disruption.
Figure 6. Relative expression levels of Cox IV and Cox Vb in NIH-3T3 mycER (N) and NIH-3T3 mycER-Bcl-2 (B) cells with and without myc activation by 1 nM tamoxifen for 24 h.

Transfection of Bcl-2 results in the prevention of c-myc-induced apoptosis. Mitochondria from Bcl-2-transfected cells fail to develop the conformational changes described above after c-myc induction and mitochondria mass remains stable. Bcl-2 expression in cells with activated c-myc also led to changes in mitochondrial protein expression by Western blot compared to the effect of c-myc alone. Steady state levels of Cox Vb protein increased 2.2 fold higher than that observed for Cox IV following c-myc activation. This was reduced to 25% in Bcl-2-transfected cells, due to both a relative increase in Cox IV expression and decrease in Cox Vb expression (Figure 6).
We have previously shown the ability of antioxidants such as N-acetylcysteine to prevent apoptosis. In recent experiments using Colo-205 colon carcinoma cells, we determined the effect of antioxidants on the mitochondrial changes induced by apoptotic triggers. Both NAC and α-tocopherol led to almost complete inhibition of the increase in mitochondrial mass as they rendered cells resistant to apoptosis.

The redox effect of Bcl-2 might act similarly in regulating mitochondrial synthesis. The transcription factor NRF-2 controls transcription of several nuclear coded mitochondrial genes, including Cox IV, in a redox-sensitive manner. We assessed the effect of Bcl-2 expression on NRF-2 transcriptional activity using an NRF-2/GA DNA binding site linked to a luciferase reporter (Chinenov and others 1998). NRF-2 activity increased 4-5 fold in Bcl-2-transfected cells compared to vector controls and was maintained at higher levels in serum-deprived cells following c-myc activation (Figure7).
Figure 7. GABP-reporter activity measured by luciferase activity in transient transfected NIH-3T3 cell lines. CEBP-reporter assay is used as control.
Task 2 - Test of bcl-2 in murine mammary carcinogenesis models

Bcl-2 and its anti-apoptotic homologues confer cellular resistance to apoptosis in a wide variety of contexts, consistent with a proposed function in a central, conserved apoptotic pathway. We evaluated the effects of transgenic Bcl-2 expression in breast epithelium on the heightened physiological apoptosis occurring after litters are weaned from post-partum females. Six to eight week old MMTV-bcl-2 transgenic female mice or control females were mated to control males and allowed to nurse for 3 weeks after birth. Females were sacrificed on the day of weaning and at 2, 7 and 14 days post-weaning. Histologic sections of mammary tissue were prepared for routine histology and TUNEL assay. Maximal levels of apoptosis in control mice were observed on the day of weaning, when widespread involution of lobular tissue was demonstrated on H & E stained sections (Figure 8 in appendix). Specimens from MMTV-bcl-2 transgenic females showed only rare TUNEL-stained cells at day 0, with retention of lobular differentiation. Although apoptosis was less prominent at later periods, transgenic mice maintained greater cellularity in small ductule/lobular remnants.

A total of 72 transgenic MMTV-bcl-2 virgin female mice and 58 control littermates were treated with a total DMBA (dimethylbenzanthracene) dose of 6 mg and
observed for the occurrence of mammary tumors. A small cooperative effect of bcl-2 on DMBA-induced mammary carcinogenesis was observed (Figure 9), however, an increase in tumor incidence/week was only present at 7-8 weeks after the initiation of drug treatment.

![Cumulative tumor incidence graph](image)

**Figure 9.** Cumulative mammary tumor incidence in MMTV-Bcl-2 mice and control littermates treated with DMBA.

As suppression of apoptosis was expected to be the mechanism of any interaction for bcl-2 with carcinogen exposure, we evaluated apoptosis within the mammary tumors for both genetic backgrounds. Mammary tumors were removed from mice at the time of sacrifice, fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial five-
micron sections were processed by hematoxylin-eosin staining for tumor histology and TUNEL staining for apoptosis. Cells with TUNEL-positive nuclei were counted at high magnification from four random fields estimated to contain >80% tumor cells in a representative section from each tumor. Mammary tumors from control B6xC3H females had 14.2 +/- 4.8 apoptotic cells per high-power field (N = 7), while MMTV-bcl-2 transgenic-derived tumors had 20.5 +/- 12.7 apoptotic cells/hpf (N= 11) (See Figure 2, Annual Report 1996). Transgenic mice were also noted to have non-uniform expression of the human Bcl-2 protein in mammary tumor sections, suggesting transgene down-regulation or silencing.

Our initial mating experience with MMTV-bcl-2 and myc mice resulted in few viable litters and we have had to set up additional mating pairs to obtain sufficient double transgenic females for analysis.

Task 3 - Analysis of the expression of genes regulating apoptosis during physiologic mammary epithelial cell death.

A cohort of virgin female FVB/N mice has been sacrificed with collection of mammary and vaginal tissues. Histologic staining of vaginal sections allows determination of the ovulatory cycle stage for paired mammary tissue blocks. Our initial examination of luteal phase mammary epithelium for Bcl-2, Bax, Bcl-x and Bak expression demonstrated distinct patterns of expression for each protein. Bcl-2 is expressed in both ductal and
lobular epithelium, although both regions are notably heterogenous. Bax is predominantly present in myoepithelial cells. Bcl-x has low expression with a fairly homogenous pattern. Finally, Bak is expressed principally in small terminal ductules. (See Figure 3, Annual Report 1996)

Task 4 - Examination of hormonally treated and transgenic mice for apoptotic setpoints in mammary epithelium.

We are still collecting and analyzing mammary and vaginal tissue blocks from MMTV-myc and MMTV-bcl-2 females.

Conclusions

The recent evidence that metastatic breast tumor DNA has both increased hydroxyl radical-induced damage as well as greater diversity of modified bases (Malins and others, 1996) is consistent with a role of oxidative stress in tumor progression. As Bcl-2 appears to modulate oxidative stress, probably via its interaction with mitochondrial functions, the relationship between the expression of Bcl-2 and related proteins and tumor progression may provide important insights into this process and its mechanisms. If tumor initiation and progression are related to suppression of apoptosis, the primary function of bcl-2, apoptotic indices should decrease in relation to tumor yield. Although synergy between Bcl-2 and carcinogen exposure was demonstrable, it
was less than anticipated, based on oncogene interactions in cell and animal cancer models. Alternatively, Bcl-2 expression and apoptosis rates may reflect a selective pressure for an "oxidative" cellular redox state to increase DNA damage in cancer cells. In this model, the accumulation of apoptotic cells might represent the "tip of the iceberg" as the overt manifestation of more widespread cellular derangements causing sublethal genomic damage.

Our observations that changes in mitochondrial mass and function are associated with activation of an apoptotic pathway may provide an in situ method to monitor "apoptotic setpoints" and determine whether cells with low apoptotic setpoints are cancer-prone.
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Manuscripts in preparation

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2. Altered histologic subtypes in mammary tumors from DMBA-treated MMTV-Bcl-2 transgenic mice. (Hockenbery, Henne, Maltzmann)

3. Interaction between c-myc and organelle biogenesis: apoptotic vs mitotic effects (Hockenbery, Morrish, Grandori, Eisenman)

4. Antioxidants prevent mitochondrial changes in apoptosis. (Hockenbery, Mancini, Anderson)

Meeting abstracts.


American Society of Clinical Oncology, Annual Meeting, Los Angeles, CA. "Bcl-2, oxidative stress, and apoptosis", 1995

International Society for Analytical Cytometry, Annual Meeting, Lake Placid, NY "Oxidative Stress and Apoptosis", 1994

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Appendix - Figure Legends

Figure 3. Electron microscopy analysis of mitochondrial morphology in apoptosis. a), NIH-3T3-mycER cells, untreated. b), NIH-3T3-mycER cells treated for 48 h with tamoxifen. c), NIH-3T3-mycER/Bcl-2 cells treated for 48 h with tamoxifen. d), NIH-3T3-mycER cells treated for 72 h with tamoxifen. Arrowheads indicate autophagy of mitochondria.

Figure 5. Transmission EM of apoptotic cells with dense mitochondrial matrix material. Paraformaldehyde/glutaraldehyde fixation. Negative control (L), treated with pronase 1 mg/ml for 30 min (R).

Figure 8. Mammary epithelial involution after weaning in control (a,b,e,g) and transgenic (c,d,f,h) mice. Specimens obtained at day of weaning (a, b, c, d), +2 days (e, f), +7 days (g, h). TUNEL staining (b, d).