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13. ABSTRACT (Maximum 200 words)

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 (73% vs 63%) 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. Staining of serial sections with a monoclonal antibody to human Bcl-2 showed scattered areas of absent Bcl-2 expression, corresponding to TUNEL-positive cells. Finally, apoptotic indices in tumors from transgenic and control animals were comparable.

Apoptosis is often associated with increased oxidative stress, a potential cause of DNA damage. An association between apoptosis, ROS, and genetic instability may explain the apparent retention of apoptotic programs in experimental and human tumors.

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David Harbaugh 6/12/97

PI - Signature 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). Many studies implicate p53 function in apoptosis following gamma irradiation and exposure to certain chemotherapy drugs. Suppression of apoptosis during tumor growth is tightly linked to inhibition of p53 function and tumor progression in SV40 T antigen-induced choroid plexus tumors in transgenic mice, suggesting a wider role for p53 in apoptotic control (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). 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 is correlated 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.

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 sublethal activation of apoptotic pathways can occur is unknown. 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 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.

Body

Task 1 - Analysis of genetic instability in cell lines. Rat-1 fibroblasts expressing the inducible myc-ER construct were examined for formation of reactive oxygen species during myc-dependent apoptosis triggered by serum withdrawal. Cellular peroxide generation was determined by flow cytometric assay of dichlorofluorescein (DCFH) in the presence and absence of serum and tamoxifen (four combinations). We observed increased DCFH fluorescence in the presence of tamoxifen (activating myc function) and low serum conditions, consistent with the generation of endogenous oxidative stress in various examples of apoptosis. We have also performed initial studies in primary keratinocytes expressing HPV E6 to suppress p53 function. These cells were provided by Dr. Denise Galloway and have been shown to be hypersensitive to several anti-cancer agents, including taxol. We also observed increased DCFH fluorescence following taxol treatment in this model, suggesting that p53 may regulate an endogenous source of reactive oxygen species (ROS). DNA adducts as well as strand breaks are produced by oxidative stress and may be a cause of genetic instability in cancer cells. During the next year we will determine the relationship between one type of genetic instability, amplification of the CAD gene resulting in PALA resistance, and apoptotic events and levels of oxidative stress.

We have also investigated the source of endogenous oxidative stress in Rat-1 cells. Constitutive activation of c-myc combined with serum withdrawal causes mitochondrial proliferation. The expanded population of mitochondria appear to be dysfunctional as they have low retention of membrane potential-sensitive mitochondrial dyes. The cytoplasmic fluorescence of ROS-sensitive dyes corresponds to the pattern of low Ψ_M mitochondria, suggesting that the newly synthesized mitochondria may leak oxygen-free radicals.

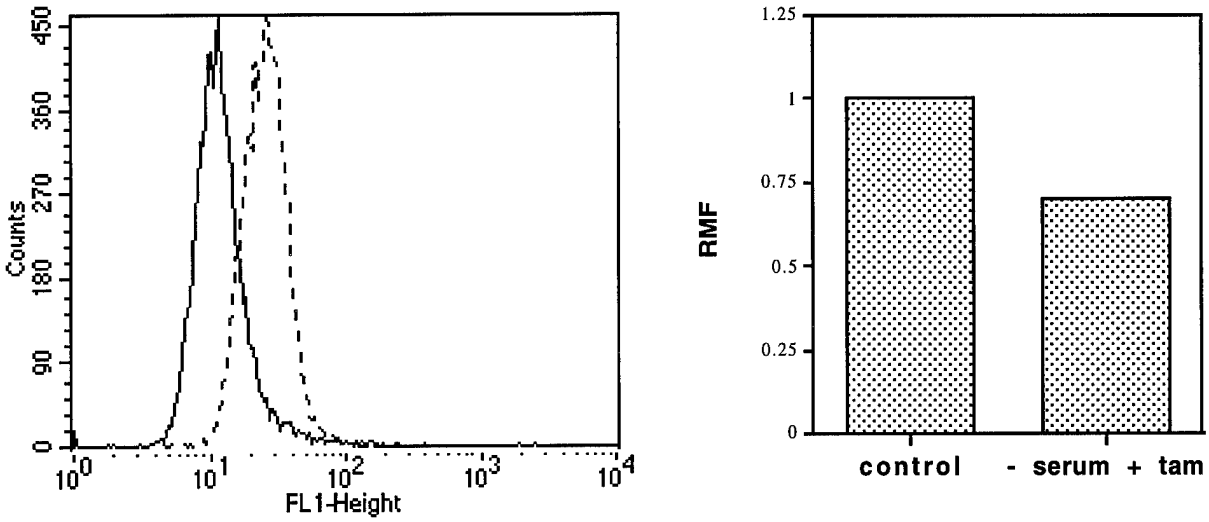

Figure 1. (left) Peroxide production by Rat-1 Myc-ER cells grown in 10% FCS (solid line) or with serum deprivation and 1 nM tamoxifen (dashed line) for 18 hrs. Cells were stained with DCFH and analyzed by flow cytometry.
(right) Relative mitochondrial function (RMF) in Rat-1 Myc-ER cells grown in 10% FCS (control) or with serum deprivation and 1 nM tamoxifen for 18 hrs. Cells were stained with the mitochondrial fluorescent dye JC-1 and analyzed for mitochondrial mass (green fluorescence) and membrane potential (red fluorescence) by flow cytometry. RMF represents ratio of mean values of red and green fluorescence.

Task 2 - Test bcl-2 in murine mammary carcinogenesis models. Work in the previous year demonstrated a small, but significant cooperative effect of bcl-2 on DMBA-induced mammary tumorigenesis. We have evaluated apoptotic rates by in situ TUNEL staining in a sample of tumors from Bcl-2-transgenic mice and control littermates. Unexpectedly, apoptotic cells were equally frequent in tumors from both groups. We subsequently performed anti-Bcl-2 immunohistochemical staining on tumors from transgenic animals, using an anti-human Bcl-2-specific antibody to detect expression of the transgene. In all tumors that we examined, areas with absent Bcl-2 staining were observed. Double-labeling demonstrated a correlation between absent Bcl-2 expression and apoptosis within the tumors. These results imply that down-regulation of transgene expression or transgene

silencing can occur at enhanced rates in cancer tissues. This may account for the low level of cooperativity seen in the DMBA study. (Figure 2, see Appendix)

In order to examine the interaction of bcl-2 and c-myc in mammary tumors in a uniform genetic background, we have completed a backcross of MMTV- bcl-2 mice into the FVB/N background. We are currently mating bcl-2 and myc transgenic mice and will compare spontaneous tumor incidences in double vs single transgene heterozygotes. It is likely that a strong synergistic effect will be seen in the double heterozygotes as has been demonstrated in a variety of cell lines. This would provide an opportunity to correlate Bcl-2 expression, apoptotic rates and tumor incidence between the DMBA and c-myc tumor models.

Task 3 - Analysis of expression of genes regulating apoptosis during physiologic mammary epithelial cell death. We have developed protocols for immunostaining Bcl-2, Bax and Bcl-x , as well as the TUNEL assay in Bouin's-fixed mouse mammary tissue. We are utilizing the 3F11 anti-murine Bcl-2 antibody (Veis and others, 199), and have obtained good results with anti-Bcl-x and Bax antibodies from Pharmingen. Bcl-2, Bcl-x and Bcl-2 are diffusely expressed at significant levels in murine mammary epithelia. (Figure 3). We have begun to collect mammary sections from virgin mice at different times in their ovulatory cycle (using vaginal cytology) for mapping of apoptosis and gene expression.

Task 4. Examination of hormonally treated and transgenic mice for apoptotic setpoints in mammary epithelium. We will commence these experiments in the next 6 months after initial analysis of the results from Task 3.

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. The availability of several models of mammary tumorigenesis that may be influenced differently by constitutive Bcl-2 expression will permit the direct testing of several hypotheses. If tumor initiation and progression are related to suppression of apoptosis, apoptotic indices will decrease in relation to tumor yield. Alternatively, if cellular redox state is maintained at an oxidative level to increase DNA damage in cancer cells, Bcl-2 expression and apoptosis rates may reflect this requirement. 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 their effects on redox state. It will also be informative to assess hydroxyl radical-induced DNA damage in breast tissue and tumors in each model. Dr. Larry Loeb at the University of Washington has considerable expertise in this area and is available to assist our experiments.

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Figure Legends for Appendix

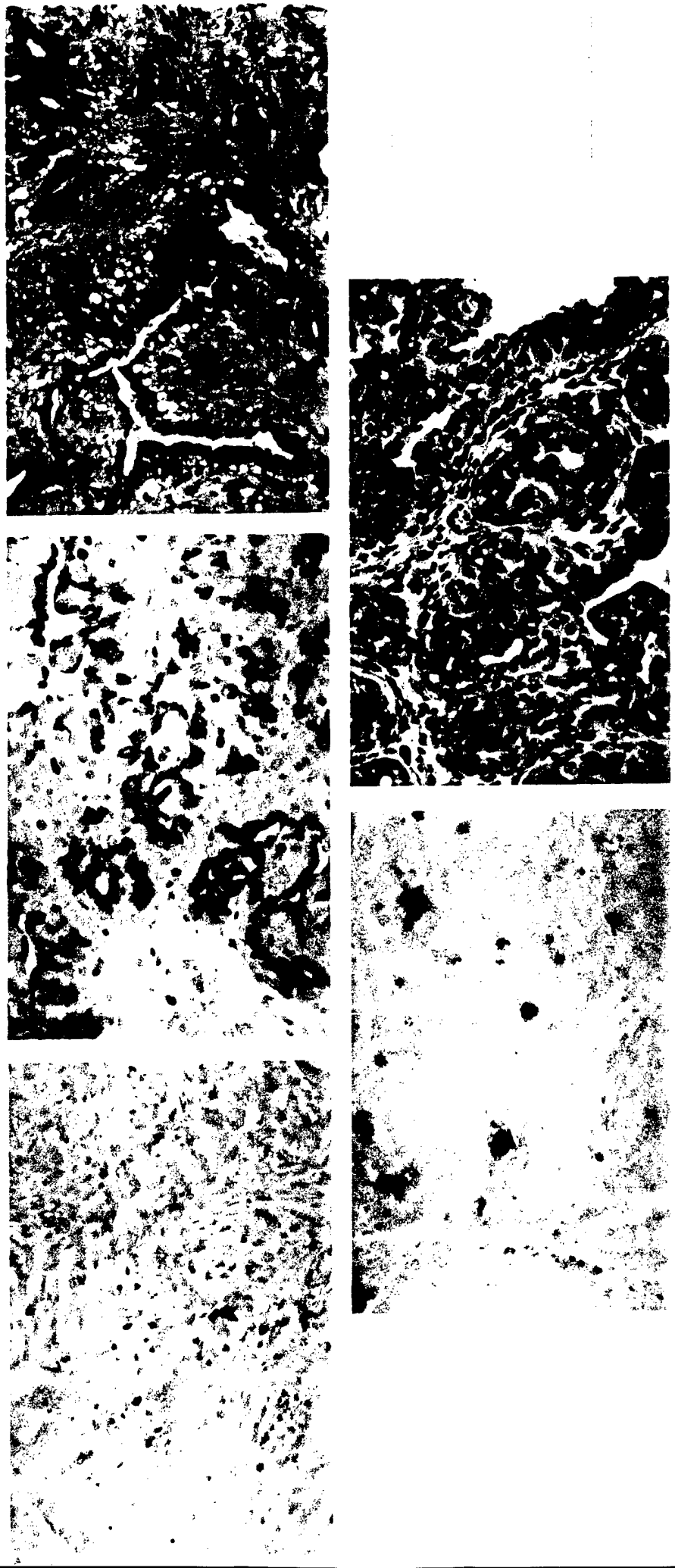
Figure 2. Photomicrographs of formalin-fixed sections of murine mammary tumors.

(Top left) TUNEL staining of mammary tumor from MMTV-bcl-2 transgenic female.
(Top center) Immunostaining for human Bcl-2 of mammary tumor from MMTV-bcl-2 transgenic female.
(Top right) H&E staining of mammary tumor from MMTV-bcl-2 transgenic female.
(Bottom left) TUNEL staining of mammary tumor from control female.
(Bottom right) H&E staining of mammary tumor from control female.

Figure 3. Photomicrographs of formalin-fixed sections of murine mammary tissue from FVB female mice.

(A) Immunostaining for Bcl-x
(B) Immunostaining for Bax
(C) Negative control

Figure 2

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