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BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death

(oncogene/lymphoma/mitochondria)

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ABSTRACT **The *BCL2* protooncogene encodes an inner mitochondrial membrane protein that blocks programmed cell death. *BCL2* was isolated from the chromosomal breakpoint of follicular B-cell lymphoma. Transgenic mice that overexpress BCL2 display extended survival of resting B cells. In this study we use a monospecific anti-human BCL2 antibody to define the distribution of BCL2 protein within organized tissues. BCL2 is restricted within germinal centers to the follicular mantle and to portions of the light zone implicated in the selection and maintenance of plasma cells and memory B cells. BCL2 is present in the surviving T cells in the thymic medulla. All hematopoietic lineages that derive from a renewing stem cell also display BCL2. A limited number of nonlymphoid tissues demonstrate BCL2 and can be grouped as (*i*) glandular epithelium in which hormones or growth factors regulate hyperplasia and involution, (*ii*) complex differentiating epithelium such as skin and intestine characterized by long-lived stem cells, and (*iii*) long-lived postmitotic cells such as neurons. Within these tissues that demonstrate apoptotic cell turnover, BCL2 is often topographically restricted to long-lived or proliferating cell zones. BCL2's function as an antidote to apoptosis may confer longevity to progenitor and effector cells in these tissues.**

BCL2 is unique among protooncogenes by being localized to the inner mitochondrial membrane (1). Moreover, *BCL2* has the oncogenic function of blocking programmed cell death (1–3). Deregulated BCL2 extends the survival of certain hematopoietic cell lines following growth factor deprivation. When pro-B-cell or promyelocyte cell lines are deprived of interleukin 3 they normally succumb to a programmed demise entitled apoptosis. This pattern of morphologic cell death is characterized by a dramatic plasma membrane blebbing, cell volume contraction, nuclear pyknosis, and internucleosomal DNA degradation following the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease (4, 5). Overexpression of mitochondrial BCL2 is an antidote to this process (1). BCL2 extends the survival of such cells independent of affecting their proliferation (3).

The *BCL2* protooncogene was discovered at the chromosomal breakpoint of the t(14;18) found in human follicular lymphoma. The t(14;18) juxtaposes the *BCL2* gene from chromosome 18 with the immunoglobulin heavy chain (*IGH*) locus on 14 (6–8). This creates a *BCL2–IGH* fusion gene that is markedly deregulated, resulting in overproduction of BCL2 RNA and protein (9–12). *BCL2–IG* transgenic mice overexpress BCL2 in lymphoid tissues and develop a polyclonal expansion of small resting B cells (13, 14). Notably, these cells accumulate because they fail to die, demonstrating prolonged survival. However, these B cells can proliferate and *BCL2–IG* transgenic mice progress to high-grade lymphomas, arguing that extended cell survival is tumorigenic (15).

In light of these findings, we used a monoclonal antibody to the human BCL2 protein to determine the distribution of BCL2 within the architecture of normal B-cell organs. Moreover, the role of apoptosis in the homeostasis of multiple cell lineages suggested that BCL2 might regulate survival in other cell types. Consequently, we defined the spatial distribution of BCL2 within various organized tissues.

MATERIALS AND METHODS

All tissue specimens were obtained from surgical resections or autopsy material and processed as frozen sections. Most somatic tissue sections were judged to be pathologically normal by routine histologic examination of adjacent sections. The sections were fixed in acetone at 4°C for 10 min and treated with 0.18% H_2O_2 in methanol for 30 min. The slides were blocked with avidin and biotin (Vector Laboratories) according to instructions and blocked with 1% goat serum for 30 min. The 6C8 hamster anti-human BCL2 monoclonal antibody or a control hamster anti-recombinant interleukin 2 monoclonal antibody, adjusted to equal concentrations, was incubated with the slides for 1 hr, followed by biotinylated goat anti-hamster IgG (United States Biochemical) at a 1:40 dilution and ABC reagent (Vector Laboratories) for 45 min each. Staining was developed using 0.05% diaminobenzidine with 0.01% H_2O_2 for 5 min. Slides were counterstained with methyl green/alcian blue, dehydrated, and mounted. Frozen sections of brain tissue were processed according to a similar method that has been extensively detailed (16).

Freshly obtained human tonsil or breast was solubilized in SDS sample buffer (2% SDS/200 mM 2-mercaptoethanol). One hundred micrograms of solubilized proteins was run on a SDS/12.5% polyacrylamide gel. Western blot was performed as described using 6C8 monoclonal antibody (14).

RESULTS

Germinal Center. The germinal center of the lymphoid follicle appears to provide a microenvironment for the generation of memory B and plasma cells. Following stimulation with antigen, B-cell blasts seed the primary follicle, which matures to a secondary germinal center with well-defined anatomic zones (17). Within human tonsils the most intense staining for BCL2 was in the follicular mantle, composed of recirculating IgM/IgD B cells (18) (Fig. 1). Lymphocytes within the interfollicular regions were also often positive. In striking contrast, the majority of cells within the germinal center were negative for BCL2. Centroblasts within the dark zone fail to stain for BCL2. BCL2 is also absent from centrocytes in the basal portion of the light zone laden with

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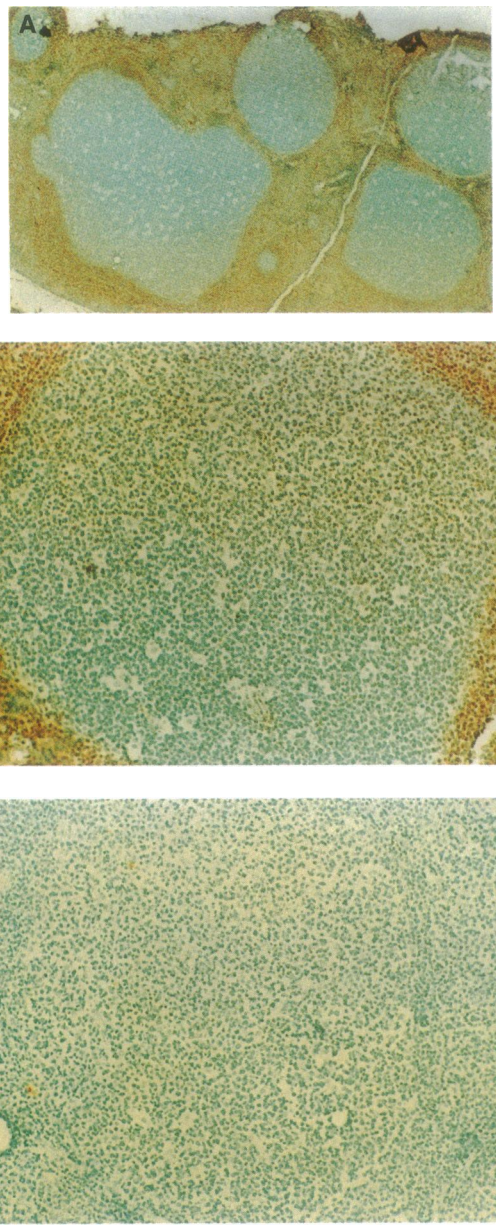

FIG. 1. Immunoperoxidase staining for BCL2 in human tonsil. (*A*) Germinal centers reveal faint staining at one pole. (×35.) (*B*) Germinal centers with apical portion of light zone at top. (×175.) (*C*) Control hamster anti-recombinant interleukin 2 monoclonal antibody does not stain tonsil.

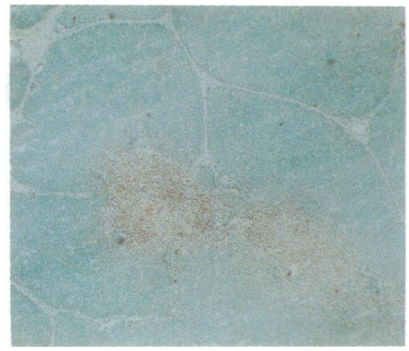

FIG. 2. BCL2 in thymus. Hassall's corpuscles stained nonspecifically.

tingible body macrophages. BCL2 protein uniformly returns at low intensity within the more apical portion of each light zone (Fig. 1 *A* and *B*), where residual antigen localized to the surface of follicular dendritic cells appears to select high-affinity B cells (17, 19). The majority of BCL2-positive cells coexpressed the CD22 B-cell marker but not the CD3 T-cell marker (data not shown).

Thymus. The thymus has distinct cortical and medullary regions that demonstrate thymocytes at serial stages of maturation. The vast majority of thymocytes within the cortex were negative for BCL2, with only rare cells displaying positivity (Fig. 2). Beginning at the juxtamedullary junction between medulla and cortex, lightly stained thymocytes were seen. Within the medulla, strong BCL2 staining was associated with thymocytes. The staining of Hassall's corpuscles was nonspecific, as control antibodies demonstrated the same pattern. Thymocytes undergo positive and negative selection on the basis of their T-cell receptor specificities and the majority of cortical thymocytes die by apoptosis (20, 21). The mature thymocytes that survive accumulate in the medulla.

Bone Marrow. Staining of bone marrow aspirates revealed that BCL2 is present in the precursor cells of all hematopoietic elements but is absent in their mature progeny. Within the myeloid series, promyelocytes and myelocytes demonstrated an intense cytoplasmic pattern of BCL2. Metamyelocytes and band forms displayed decreased levels of staining, whereas mature polymorphonuclear cells were essentially negative (Fig. 3*A*). Among erythroid precursors, normoblasts and nucleated erythrocytes exhibited BCL2 staining, but reticulocytes were negative (Fig. 3*B*). Megakaryocytes were strongly positive for BCL2 (Fig. 3*C*).

Breast. Breast epithelium undergoes hyperplasia or involution in response to hormone supplementation or withdrawal, respectively (22). Immunohistochemical staining of

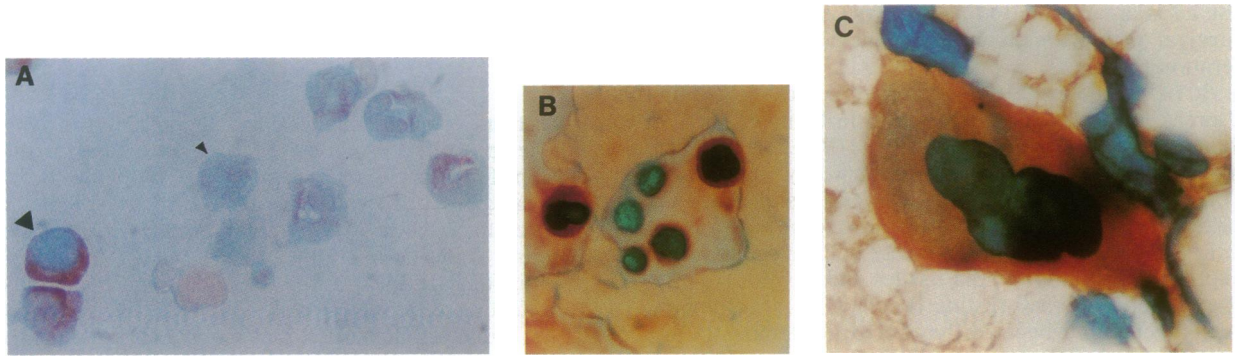

FIG. 3. BCL2 expression in bone marrow cells. (*A*) Within myeloid precursors promyelocytes stained intensely (large arrowhead), whereas granulocytes had weak staining (small arrowhead). (×600.) (*B*) BCL2 expression in the erythroid series. (×600.) Normoblasts and nucleated erythrocytes demonstrated strongest staining. (*C*) Megakaryocyte stained intensely for BCL2. (×600.)

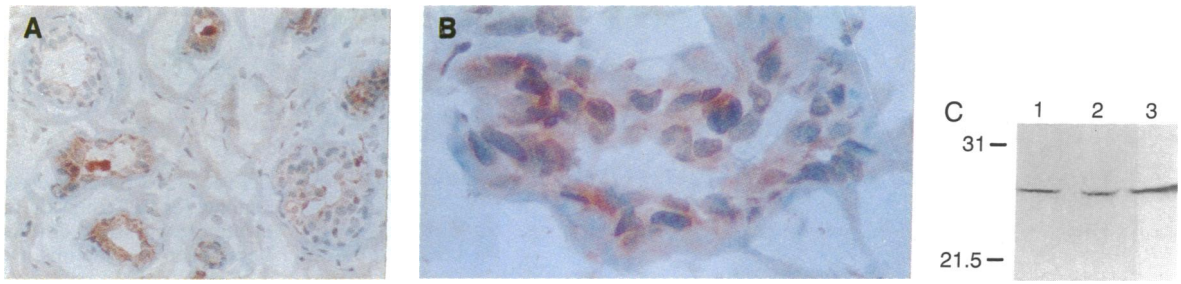

FIG. 4. BCL2 expression in breast. (*A*) Immunolocalization of BCL2 protein in a breast lobule showing BCL2 expression within epithelial cells. (×240.) Proteinaceous material within ducts stained nonspecifically. (*B*) High magnification shows uniform staining of epithelial cells. (×600.) (*C*) Western blot of 100 μg of breast tissue lysate (lanes 1 and 2) and tonsil lysate (lane 3) developed with the 6C8 monoclonal antibody demonstrates 25-kDa BCL2 protein.

breast tissue from a premenopausal nongravid woman was performed. Epithelial cells of large and small ducts were noted to have abundant cytoplasmic BCL2 (Fig. 4 *A* and *B*). Although some variability in intensity of staining was seen, all epithelial cells examined were positive. The reactivity with proteinaceous material within duct lumens was nonspecific. A Western blot of cell lysates of normal breast tissue compared to tonsil demonstrated an identically sized 25-kDa BCL2 protein recognized by the 6C8 antibody (Fig. 4*C*). The level of BCL2 protein in breast tissue is approximately half that found in tonsil.

Thyroid. The thyroid gland is another hormone-dependent tissue in which hyperplasia and involution are known to occur (23). Thyroid gland epithelium had prominent cytoplasmic BCL2 staining (Fig. 5). All cells in the follicular epithelium appeared to be stained.

Prostate. Prostate epithelium is composed of a basal layer of low cuboidal epithelium and overlying mucous-secreting columnar cells. The prostate sections examined included normal areas as well as benign prostatic hypertrophy. BCL2 staining was confined to basal cells of the pseudocolumnar prostate epithelium, whereas columnar cells, including hyperplastic regions, lacked staining (Fig. 6).

Pancreas. Exocrine and endocrine glands of the pancreas are capable of hyperplasia and involution in response to physiologic stimuli (24, 25). Pancreatic acinar cells and islet cells exhibited moderate amounts of BCL2 in a cytoplasmic distribution (Fig. 7). Pancreatic duct cells seen in some sections were not stained.

Gastrointestinal System. The gastrointestinal tract is comprised of a self-renewing stem cell population that generates four epithelial cell types: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (26). Small intestinal epithelium is highly structured topographically, with differentiation extending up the villus from the small pool of stem cells at the base of crypts. A larger pool of proliferating cells extends to mid-crypt. Cells differentiate after leaving the stem cell zone and continuously migrate up the crypt and villus. At the villus tip cells complete their life-span and are extruded. Examination of human small intestine sections with anti-BCL2 antibody demonstrated staining of crypt epithelial cells. The same intensity of staining was noted in colonic crypts. BCL2 was confined to the lower half of crypts in small and large intestine (Fig. 8 and data not shown). The staining pattern appeared diffusely cytoplasmic in enterocytes as well as goblet cells. Villi and the absorptive surface of the colon lacked demonstrable BCL2. In this highly organized epithelium, BCL2 expression is restricted along the vertical crypt–villus axis to stem cells and proliferation zones.

Skin. The epidermis is a highly organized squamous epithelium. The basal layer of the epidermis is responsible for the proliferation and regeneration of the epithelium. Progressive differentiation, as typified by novel keratin synthesis, occurs as cells move upward in this epithelium. Fully keratinized cells in the stratum corneum are anucleate and eventually detach from the epithelium. Immunohistochemical analysis revealed that BCL2 was present exclusively in the basal layer of the skin (Fig. 9).

Nervous System. Adult neurons are long-lived postmitotic cells. Immunohistochemical staining of cerebral cortex obtained from the temporal lobe demonstrated that BCL2 was present within neurons. The characteristic granular staining pattern of BCL2 was noted in the dendritic cytoplasm surrounding many of the large, round nuclei of neurons (Fig. 10).

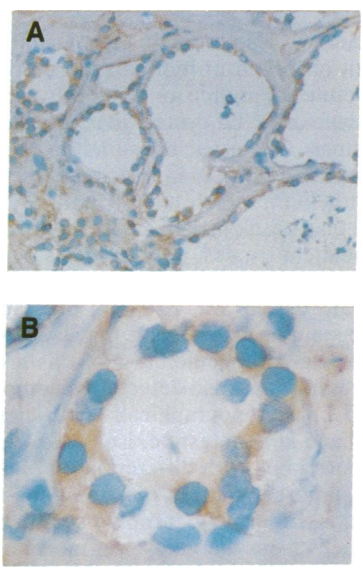

FIG. 5. Immunoperoxidase staining for BCL2 in thyroid. BCL2 expression is noted in thyroid follicular epithelial cells (*A*; ×240) and in a cytoplasmic pattern (*B*; ×600).

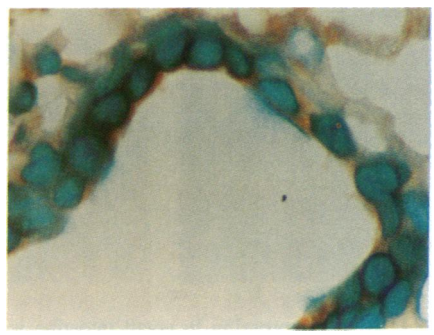

FIG. 6. Immunoperoxidase staining in prostate detects BCL2 within basal epithelial cells. (×600.)

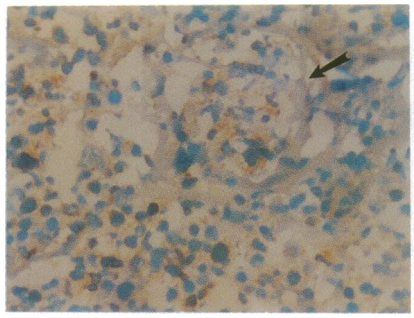

FIG. 7. BCL2 expression within pancreatic acini and islet (arrow) detected by immunoperoxidase staining. (×240.)

Neither the axonal tracts nor glial cells demonstrated BCL2 protein.

A number of tissues examined lacked BCL2, as assessed by antibody staining. These included liver, lung, heart, kidney, cervix, myometrium, testis, ovary, and bladder. Fibroblasts that were present in sections lacked BCL2 reactivity. Smooth muscle cells in intestine and bladder displayed no detectable BCL2.

DISCUSSION

The *BCL2* gene has two unique properties among oncogenes: the oncoprotein localizes to the inner mitochondrial membrane and it produces dramatic extension of cell survival when it is overexpressed (1). As an initial step to assess the role of BCL2 as a regulator of physiologic cell death, we have determined the normal tissue distribution of BCL2. BCL2 protein is found within various cell lineages and tissues that are all characterized by apoptotic cell turnover. BCL2 is topographically restricted to the long-lived progenitor cells that renew these lineages and select postmitotic cells that require an extended life-span.

Immunohistochemical examination of lymphoid germinal centers using 6C8 antibody demonstrated a complex pattern of BCL2 distribution in mature B cells. BCL2 was strongly positive in the small recirculating lymphocytes of the follicular mantle zone. In contrast, germinal center development appeared to involve a "resetting" of BCL2 expression. Proliferating centroblasts and the majority of centrocytes lacked BCL2, which may allow their death by apoptosis. Importantly, BCL2 protein returned within a subset of the

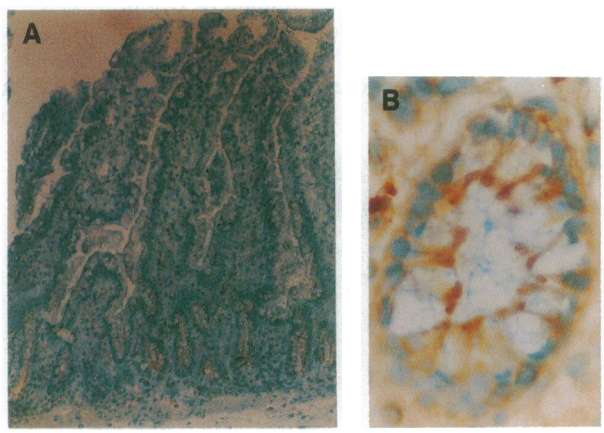

FIG. 8. (*A*) BCL2 expression in small intestine is confined to lower crypts. (×24.) (*B*) Cross-section of small intestinal crypt demonstrating BCL2 expression in enterocytes and goblet cells. (×600.)

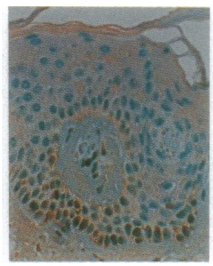

FIG. 9. Immunoperoxidase staining for BCL2 in skin demonstrates expression confined to basal layer of epidermis. (×240.)

light zone B cells, which may enable the survival of memory B cells.

Thymus sections revealed a parallel topographic distribution of BCL2 among developing T cells. A corticomedullary gradient of BCL2 staining was also noted by Pezella *et al.* (18). The cortex is dominated by double-positive ($CD4^+$ $CD8^+$) T-cell precursors. Most of these thymocytes appear to undergo a suicide program, termed negative selection, resulting from avid autoreactivity with self determinants. To survive, cells are also felt to require a positive selection step representing a low-affinity recognition of self. The surviving single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) mature thymocytes are found within the medulla. Thus, the thymus and germinal centers are organs of selective apoptosis in which BCL2 is restricted to the sites of mature, surviving lymphocytes.

Beyond lymphocytes, the other hematopoietic lineages that derive from a renewing stem cell population also demonstrate BCL2. Staining of bone marrow aspirates displayed BCL2 in proliferating precursor cells of all lineages but not in postmitotic maturation stages. Aging neutrophils die by apoptosis *in vitro*, and perhaps *in vivo*, as evidenced by characteristic morphologic changes (27). In addition, the induction of differentiation in the HL-60 promyelocytic cell line to a mature neutrophil phenotype results in death by apoptosis (28).

A secondary category of tissues that demonstrated BCL2 protein is glandular epithelium that is regulated by hyperplasia or involution, usually in response to hormonal stimuli. BCL2 protein was detected in breast duct epithelium by immunohistochemistry and Western blotting. Apoptotic cell death has been noted during involution of lactating breast (29). Morphologic evidence of apoptosis has been detected, to a lesser extent, in normal breast tissue during ovulatory cycles (30).

BCL2 protein was also found in thyroid epithelium. Apoptosis has been observed in hyperplastic murine thyroid induced to involute by iodide or triiodothyronine (31). The uniform presence of BCL2 protein in thyroid epithelium may prevent apoptosis at normal hormone levels. The prostate gland demonstrated BCL2 in basal cuboidal epithelium. Columnar epithelium, which ultrastructurally appears more differentiated than basal, lacked detectable BCL2. Like other hormone-dependent tissues, apoptosis has been observed in prostate epithelium upon castration and to a lesser extent in healthy prostate tissue (32). Areas of prostatic hyperplasia lacked BCL2 staining, suggesting that BCL2-mediated cell survival is not the pathogenic basis of this lesion.

BCL2 expression was also detected in complex, organized epithelia. BCL2 was topographically restricted to the lower crypt in small and large intestine. Loss of BCL2 in migrating enterocytes correlates with differentiation, loss of proliferative capacity, and reduction of remaining life-span along the crypt–villus axis. Enterocyte turnover occurs mostly through extrusion of cells from the villus, yet morphologic evidence of apoptosis is seen in healthy villi (33). This suggests that enterocytes undergo a programmed cell death after terminal differentiation. In addition, enterocytes that are extruded bear ultrastructural changes of microvillus disorganization

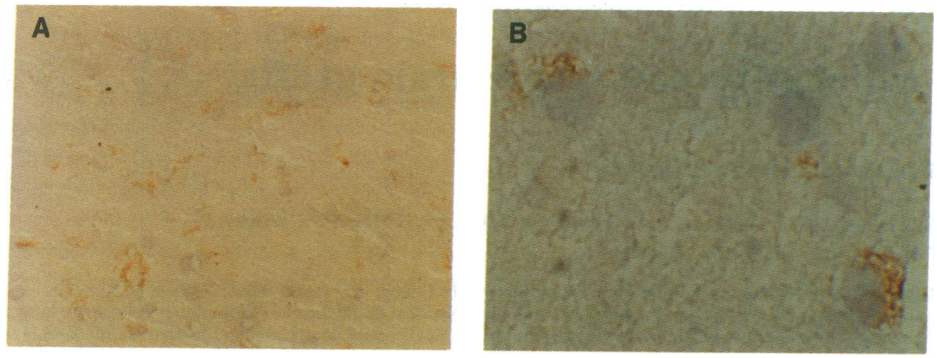

FIG. 10. (*A*) BCL2 expression in cerebral cortex is localized to neurons by immunoperoxidase staining. (×240.) (*B*) Higher magnification. (×600.)

seen with frankly apoptotic cells (33). Thus, BCL2 down-regulation may constitute a biochemical marker of a commitment to cell death that follows differentiation.

BCL2 was also localized to the basal layer of the epidermis. Loss of BCL2 expression in suprabasal layers is analogous to intestinal differentiation with eventual cell loss by means of sloughing. Apoptosis is a commonly recognized mode of epidermal cell death in pathologic conditions such as UV-induced skin damage (34) and graft-versus-host disease (35). The geographic restriction of BCL2 in complex epithelium and immune organs argues for important mechanisms to down-regulate as well as up-regulate this gene.

RNA analysis detected BCL2 expression in newborn and adult brain (ref. 36; D. Veis and S.J.K., unpublished observations). We have now localized BCL2 protein to neurons by immunohistochemical staining of human adult neocortex. Adult neurons are long-lived postmitotic cells; however, during neural development neurons undergo extensive cell death, >50% in some regions (37). This appears to eliminate aberrant neural connections and adjust neuronal projections to the size of their synaptic targets. It has been postulated that neuronal cell survival depends on neurotrophic factors supplied by the target cells. Nerve growth factor (NGF) is the prototypic factor necessary for *in vivo* and *in vitro* survival of sympathetic and sensory ganglion cells. Neuronal cell death after NGF withdrawal has features of an active cell death, including dependence on RNA and protein synthesis (38). BCL2 protein in neurons may reflect the effect of neurotrophic factors to ensure a stable neuronal population.

Transgenic mice overproducing BCL2 established that prolonged B-cell survival resulted in high-grade neoplasia. Protracted cell life-spans increase the acquisition of additional secondary genetic alterations responsible for tumor progression (15). Of note, a number of the cell lineages shown here to express BCL2 have a high incidence of cancer. Skin, colon, breast, prostate, and pancreas rank first, third, fourth, fifth, and ninth, respectively, in the incidence of all carcinomas (39). The extended survival of such cell types by BCL2 may prove to be a factor in their predisposition to malignancy.

Importantly, not all long-lived cell types displayed BCL2 expression. In particular, muscle and other mesodermal origin tissues were frequently negative. BCL2 appears to be restricted to tissues in which apoptosis molds developing structures or accounts for cell turnover. BCL2's role as an antidote to programmed death may be required to save the progenitor and long-lived cells in these lineages.

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