American Journal of Pathology, Vol. 146, No. 6, June 1995

Short Communication

Immunohistochemical Analysis of Mcl-1 Protein in Human Tissues

Differential Regulation of Mcl-1 and Bcl-2 Protein Production Suggests a Unique Role for Mcl-1 in Control of Programmed Cell Death In Vivo

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***The* mcl-1 *gene encodes an approximately 37-kd protein that has significant homology with Bcl-2, an inhibitor of programmed cell death that is expressed in many types of long-lived cells. In this study we determined the* in vivo *patterns of Mcl-1 protein production in normal human tissues by immunohistochemical means, using specific polyclonal antisera, and made comparisons with Bcl-2. Like Bcl-2, Mcl-1 immunostaining was observed in epithelial cells in a variety of tissues, including prostate, breast, endometrium, epidermis, stomach, intestine, colon, and respiratory tract. However, often the expression of* mcl-1 *and* bcl-2 *in complex epithelia occurred in gradients with opposing directions, such that Bcl-2 immunostaining tended to be higher in the less differentiated cells lining the basement membrane, whereas Mcl-1 immunostaining was more intense in the differentiated cells located in the upper layers of these epithelia. The* in vivo *patterns of* mcl-1 *and* bcl-2 *expression were also strikingly different in several other tissues as well. Within the secondary follicles of lymph nodes and tonsils, for example, germinal center lymphocytes were Mcl-1 positive but mostly lacked Bcl-2; whereas mantle zone lymphocytes expressed* bcl-2 *but not* mcl-1. *Intense Mcl-1 immunoreactivity was also detected in several types of neuroendocrine cells, including the adrenal cortical cells that are Bcl-2 negative, sympathetic neurons that also contain Bcl-2, a subpopulation of cells in the pancreatic islets, Leydig cells of the testis, and granulosa lutein cells of the ovarian corpus luteum but not in thyroid epithelium, which is strongly Bcl-2 positive. Little or no Mcl-1 was detected in neurons in the brain and spinal cord, in contrast to Bcl-2, which is present in several types of central nervous system neurons. Conversely, strong Mcl-1 immunostaining was found in cardiac and skeletal muscle, which contain comparatively less Bcl-2. Additional types of cells that are Bcl-2-negative but that expressed* mcl-1 *include chondrocytes and hepatocytes. These findings demonstrate that* mcl-1 *expression is widespread* in vivo *and imply that the Mcl-1 and Bcl-2 proteins fulfill different roles in the over-*

Supported in part by grants from the National Institutes of Health (CA-60181) and American Cancer Society (DHP-32C). Dr. Reed is a Scholar of the Leukemia Society of America.

Accepted for publication March 1, 1995.

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all physiology of cell death regulation. (Am J Pathol 1995, 146:1309–1319)

The protein encoded by the *bcl-2* (B-cell lymphoma/leukemia-2) gene is a critical regulator of programmed cell death and has been shown to be capable of blocking apoptosis induced by an impressive array of physiological, pathological, and experimental stimuli and insults (reviewed in Ref. 1). It has been suggested therefore that Bcl-2 controls a distal event in a final common pathway involved in cell death regulation, the molecular details of which remain sketchy at present. Although the Bcl-2 protein has no homology with any other protein with a known biochemical mechanism of action, recently, at least four novel cellular genes have been discovered that encode proteins that share significant amino acid sequence homology with Bcl-2. Some of these proteins, such as Bcl-X_L and A1, have been shown through gene transfer experiments to function as blockers of apoptotic cell death, whereas others, such as Bax and Bcl-X_S, abrogate Bcl-2 function and promote cell death[2–4] (unpublished data). Using a combination of biochemical and functional assays, we and others have shown that many of these Bcl-2 family proteins can physically interact, forming homo- and heterodimers (or possibly multimers) with each other.[5,6] Although the significance of these protein-protein interactions remains to be fully elucidated, a model has been proposed in which Bax/Bax homodimers are envisioned as active inducers or enhancers of an evolutionarily conserved cell death pathway and the interaction of Bcl-2 with Bax in the form of Bax/Bcl-2 heterodimers is required to promote cell survival.[5] Consistent with this idea, the Bcl-X_L protein, which shares 47% homology with Bcl-2 and has been reported to function as a blocker of apoptosis, can also form heterodimers with Bax and neutralize its effects.[5] This model for relating Bcl-2 family protein interactions to function, however, is complicated by the observation that Bcl-2 can also form protein complexes with itself and Bcl-X_L, the biological significance of which remains unknown at present.

One of the newly described members of the Bcl-2 protein family, Mcl-1 (myeloid cell leukemia-1), was fortuitously identified by differential screening of cDNA libraries derived from a human myeloid leukemia cell line induced to undergo differentiation in culture.[7] Expression of *mcl-1* is transiently induced in these leukemic cells after stimulation with agents that promote terminal differentiation. Recently, it was shown that gene transfer-mediated elevations in *mcl-1* expression can partially abrogate apoptosis induced by conditional production of the c-Myc oncoprotein in Chinese hamster ovary cells.[8] Furthermore, we have shown that Mcl-1 can function similar to Bcl-2 in neutralizing Bax-mediated cytotoxicity in yeast,[5] providing further support that Mcl-1 is a blocker of cell death. The amino acid sequence of the human Mcl-1 protein, as predicted by cDNA cloning, reveals a polypeptide of 350 amino acids (~37 kd) that is ~35% homologous with the 239-amino-acid human Bcl-2 (~26 kd) over a stretch of 139 amino acids. This homology resides primarily in the carboxyl halves of these proteins, with the amino-terminal portions of the proteins diverging in sequence and length. Both Bcl-2 and Mcl-1 contain a stretch of hydrophobic residues at their carboxy termini. In the case of Bcl-2, this hydrophobic segment has been shown to constitute a transmembrane domain that is responsible for post-translational insertion of Bcl-2 protein into membranes[9] and that appears to be necessary for optimal function as a blocker of programmed cell death in some settings but not others.[10–12] The intracellular membranes in which the Bcl-2 protein is predominantly located include the outer mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum,[13–16] which, when viewed at the level of conventional light microscopy, typically results in a granular or punctate cytosolic immunostaining pattern with perinuclear membrane and nuclear envelope accentuation.

Taking advantage of the unique amino-terminal domains of the Mcl-1 and Bcl-2 proteins, we have recently described the preparation of Mcl-1-specific antisera and applications of these antibody reagents for immunohistochemical and immunoblot analysis of Mcl-1 protein in normal and neoplastic lymph nodes.[17] Those studies revealed a provocative reciprocal distribution of Mcl-1 and Bcl-2 proteins within the cells of the secondary lymphoid follicles of normal and hyperplastic nodes, with Bcl-2 residing in the long-lived recirculating population of small resting lymphocytes found in the mantle zone region and Mcl-1 immunostaining occurring in the apoptosis-prone lymphocytes located in the germinal centers. Other than lymph nodes, however, the expression of *mcl-1* has never been examined in other normal tissues. Here we have extended our previous investigations by performing a comprehensive immunohistochemical analysis of the patterns of *mcl-1* expression in human tissues, making comparisons with *bcl-2*.

Materials and Methods

Normal human tissues were obtained from combinations of fresh autopsy and biopsy materials. For immunohistochemical assays, tissues were either fixed in Bouin's solution or 10% neutral-buffered formalin for subsequent embedding in paraffin and sectioning. For immunoblotting, tissue fragments were either frozen in liquid nitrogen and processed later or were placed immediately into a lysis buffer for preparation of protein samples.[17] The polyclonal antisera employed here were raised in rabbits against synthetic peptides corresponding to amino acids 121 to 139 of the human Mcl-1 protein[17] and 41 to 54 of the human Bcl-2 protein.[18] These synthetic peptides corresponded to unique regions in the Mcl-1 and Bcl-2 proteins that distinguish them from all other known members of the Bcl-2 protein family. The specificity of the resulting antisera was confirmed by a number of methods, including immunoblot analysis of *in vitro* translated proteins and cell lysates prepared from *mcl-1*- or *bcl-2*-transfected cell lines, immunoprecipitation assays with [^{35}S]methionine-labeled proteins, and peptide immunoadsorption assays that demonstrated complete abrogation of reactivity of antibodies with the Mcl-1 and Bcl-2 proteins by the appropriate competing peptide.[17]

Immunohistochemical analysis of Mcl-1 and Bcl-2 proteins in tissue sections was performed exactly as described previously[17] and involved use of a diaminobenzidine detection method with either hematoxylin or methyl green counterstaining of nuclei. The intensity of the immunostaining was evaluated on a 5-point scale (0, 1^+, 2^+, 3^+, 4^+), with immunostained sections from human tonsils as a control and arbitrarily setting the Mcl-1 immunostaining of germinal center lymphocytes at 3^+ and the Bcl-2 immunostaining of mantle zone lymphocytes at 4^+.

Immunoblot analysis was accomplished by previously described methods[17,18] with aliquots of cell lysates that were normalized for total protein content (typically 50 μg/lane). As an internal control, lysates from an *mcl-1*-transfected murine hemopoietic cell line 32D-MCL-1 were included on every blot,[17] and the intensity of the Mcl-1 protein band was arbitrarily set at 4^+ for purposes of comparison with protein-containing lysates derived from various human tissues. To verify integrity of the protein samples, the top portion of blots was excised and stained with Ponceau S.

RNA blot analysis of *mcl-1* mRNA levels in various mouse tissues was performed essentially as described previously[19] with a human *mcl-1* cDNA probe.[5,17]

Results

The Mcl-1 protein was examined by use of an immunohistochemical assay in a wide variety of paraffin-embedded tissue specimens. Table 1 summarizes the immunohistochemistry results and Figure 1 shows some examples. The specificity of the immunostaining was confirmed in every case by staining of sequential sections with preimmune serum and staining with antiserum that had been preabsorbed with either Mcl-1 or Bcl-2 peptide, both of which produced negligible immunoreactivity (not shown).

Skin

In contrast to Bcl-2, which has been reported primarily in the basal cell layer of keratinocytes in the epidermis,[20] Mcl-1 immunoreactivity was strongest in the upper layer of the this stratified epithelium. Specifically, the granular layer of cells contained 2^+ to 4^+ Mcl-1 immunostaining compared with only 1^+ to 2^+ immunostaining in the spinous and basal cell layers. The layer of cornified epithelial cell remnants at the body surface were Mcl-1 negative (Figure 1a). In the dermis, weak to moderate (1 to 2^+) immunostaining for Mcl-1 was detected in the sweat glands, whereas hair follicles and connective tissue fibroblasts contained little or no Mcl-1 immunoreactivity (0 to 1^+), and peripheral nerves were completely negative.

Gastrointestinal Tract

The epithelial cells lining the gastric pits in the stomach (mucous neck cells) were Mcl-1 positive (2 to 3^+), whereas the parietal and chief cells located in the base of the gastric glands were either negative or only weakly immunostained (0 to 1^+). In contrast, Bcl-2 immunostaining was detected in all of these cell types in the stomach and tended to be more intense in the mucous cells located lower in the gastric pits than in those at the luminal surface. Strong Mcl-1 immunostaining was seen in the small intestine. In the proximal duodenum, for example, the intestinal epithelial cells contained intense (4^+) cytosolic immunoreactivity associated with punctate structures that probably represent organelles (Figure 1b), possibly mitochondria by analogy to Bcl-2.[13–16] In the distal

Table 1. *Summary of Results for Immunohistochemical Analysis of Bcl-2 and Mcl-1 Proteins in Human Tissues*

Tissue/organ	Cell type	Mcl-1	Bcl-2
Skin	Keratinocytes		
Epidermis			
Basal cell layer		1–2$^+$	2–3$^+$
Spinous layer		1–2$^+$	1–2$^+$
Granular layer		2–4$^+$	2–4$^+$
Cornified layer		0–1$^+$	0
	Melanocytes	0-1$^+$	2-4$^+$
Dermis			
Sweat glands		1–2$^+$	1–3$^+$
Sebaceous glands hair follicles		0–1$^+$	1–3$^+$
Fibroblasts		0–1$^+$	0–1$^+$
Peripheral nerve fibers		0	1–3$^+$
Digestive			
Stomach			
	Surface mucous cells	1–3$^+$	1–3$^+$
	Mucous neck cells	2–3$^+$	1$^+$
	Parietal cells	1–2$^+$	0–1$^+$
	Chief cells	0–1$^+$	0–1$^+$
Small intestine			
Duodenum:	Enterocytes	2–4$^+$	0–2$^+$
Jejunum/ileum:	Absorptive epithelial cells	2–3$^+$	0–1$^+$
	Crypt stem cells	0–1$^+$	0
	Paneth's cells	0	0
Colon:	Absorptive epithelium	2–3$^+$	0–1$^+$
	Crypt stem cells	0	2–4$^+$
Liver:	Hepatocytes	0	1–2$^+$
Pancreas:	Exocrine cells	0–1$^+$	0–1$^+$
Respiratory			
Nasopharynx			
	Basal cells	0–1$^+$	2–3$^+$
	Squamous cells	2–3$^+$	0–1$^+$
Trachea			
	Basal cells	1–2$^+$	1–3$^+$
	Ciliated columnar cells	2–4$^+$	1–2$^+$
	Chondrocytes	2–4$^+$	0
Bronchi			
	Epithelium	1–2$^+$	1$^+$
Alveoli			
	Pneumocytes	0	0
	Alveolar macrophages	3–4$^+$	0–1$^+$
Cardiovascular			
Heart			
Endocardium		0	0–1$^+$
Myocardium		1–3$^+$	1–2$^+$
Aorta			
Tunica intima		0	0
Tunica media		0–2$^+$	1–3$^+$
Tunica adventitia		0	0
Reproductive			
Ovary			
Germinal epithelium		1–2$^+$	0
Corpus luteum		1–2$^+$	2–4$^+$
Stroma		0	0
Oviduct			
	Columnar epithelium	2–4$^+$	2–3$^+$
Uterus			
Endometrium		0–1$^+$	3–4$^+$
Myometrium		2–4$^+$	1–2$^+$
Vagina/cervix			
	Basal cells	0–1$^+$	2–3$^+$
	Squamous epithelium	2–3$^+$	0–1$^+$
Mammary gland			
Alveoli,	cuboidal epithelium	1–2$^+$	2–4$^+$
Ducts,	cuboidal epithelium	1–2$^+$	2–4$^+$
Prostate			
Epithelium:	Basal cells	0–1$^+$	2–4$^+$
	Secretory cells	1–3$^+$	0
	Smooth muscle	1–3$^+$	1–3$^+$
Testis			
	Spermatogonia	0	0
	Spermatocytes	0–1$^+$	0–1$^+$
	Spermatids	0–1$^+$	2–4$^+$
	Sertoli cells	0	0–1$^+$
	Leydig cells	2–4$^+$	1$^+$
Musculoskeletal			
Skeletal muscle			
	Myotubules	1–2$^+$	0–3$^+$
Bone			
	Osteoblast/osteoclasts	0	0–1$^+$
Hemopoietic/lymphoid			
Spleen			
White pulp:	Small lymphocytes	0$^+$	3–4$^+$
	Large lymphocytes	1–4$^+$	0–1$^+$
Red pulp:	Granulocytes	0–1$^+$	0–1$^+$
Tonsil/lymph node			
Follicles:	Germinal center/lymphs	1–4$^+$	0
	Germinal center/ Small cleaved lymphs	2–4$^+$	1–4$^+$
	Mantle zone/small lymphs	0–1$^+$	3–4$^+$
Interfollicular regions:	Large lymphocytes	2–4$^+$	0–1$^+$
Mantle/interfollicular	Small lymphocytes	0–1$^+$	3–4$^+$
	plasma cells	2–4$^+$	2–4$^+$
Thymus			
Cortex:	Thymocytes	0–4$^+$	0–1$^+$
Medulla:	Thymocytes	0–1$^+$	3–4$^+$
	Epithelioreticular cells	3–4$^+$	0–1$^+$
Bone Marrow			
Myeloid:	Promyelocytes	0–2$^+$	2–3$^+$
	Myelocytes	0–2$^+$	2–3$^+$
	Metamyelocytes	0–2$^+$	0–1$^+$
	Bands	0	0
	Mature neutrophils	0	0
Erythroid:	Early normoblasts	1–2$^+$	3$^+$
	Intermediate	1–2$^+$	2–3$^+$
	Late	0–1$^+$	0–2$^+$
	Erythrocytes	0	0
Megakaryocytes		1$^+$	1–3$^+$
Urinary Tract			
Kidney			
Proximal thick and thin segments		0–1$^+$	1–2$^+$
Distal convoluted tubule		1–3$^+$	0
Henle's loop		1–3$^+$	1–2$^+$
Collecting ducts		1–3$^+$	0
Glomeruli		0	0
Bladder			
	Epithelium	0–4$^+$	2–4$^+$
	Smooth muscle	0–1$^+$	1–3$^+$
Endocrine			
Adrenal gland			
Adrenal cortex:	Zona glomerulosa	4$^+$	0–2$^+$
	Zona fasciculata	2$^+$	0–2$^+$
	Zona reticularis	1–1$^+$	1$^+$
Adrenal medulla:	Chromaffin cells	0–1$^+$	2–4$^+$
Thyroid			
Follicular epithelium		0–1$^+$	3–4$^+$
Endocrine pancreas			
Islets of Langerhans		0–4$^+$	0–1$^+$
Nervous system			
Brain			
Cortex:	Large neurons	0–1$^+$	0–2$^+$
	Neuropil	1$^+$	1–2$^+$
	Neuroglia	0	0
	Myelin fibers	0	0
	Axons in white matter	0–1$^+$	0
Hippocampal neurons		0–1$^+$	1–3$^+$
Spinal cord			
	Neurons	0–1$^+$	0–2$^+$
	Neuroglia	0	0
	Myelin fibers	0	0
	Neuropil	1–2$^+$	1$^+$
Dorsal root/sympathetic ganglia			
	Ganglion cells	1–3$^+$	2–4$^+$
	Schwann cells	0–1$^+$	1–3$^+$
	Axons	0–1$^+$	0–2$^+$

Results of immunostaining experiments are summarized. Numbers represent relative intensity of cells within the stated organ or among the specified cell types. Immunostaining results were scored on a 4-point scale (0 to 4$^+$). During each experiment, a section of tonsil tissue was stained as a standard, with Mcl-1 immunoreactivity in germinal center lymphocytes arbitrarily set at 3$^+$ and Bcl-2 in mantle zone lymphocytes at 4$^+$. Data are representative of two to four independent experiments.

duodenum and jejunum, cytosolic Mcl-1 immunoreactivity was more evenly distributed but still present in a fine, granular pattern, again suggestive of an association with organelles. In the villi of the small intestine and in the colonic crypts, a gradient of Mcl-1 immunostaining was seen with more intense immunostaining occurring in the epithelial cells located in the upper portions than in the bases of the intestinal and colonic glands (see Figure 1c for example). This pattern is the opposite of that previously reported for Bcl-2,[21,22] which is present at higher levels in the epithelial cells in the crypts than at the luminal surface. Hepatocytes contained 1^+ to 2^+ intensity Mcl-1 immunostaining in the cytosol, which again was present in association with what appeared to be organelles. The exocrine cells of the pancreas were either negative for Mcl-1 or only weakly (1^+) positive, whereas the epithelial cells lining the pancreatic ducts were more intensely stained (1^+ to 2^+).

Pulmonary

Mcl-1 immunostaining in the epithelium of the nasopharynx occurred in a gradient opposite to that previously reported for Bcl-2,[23] with the highest levels of Mcl-1 appearing in the cells at the surface and the lowest in the cells lining the basement membrane (Figure 1d). The pseudostratified columnar epithelium of the trachea and large airways also contained Mcl-1, with the larger columnar cells at the luminal surface having stronger intensity immunostaining (3^+ to 4^+) than the less differentiated smaller rounder cells (1^+ to 2^+) located mostly along the basement membrane (Figure 1e). In contrast, Bcl-2 immunostaining tended to be stronger in the less differentiated cells than in the tall columnar cells (not shown). Alveolar pneumocytes were negative or only weakly stained for Mcl-1, whereas alveolar macrophages were strongly Mcl-1-positive (Table 1).

Cardiovascular

Cardiomyocytes contained moderate Mcl-1 immunoreactivity (2 to 3^+). The immunoreactivity often had a granular quality and was sometimes stronger in the vicinity of the nucleus (Figure 1f). The endocardium was Mcl-1 negative. Arterial smooth muscle cells generally contained only low levels of Mcl-1 immunoreactivity (0 to 1^+), whereas intimal endothelial cells and adventitial connective tissue fibroblasts were negative.

Musculoskeletal

Skeletal muscle was heterogeneously stained for Mcl-1. Some fibers contained moderate (2^+ to 3^+) Mcl-1 immunoreactivity whereas others were nearly Mcl-1 negative (Figure 1g). It is currently unknown whether this heterogeneity in Mcl-1 immunostaining correlates with fast (mitochondria-rich) or slow (mitochondria-poor) twitch fiber status. Similar to cardiac muscle, the Mcl-1 immunoreactivity had a granular appearance, suggestive of organelle staining. In some cross-sections of striated muscle fibers, the Mcl-1 immunoreactivity was heterogeneously distributed (Figure 1h). In cartilage, chrondrocytes contained 2^+ to 4^+ Mcl-1 immunostaining (Figure 1i) but were completely negative for Bcl-2 (not shown). Osteoblasts and osteoclasts were Mcl-1 negative (Table 1).

Hematolymphoid

Variable levels of Mcl-1 immunoreactivity were seen in hemopoietic cells in the bone marrow (a total of three biopsies and three smears derived from six persons were examined). Somewhat less than half of all promyelocytes, myelocytes, and metamyelocytes contained weak to moderate intensity (1 to 2^+) Mcl-1 immunoreactivity, whereas bands were only occasionally Mcl-1 positive, and mature neutrophils were only rarely immunostained for Mcl-1. Early and intermediate stage normoblasts were almost uniformly immunostained for Mcl-1 (1 to 2^+), but later stage normoblasts were less frequently Mcl-1 positive, and erythrocytes were negative. Megakaryocytes contained diffuse weak (1^+) cytosolic Mcl-1 immunoreactivity. The immunostaining of immature myeloid and erythroid cells with anti-Bcl-2 antibodies was generally more intense than for Mcl-1, but, like Mcl-1, diminished in frequency and intensity as these hemopoietic cells matured.[20,21,23]

In lymph nodes and tonsils, intense (3 to 4^+) Mcl-1 immunostaining was present in both small cleaved and large noncleaved lymphocytes of germinal centers, whereas the small lymphocytes that comprised the mantle zone region of these lymphoid follicles were completely Mcl-1 negative (Figure 1l). As reported previously, this pattern of *mcl-1* expression in the secondary lymphoid follicles of nodes and tonsils is the opposite of that seen with Bcl-2, which is present at high levels in the mantle zone lymphocytes and absent from most germinal center cells.[17,20,24] Large activated lymphocytes and mature plasma cells in the interfollicular regions of nodes were also strongly stained for Mcl-1.

Similarly, in the spleen, most lymphocytes in germinal centers were Mcl-1 positive, whereas the surrounding cuff of small lymphoid cells of both the mantle zone and marginal zone were negative. In other areas of the white pulp, including the periarterial lymphatic sheath, most lymphocytes were weakly stained for Mcl-1 (1^+), but occasional larger activated lymphoblasts contained high levels (3 to 4^+) of Mcl-1 immunoreactivity (not shown). In the red pulp, granulocytes exhibited 0 to 1^+ Mcl-1 immunoreactivity. Scattered mature plasma cells throughout the spleen were Mcl-1 immunoreactive.

In the thymus, prominent Mcl-1 immunostaining was present in the epithelioreticular cells and Hassall's corpuscles (3 to 4^+; Figure 1j). Most thymocytes in the cortex and medulla were either negative or only weakly immunostained (1^+), but rare cells in the cortex contained a rim of intense (3 to 4^+) cytosolic Mcl-1 immunoreactivity.

Genitourinary

In the kidney, Mcl-1 immunoreactivity was present at moderate levels (1 to 3^+) in the collecting ducts and most of the tubules of the nephrons but was largely absent from the glomeruli (Figure 1m). In bladder epithelium, Mcl-1 immunostaining was strongest in the luminal epithelial cells and diminished in intensity toward the basal layers of cells (Figure 1n). Bcl-1 immunoreactivity, in contrast, had an opposite orientation, with stronger immunostaining of the basal than the luminal layer (not shown).

The epithelium of the prostate contained weak to moderate Mcl-1 immunostaining (1^+ to 3^+), with the differentiated luminal secretory epithelial cells exhibiting stronger immunostaining than the basal cells along the basement membrane, which were mostly Mcl-1 negative (Figure 1o). In contrast, intense Bcl-2 immunostaining was present in the basal cells, with little or no staining detected in the secretory cells. In the testis, moderate to strong (2 to 4^+) Mcl-1 immunostaining was seen in the Leydig cells (Figure 1p) as well as cytoplasmic debris around spermatozoa. The Sertoli cells, spermatogonia, primary and secondary spermatocytes, and spermatids were mostly negative. In contrast, Bcl-2 immunostaining has been reported in the spermatids but not the other cells of the testis.[23]

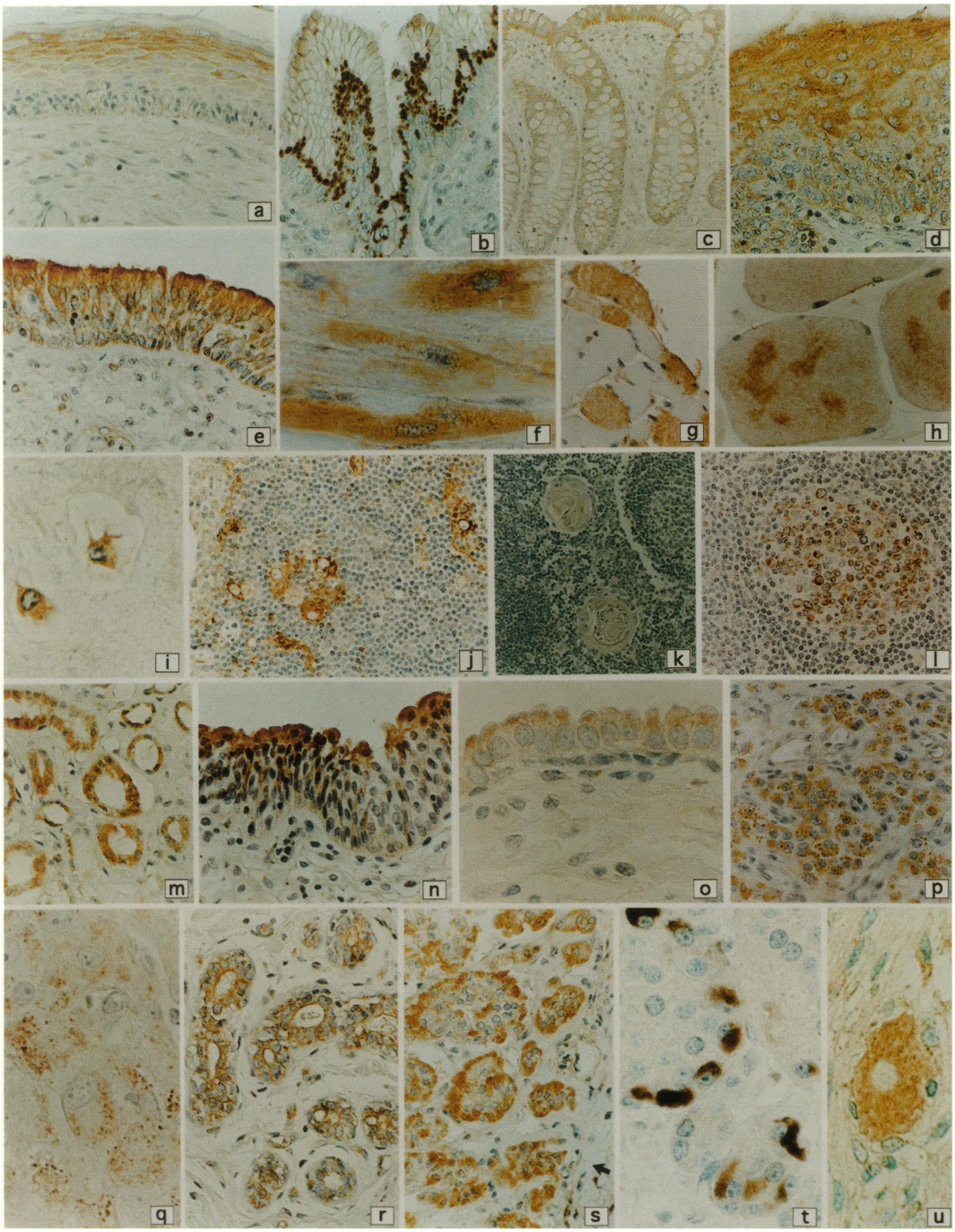
In the uterus, Mcl-1 immunostaining was present at moderate to high levels in the epithelial cells that comprise the endometrium. The smooth muscle cells of the myometrium contained variable levels of Mcl-1 immunoreactivity (1–3^+). In ovarian tissues, Mcl-1 immunostaining was most pronounced in the granulosa lutein cells of the corpus luteum (Figure 1q), with either no or only low (1^+) levels of Mcl-1 immunoreactivity detected in other ovarian cells at other stages of the ovulatory cycle. The Mcl-1 immunostaining of these lutein cells was distinctly granular in appearance. Interestingly, unlike other locations examined, arterioles within ovarian stromal tissue contained moderate to strong Mcl-1 immunostaining in the elastic lamina (not shown). Mcl-1 was present in mammary epithelium (1 to 3^+) but was largely absent from the surrounding connective tissue (Figure 1r).

Neuroendocrine

The cells of the adrenal cortex exhibited strong Mcl-1 immunostaining, with the aldosterone-producing cells of the zona glomerulosa in the outermost portion containing higher levels of Mcl-1 immunoreactivity (4^+) than the cells of the zona fasciculata (2^+) and the zona reticularis (1 to 3^+), which are responsible for glucocorticoid and androgen secretion (Figure 1s and not shown). The chromaffin cells of the adrenal medulla were only weakly immunostained (0 to 1^+) for Mcl-1.

In the islets of Langerhans in the pancreas, a subpopulation of cells was observed that contained intense Mcl-1 immunoreactivity (3 to 4^+; Figure 1t). Typically, these cells were located at the outer border of the islets, the reported position of the glucagon-producing α-cells.[25] Indeed, double staining tech-

Figure 1. *Immunostaining of human tissues for Mcl-1 or Bcl-2. Various paraffin-embedded tissues were sectioned and immunostained with antiserum specific for the Mcl-1 protein. Antibodies were detected by a diaminobenzidine colorimetric method, which produces a brown color. Nuclei were counterstained with either hematoxylin or methyl green.* **a:** *Epidermis (×400 original magnification).* **b:** *Duodenum (×400).* **c:** *Colon (×200).* **d:** *Nasopharnyx (×400).* **e:** *Trachea (×400).* **f:** *Cardiac myocytes (×1000).* **g:** *Skeletal muscle (pectoralis) (×100).* **h:** *Cross-section through muscle fibers taken from same muscle sample shown in preceeding panel (×1000).* **i:** *Chrondrocytes (×1000).* **j:** *Thymus, showing mostly medulla (×400).* **k:** *Same thymus as in* (j)*, immunostained with anti-Mcl-1 antiserum that had been preadsorbed with Mcl-1 peptide to verify specificity of the immunoreactivity (×200). At right, an ectopic germinal center can be seen in addition to the thymic tissue with Hassal's bodies in the center of the photomicrograph.* **l:** *Tonsil, showing a secondary lymphoid follicle with germinal center with surrounding mantle zone (×200).* **m:** *Kidney, showing Mcl-1 staining in collecting ducts and thin segments of descending limb of Henle, but not in surrounding stromal tissue (×200).* **n:** *Bladder epithelium (×400).* **o:** *Prostate epithelium (×1000).* **p:** *Leydig cells within the testis (×400).* **q:** *Granulosa lutein cells within a corpus luteum in ovary (×1000).* **r:** *Mammary gland, showing Mcl-1 immunostaining in epithelial duct cells but not surrounding stroma (×400).* **s:** *Adrenal cortex, showing cells within the zona glomerulosa where mineralocorticoids are produced. The adrenal capsule is indicated by an arrow (×400).* **t:** *pancreatic islets surrounded by exocrine cells (×1000).* **u:** *Sympathetic neuron within a paraspinal ganglion, counterstained with methyl green (×1000).*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niques suggested that at least some of these cells also contained glucagon but not insulin or somatostatin (unpublished data). Expression of *mcl-1* therefore may be limited to the α-cells of the islets and absent from the β- and δ-cells, although more definitive studies must be performed before firm conclusions can be reached. Little Mcl-1 immunostaining was detected in the thyroid epithelium (not shown). In contrast, these cells stained intensely for Bcl-2.

Mcl-1 immunostaining was detected at variable levels (2^+ to 4^+) in the neurons of sympathetic ganglia and the paraspinal dorsal root ganglia, with many cells exhibiting moderate to strong immunoreactivity in a granular cytosolic distribution (Figure 1u and data not shown). In the spinal cord and brain, only weak (1^+) Mcl-1 immunostaining was detected in some populations of neurons.

Immunoblot Analysis of Mcl-1 Protein

The results of immunoblot assessment of Mcl-1 and Bcl-2 protein levels in various tissues are summarized in Table 2. In general, the immunoblot analysis of Mcl-1 protein levels in tissue lysates was in agreement with the immunostaining data, particularly given the heterogeneity of Mcl-1 production among the various cell types found in different organs. The highest relative levels of Mcl-1 protein were found in the adrenal glands, oviduct, and testes, with somewhat lower levels in the skin, liver, ovary, and trachea. All of these tissues contained cells with strong Mcl-1 immunostaining. In all tissues examined, the Mcl-1 antibody reacted with a ~37- to 39-kd protein, consistent with our previous characterization of the Mcl-1 protein.[17] In some specimens, an additional ~30-kd band was also seen, which we have shown probably represents a partial degradation product of Mcl-1.[17] In this regard, the Mcl-1 protein contains two PEST sequences, which are known to target proteins for rapid degradation.[26] Nevertheless, the bulk of proteins in the tissue lysates analyzed here were determined to be intact, on the basis of the use of a protein stain (see Materials and Methods for details), and reanalysis of the same blots with anti-Bcl-2 antibodies demonstrated undegraded 26-kd Bcl-2 protein (data not shown). Thus, the presence of the lower molecular weight Mcl-1 protein species does not represent a general problem with protein integrity and presumably therefore reflects the specific regulation of Mcl-1 protein turnover whether physiological or a result of postmortem processes. The failure to detect Mcl-1 protein in some tissues such as pancreas and breast presumably reflects the presence of many Mcl-1-negative cells that dilute the contribution of the *mcl-1*-expressing cells. However, we cannot entirely exclude the possibility of rapid and selective degradation of Mcl-1 protein during tissue harvesting and processing, and thus these data should be regarded as a minimal estimate of the relative amounts of Mcl-1 protein present in the various tissues analyzed.

Table 2. *Summary of Immunoblot Analysis of Mcl-1 and Bcl-2 Protein Levels in Human Tissues*

Tissue	Mcl-1	Bcl-2
Skin	$0–4^{+*}$	$2–3^+$
Stomach	$0–1^+$	$2–3^+$
Intestine	0	$3–4^+$
Liver	$0–2^{+*}$	$0–1^+$
Pancreas	0	1^+
Lung	$0–1^+$	0
Trachea	2^+	2^+
Heart	$0–2^+$	$0–2^+$
Aorta	$0–2^+$	$2–3^+$
Skeletal muscle	$0–2^+$	$1–2^+$
Prostate	$0–1^+$	$1–3^+$
Testes	$2–4^{+*}$	$1–2^+$
Ovary	$1–3^+$	4^+
Kidney	$0–2^+$	$0–3^+$
Bladder	$0–1^+$	$2–3^+$
Breast	0	$2–3^+$
Lymph node	$0–1^+$	2^+
Tonsil	$0–1^+$	$3–4^+$
Spleen	$0–1^+$	$3–4^+$
Brain (cortex)	0	$0–1^+$
Adrenal	$2–3^+$	$0–2^+$

Results of immunoblot experiments are summarized with protein-containing lysates prepared from various tissues and normalized for total protein content. Numbers represent relative intensity of bands. Immunoblot results were scored on a 4-point scale (0 to 4^+). During each experiment, an internal control lysate was included, consisting of either a murine hemopoietic cell clone 32D.3 transfected with a human *mcl-1* expression plasmid[15] or a human B cell lymphoma cell line RS11846 that contains a t(14;18) translocation involving the *bcl-2* gene, both of which were arbitrarily set at 4^+. All data are representative of two to four independent experiments. *Samples for which the predominant Mcl-1 immunoreactive bond was approximately 30 k.

RNA Blot Analysis of mcl-1 mRNA in Mouse Tissues

RNA was isolated from a variety of adult mouse tissues, and the relative levels of *mcl-1* mRNA were determined by Northern blotting in an effort to further test the validity of the immunostaining results. As shown in Figure 2, transcripts of ~4.5 and 2.7 kb were detected in several tissues by hybridization with the *mcl-1* probe. In particular, relatively high levels of *mcl-1* mRNA were seen in spleen, heart, lung, and kidney, with moderate levels in skeletal muscle and lower levels in brain and liver. Reprobing of the RNA blots with a probe for GAPDH served as a control to verify loading of essentially equivalent amounts of in-

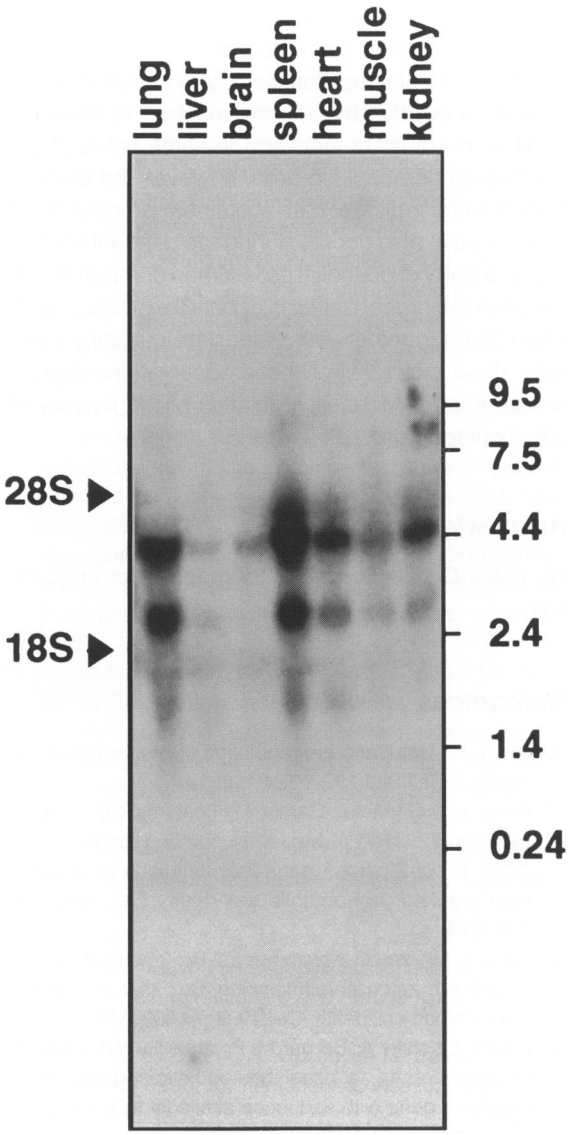

Figure 2. *RNA blot analysis of* mcl-1 *in mouse tissues. Total cellular RNA was isolated from various adult mouse (~8 weeks old) tissues, and subjected to Northern blotting assay (20 μg/lane) with a* ^{32}P-*labeled human* mcl-1 *cDNA probe.*[5,15] *The relative positions of RNA standards are indicated, as well as the 28S and 18S rRNA bands.*

tact RNA for all samples (not shown). In contrast to *mcl-1*, in the tissues examined here, *bcl-2* mRNA has been reported to be detectable by Northern blotting in mouse spleen and brain but not heart, muscle, kidney, liver, or lung.[26] These results thus confirm our immunohistochemical data that suggest that *mcl-1* is widely expressed *in vivo*. The relatively high levels of *mcl-1* mRNA seen in spleen may be accounted for by the extramedullary hematopoiesis typically found in mice, thus explaining the discrepancy with the relatively low levels of Mcl-1 protein detected by immunoblot analysis of human spleen.

Discussion

In this report, we have delineated for the first time the *in vivo* patterns of *mcl-1* expression by immunohistochemical, immunoblot, and RNA blotting approaches and have made comparisons with *bcl-2*, a gene that is known for its ability to block programmed cell death.[1] In contrast to *bcl-2*, which is expressed in many epithelial tissues in the less differentiated, self-renewing stem cell population typically found along the basement membrane,[20–23] expression of *mcl-1* was often highest in the differentiated cells located in the upper portions of complex epithelia, including those of the epidermis, intestine, colon, prostate, nasopharynx, and upper airway. The opposing gradients of *mcl-1* and *bcl-2* expression seen in these tissues, therefore, suggests that *mcl-1* and *bcl-2* fulfill different functions in the *in vivo* regulation of programmed cell death in many epithelia. As the Bcl-2 protein is an inhibitor of physiological cell death, presumably it participates in maintaining the long-term survival of the self-renewing stem cell populations in these epithelial tissues. Consistent with this notion are data from some lines of transgenic mice that have homozygous disruptions of their *bcl-2* genes (knockout mice) and that have abnormalities in the small intestine including increased numbers of apoptotic cells in the crypts and shortened microvilli.[28] In contrast to Bcl-2, the location of Mcl-1 in many epithelial tissues implies a different function for this member of the Bcl-2 protein family.

Until the function of the Mcl-1 protein is better established through gene transfer, targeted gene disruption, and other types of experiments, we can only speculate about the contribution that the Mcl-1 protein might make to the *in vivo* regulation of programmed cell death. Comparisons of Mcl-1 and Bcl-2 function in Chinese hamster ovary cells have suggested that Mcl-1 is considerably less potent than Bcl-2 at preventing c-Myc-induced apoptosis.[8] Similarly, in the myeloid progenitor clone 32D.3, we have found that gene transfer-mediated elevations in Bcl-2 protein confer upon these cells an ability for prolonged survival in the absence of growth factors, whereas Mcl-1 does not[10] (SB and JCR, unpublished data). Mcl-1 therefore may provide only limited protection against apoptosis compared with Bcl-2, thus allowing for physiological cell death mechanisms to more easily override Mcl-1 protection so that terminally differentiated cells can expire in an appropriate fashion.

Previously, we showed that Mcl-1 can physically interact with the Bcl-2 protein.[5] Although the functional significance of this protein-protein interaction

remains unclear, one implication of the data shown here is that the potential for interactions between the Mcl-1 and Bcl-2 proteins is markedly limited *in vivo* by the differential patterns of expression of the *mcl-1* and *bcl-2* genes. Although Mcl-1 and Bcl-2 were presumably produced within the same cells in some tissues, such as mammary epithelium and in sympathetic ganglia, in many cases the patterns of Mcl-1 and Bcl-2 protein production were strikingly different. For example, the opposing gradients of Mcl-1 and Bcl-2 protein production seen in the epithelia of the nasopharynx, trachea, colon, parts of the intestine, and prostate reduce opportunities for interactions of the Mcl-1 and Bcl-2 proteins in these tissues. Similarly, in the secondary follicles of lymph nodes and tonsils, Mcl-1 was present in most lymphocytes within germinal centers, whereas Bcl-2 was predominantly located in the lymphocytes of the mantle zone region, again limiting possibilities for interactions between Mcl-1 and Bcl-2. These findings suggest therefore that the differential expression of *mcl-1* and *bcl-2* in various tissues has important implications for determining the repertoire of Bcl-2-like proteins that are available for participation in protein-protein interactions in any particular type of cell at its various stages of differentiation or activation. Thus, although at the biochemical level the Mcl-1 and Bcl-2 proteins are capable of interacting with each other, the data presented here suggest that Mcl-1/Bcl-2 protein-protein interactions are probably not physiologically relevant for regulating programmed cell death in many types of cells *in vivo*. In contrast, many of the same types of cells that lack Bcl-2 contain Bax protein,[23] including the more differentiated cells in the epidermis, colon, and prostate, as well as in the large noncleaved cells located in the germinal centers of lymph nodes. This therefore raises the possibility that interactions of Mcl-1 with Bax could be more physiologically relevant in several types of cells that fail to express *bcl-2*, as the *bax* gene is more widely expressed than *bcl-2*.[23]

The *mcl-1* gene was first discovered because of its transient induction during phorbol ester-stimulated differentiation of a myeloblastic leukemia cell line,[7] implying that this member of the *bcl-2* gene family plays some specific role in the regulation of programmed cell death in more differentiated hemopoietic cells. The immunostaining data described here, however, suggest that, *in vivo*, Mcl-1 is preferentially expressed in immature myeloid precursors in the bone marrow, with considerably lower levels of Mcl-1 immunoreactivity occurring in fully differentiated neutrophils. Similar to the leukemic cells from which *mcl-1* was initially cloned, possibly the expression of Mcl-1 is only transiently induced in these hemopoietic cells during their differentiation. This idea is consistent with the observation that less than half of all immature myeloid cells contained sufficient Mcl-1 protein to be scored as positive by our immunostaining assay. In addition to transient induction in some types of differentiating hemopoietic cells, however, the data reported here indicate that sustained production of Mcl-1 protein also occurs during the differentiation of a wide variety of epithelial cells. When compared with the often reciprocal patterns of *bcl-2* expression and, when taken together with other data currently available about mcl-1,[5,7,8,17] these observations imply a unique role for Mcl-1 in regulation of the survival of differentiated cells.

Acknowledgments

We thank Cecilia Stephens for manuscript preparation.

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