

Bcl-2, Bcl-x, and Bax Expression by Immunohistochemistry in Inclusion Body Myositis

A Study of 27 Cases

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• **Context.**—Bcl-2, Bcl-x, and Bax are among the variety of proteins that have been described as being involved in the regulation of apoptotic cell death. Bcl-2 and Bcl-x_L inhibit apoptosis, and Bax is proapoptotic.

Objective.—To evaluate the expression of Bcl-2, Bcl-x, and Bax in inclusion body myositis (IBM).

Design.—We examined muscle specimens from 27 patients (17 men, 10 women) with IBM to evaluate Bcl-2, Bcl-x, and Bax expression by immunohistochemistry.

Results.—Patient ages ranged from 29 to 80 years (mean 62.2 years). All biopsies were marked by endomysial chronic inflammation, muscle fiber necrosis, and regeneration. Rimmed (autophagic) vacuoles were present in all cases. Ragged red fibers were noted in 4 biopsies (15%), and cytochrome oxidase-deficient fibers were found in 10 biopsies (37%). Ultrastructural evidence of intranuclear or cytoplasmic tubulofilamentous inclusions, confirming the diagnosis of IBM, were noted in all cases. Paracrystalline mitochondrial inclusions were seen in 5 biopsies (18.5%). Inflammatory cells stained positively with Bcl-2 in all biopsies, Bax in 26 biopsies (96%), and Bcl-x in 8 biopsies

(30%). Degenerating muscle fibers were highlighted with Bax in 24 biopsies (89%), Bcl-2 in 2 biopsies (7%), and Bcl-x in 3 biopsies (11%). Regenerative muscle fibers were noted to stain with Bax in 24 muscles (89%), Bcl-2 in 21 muscles (78%), and Bcl-x in 4 muscles (15%). Rimmed vacuoles were highlighted by Bax in 24 biopsies (89%) and only rarely by Bcl-2 (n = 2, 7%) and Bcl-x (n = 3, 11%). A subsarcolemmal staining pattern was observed in 21 biopsies (78%) with Bax, 6 biopsies (22%) with Bcl-2, and only 1 biopsy (4%) with Bcl-x.

Conclusions.—(1) Bax (proapoptotic) immunostaining highlighted most autophagic vacuoles; (2) subsarcolemmal Bax and Bcl-2 immunoreactivity may be associated with mitochondrial defects that are commonly noted in IBM; (3) Bcl-2 and Bax immunoreactivity were not confined to degenerating muscle fibers and in fact appeared to be expressed more commonly in regenerating fibers, suggesting that their expression may be independent of apoptosis in the setting of IBM.

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In 1967, Samuel Chou described myxovirus-like tubulofilamentous structures in a patient who was presumed to have polymyositis.¹ This finding subsequently resulted in the recognition of inclusion body myositis (IBM) as a unique pathologic entity. Clinically, patients with IBM typically present with slowly progressive, painless distal muscle weakness without significant associated systemic autoimmune or connective tissue diseases.^{2–8} Histopathologically, the identification of rimmed or autophagic vacuoles and tubulofilamentous inclusions, measuring 15 to 18 nm in diameter, in the background of inflammatory myopathic-appearing biopsy material are diagnostic of the entity. The importance of making the diagnosis rests on the therapeutic implications. Most cases of IBM appear to be refractory to corticosteroid therapy. This characteristic is in contrast to many of the other inflammatory myo-

pathic conditions, most notably polymyositis and dermatomyositis, which are typically responsive to steroids.

Despite the rather distinctive histopathologic features of this disease, the underlying etiology and pathophysiological mechanisms that give rise to IBM are not well defined. The consistent finding of myofiber necrosis raises a question about the potential role of apoptosis in the pathogenesis. In 1972, Kerr et al⁹ initially defined apoptosis histopathologically as the breakup of individual cells into smaller, often round to ovoid, cytoplasmic fragments containing pyknotic nuclear debris. Since its initial descriptions, much has been learned regarding the underlying mechanisms and proteins that modulate this form of cell death. The role of apoptosis in the development of skeletal muscle has been well described,^{10,11} but its role in the pathogenesis of human neuromuscular diseases has yet to be fully elucidated.

A variety of proteins have been recognized as important in the modulation of apoptosis. Bax promotes apoptosis, while Bcl-2 and Bcl-x_L inhibit it.^{12–16} These proteins and their potential function in the setting of IBM have not been examined extensively. This study evaluates the immunohistochemical expression of Bcl-2, Bcl-x, and Bax in a series of 27 patients with biopsy-proven IBM.

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METHODS AND MATERIALS

The surgical pathology files at the Cleveland Clinic Foundation (Cleveland, Ohio) were searched for cases of IBM diagnosed on skeletal muscle biopsy. Cases that were studied included those in which all light and electron microscopic materials were available for review and a paraffin block of formalin- or Hollander-fixed tissue was available for immunohistochemistry. All light and electron microscopic materials were reviewed, and cases with ultrastructural evidence of diagnostic tubulofilamentous inclusions, measuring 15 to 18 nm in diameter, were included for study. A total of 27 patients with biopsies formed the study group. Routine light microscopic sections reviewed in each case included sections stained with hematoxylin-eosin, Gomori trichrome, esterase, adenosine triphosphatase (pH 4.6 and 9.8), cytochrome C oxidase, acid phosphatase, alkaline phosphatase, nicotinamide-adenine dinucleotide (reduced form), sulfonated Alcian blue, periodic acid-Schiff, and oil red O.

For each biopsy, the light microscopic findings were reviewed to document the presence of muscle fiber degeneration (myonecrosis), regeneration, and chronic endomysial inflammation. In addition, rimmed (autophagic) vacuoles were noted when present. Myofibers with rimmed vacuoles were not necessarily considered degenerating. Evidence of neurogenic atrophy in the form of angular atrophic esterase-positive muscle fibers or fiber-type grouping were also noted. The presence of ragged red fibers or a partial absence of cytochrome C oxidase staining, suggestive of mitochondrial defects, was also documented. In all cases, electron micrographs were reviewed for evidence of tubulofilamentous inclusions, autophagic vacuoles, and mitochondrial defects.

Paraffin immunohistochemistry was performed on serially cut tissue sections utilizing an avidin-biotinylated immunoperoxidase methodology.¹⁷ For each stain, appropriate positive and negative controls were performed. Immunostaining was performed with antibodies to Bcl-2 (1:5 dilution, Dako Corporation, Carpinteria, Calif), Bcl-x (1:50 dilution, Dako), and Bax (1:30 dilution, Dako). The Bcl-x antibody corresponds to amino acids 46 through 66 of human Bcl-x protein and by Western blot analysis to Bcl-x_L. In addition, a small comparison group of 8 muscle biopsies in patients with other inflammatory conditions of muscle (polymyositis, n = 4; dermatomyositis, n = 2; granulomatous myositis, n = 1; and necrotizing vasculitis, n = 1) were evaluated with the same set of antibodies.

RESULTS

Twenty-seven patients with a skeletal muscle biopsy diagnosis of IBM comprised the study group. The patients consisted of 17 men (63%) and 10 women (37%). The patients ranged in age from 29 to 80 years (mean 62.2 years). Light microscopic findings in all biopsies were consistent with those of IBM. All biopsies showed evidence of muscle fiber degeneration and regeneration. Scattered foci of chronic, predominantly endomysial inflammation consisting mainly of lymphocytes and macrophages were noted. Rimmed or autophagic vacuoles were identified in all cases and were best highlighted on the Gomori trichrome stain (Figure 1). All biopsies showed evidence of neurogenic atrophy in the form of angular atrophic esterase-positive muscle fibers. Seven biopsies showed focal areas of increased endomysial fibrous tissue, particularly in ar-

reas of prominent muscle fiber atrophy. Fiber-type grouping was not observed in any of the biopsies on the adenosine triphosphatase stains. One biopsy showed rare nemaline rod formation, highlighted on the Gomori trichrome stain. Ragged red fibers, defined by increased peripheral rim of eosinophilic-staining granular material on the trichrome stain, were noted in 4 biopsies (15%). Scattered cytochrome C oxidase-negative fibers were identified in biopsies from 10 patients (37%).

Electron micrographs were reviewed in all biopsies that showed evidence of tubulofilamentous intranuclear and intracytoplasmic inclusions, which typically ranged in diameter from 15 to 18 nm (Figure 2). In most cases, these inclusions were identified within muscle fibers that contained autophagic vacuoles. Evidence of mitochondrial derangements as manifested by paracrystalline mitochondrial inclusions were noted in 5 biopsies (19%) (Figure 3).

The Table summarizes the immunohistochemical staining results with antibodies to Bcl-2, Bcl-x, and Bax. Inflammatory cells, which were identified in all but 1 biopsy, were positive for Bax and Bcl-2 (Figure 4). Five (30%) biopsies showed rare positive-staining inflammatory cells with Bcl-x. Twenty-four of 27 biopsies demonstrated evidence of Bax-positive staining in degenerating and regenerating muscle fibers (Figure 5). Twenty-one biopsies demonstrated positive staining of regenerating muscle fibers with Bcl-2, and 4 biopsies with Bcl-x. Only 2 and 3 biopsies demonstrated positive staining of degenerating fibers with Bcl-2 antibody and Bcl-x, respectively. Normal-appearing myofibers did not stain with any of the antibodies. Focal subsarcolemmal staining was observed in scattered muscle fibers with Bax antibody in 21 biopsies (Figure 6). A similar pattern of staining was observed in only 6 biopsies with Bcl-2 antibody and in 1 biopsy with Bcl-x antibody. The vast majority of autophagic vacuoles were highlighted with Bax antibody in all cases (Figure 7). Only rare autophagic vacuoles were noted to stain positively with antibodies to Bcl-2 (n = 2) and Bcl-x (n = 3).

In the biopsies from the 8 comparison group cases, inflammatory cells (principally lymphocytes) stained positively with Bax and Bcl-2 antibodies in all cases and with Bcl-x in 2 cases of polymyositis. Staining was observed in degenerating myofibers with Bax in 4 cases, Bcl-x in 1 case, and Bcl-2 in none of the cases. Regenerating muscle fibers were highlighted in all 8 cases with Bax, in 7 cases with Bcl-2, and in 1 case with Bcl-x.

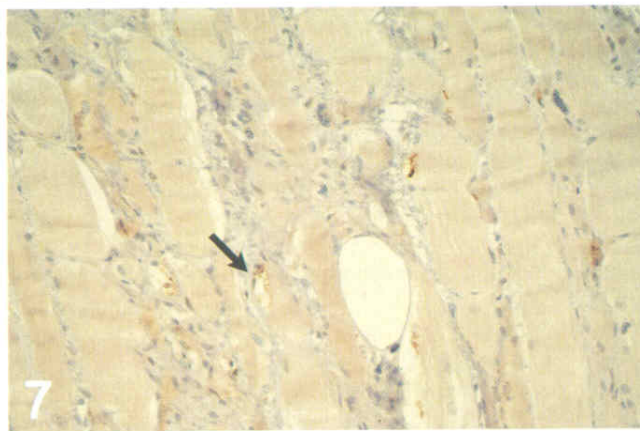
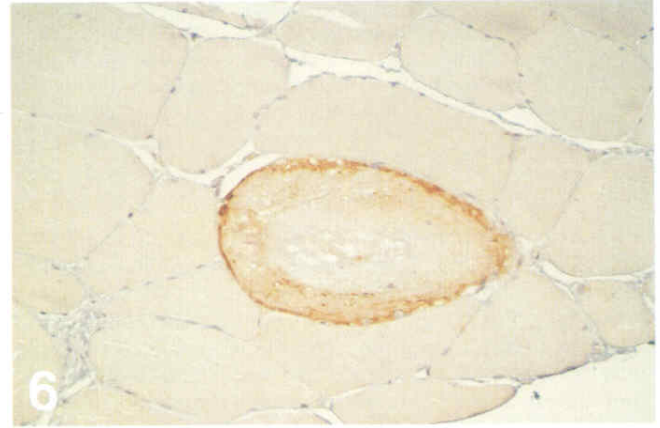
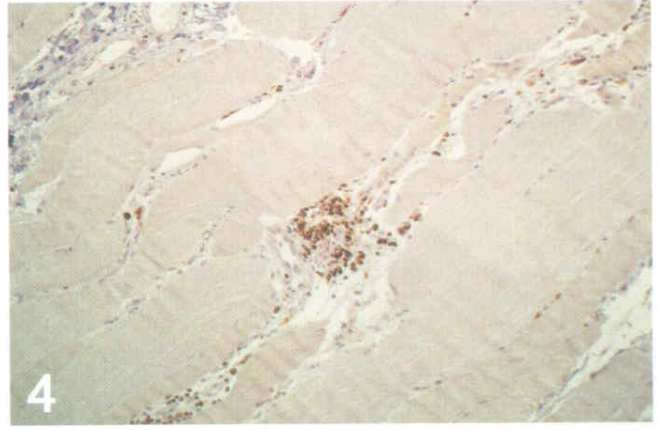
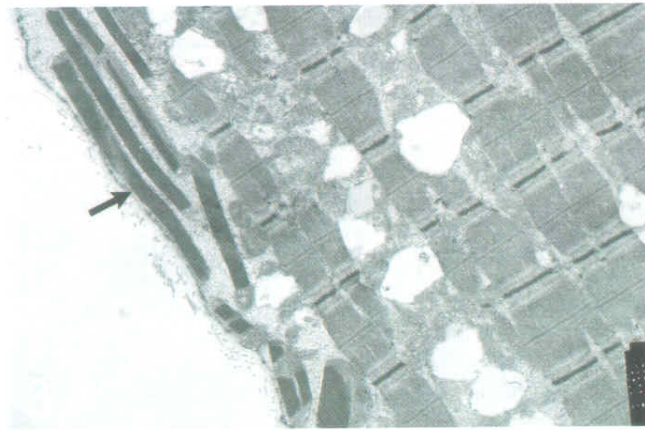
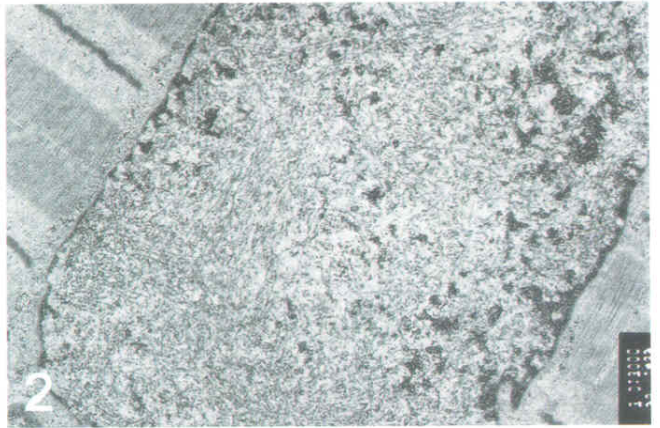
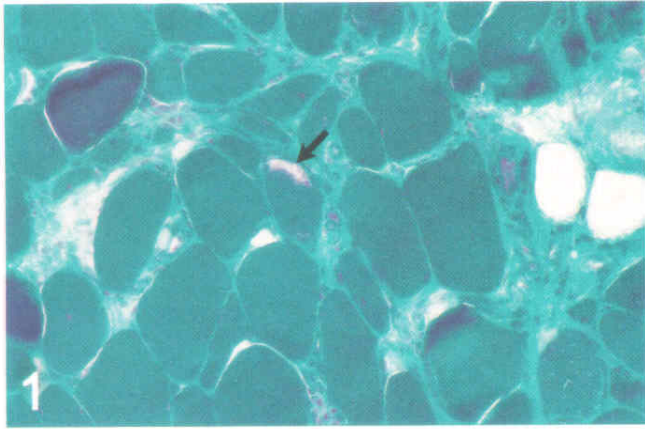
COMMENT

The slowly progressive clinical course in IBM and poor therapeutic response to anti-inflammatory medications suggest that the inflammatory component of the lesion may not be the main process mediating myofiber necrosis. Apoptosis, as a mechanism of muscle fiber death, has been well documented to occur in certain pathologic conditions involving skeletal muscle, including muscular dystrophy

Summary of Bcl-2, Bcl-X, and Bax Immunoreactivity in Inclusion Body Myositis (N = 27)

Antibody	Inflammation	Degenerating Fibers	Regenerating Fibers	Autophagic Vacuoles	Subsarcolemmal Staining
Bax	26 (96)*	24 (89)	24 (89)	24 (89)	21 (78)
Bcl-2	27 (100)	2 (7)	21 (78)	2 (7)	6 (22)
Bcl-x	8 (30)	3 (11)	4 (15)	3 (11)	1 (4)

* Values are expressed as number positive (%). Inflammatory cells were not identified on the slide in 1 case immunostained with Bax antibody.



and spinal muscular atrophy.¹⁸⁻²⁰ Several proteins have been identified to play a role in modulating apoptosis. Of the apoptosis-associated antibodies that were examined in the current study, the most widespread immunoreactivity was observed with the Bax antibody. In the majority of biopsies, scattered degenerating and regenerating muscle fibers were observed to stain positively with the Bax antibody. In addition, prominent staining was observed in association with autophagic rimmed vacuoles and in the subsarcolemmal region, the latter likely corresponding to mitochondria in this location. Whether apoptosis occurs or not in a given cell is related to the ratio of Bcl-2 to Bax and the ability of Bax to block Bcl-2 activity. In the degenerating fibers in the current study, the number of fibers expressing Bax far exceeded those that immunophenotypically expressed Bcl-2, which would favor apoptosis in susceptible cells. Bax expression is not typically observed in normal muscle fibers, as evidenced in the current study, a finding that has also been observed by others.²¹ The expression of Bax, particularly in the degenerating muscle fibers as compared with Bcl-2, implies that the protein may play a role in the myonecrosis observed in IBM. However, the expression of Bax antibody and almost comparable Bcl-2 expression in regenerating myofibers would indicate that the expression of these antibodies in this scenario may be independent of apoptosis. Bcl-x expression in both degenerating and regenerating muscle fibers was negligible. This may indicate no increased expression or only a minimal increase in protein expression to a degree that cannot be detected by immunohistochemistry. Interestingly, a comparison group of 8 other inflammatory conditions of muscle showed no obvious difference in terms of antibody expression in inflammatory cells, degenerating myofibers, or regenerating myofibers versus IBM. Whether this implies a mechanistic similarity between these inflammatory conditions or whether a larger number of cases in the control group (or some subset of this group) would uncover a difference is not known.

In 1999, Olivé and Ferrer²² examined Bcl-2 and Bax protein expression in biopsies from 13 patients with a variety of muscle diseases, including 4 patients with IBM, 3 patients with polymyositis, 4 patients with Becker muscular dystrophy, and 2 patients with necrotizing myopathy. Similar to the current study, immunoreactivity for Bcl-2 and Bax was not observed in normal muscle fibers.²² In the 4 patients who had IBM, 18% to 24% of muscle fibers stained positively with Bcl-2 antibody, and 27% to 35% of myofibers stained with Bax antibody. In all 4 cases, staining was observed in the cytoplasm, sarcoplasmic reticulum, rimmed vacuoles, and the inflammatory infiltrates. Most of the immunostaining reported by Olivé and Fer-

rer²² in these 4 cases corresponded to rimmed vacuole or probable mitochondrial-associated staining patterns. The immunoreactivity with both antibodies was observed in "some necrotic fibers" and "some regenerating fibers" of patients in all the examined conditions, including IBM.²² These authors concluded that Bcl-2 and Bax immunoreactivity was not disease specific, but appeared to relate somewhat to the severity of the disease.²²

It is well known that patients with IBM are susceptible to developing DNA mitochondrial mutations and may demonstrate evidence of ultrastructural mitochondrial alterations (increased numbers of mitochondria and mitochondrial dysmorphology, including paracrystalline mitochondrial inclusions).^{6,23-26} Apoptosis-associated proteins are localized to a variety of cellular membrane structures, including mitochondrial membranes, endoplasmic reticulum, and nuclear membranes.²⁷ Prior to cell death, many of the proapoptotic proteins are localized to the cytosol or cytoskeleton. Bax is normally found either in the cytosol or loosely attached to cellular membranes. When activated, Bax translocates to mitochondria.^{27,28} In contrast to inactive Bax, Bcl-2 is an integral membrane protein heavily localized to mitochondria in the normal state.²⁷ The fact that subsarcolemmal staining was observed in only 22% of biopsies with Bcl-2 antibody suggests that it may be present at very low levels in this location. Perhaps alteration of the mitochondria, as part of the IBM disease process, may serve as a signal for Bax activation and increased localization of protein to the subsarcolemmal region, where the mitochondria typically reside in greatest concentration.

Bax immunostaining was also prominently identified in association with autophagic rimmed vacuoles. Autophagic vacuoles typically consist of a mixture of glycogen material, amorphous granular and fibrillary material, dense bodies, and multilaminated membranous structures.²⁹ These vacuoles are felt to represent a degenerative phenomenon within the cell. Whether localization of Bax immunostaining to these vacuoles represents true up-regulation of the protein or a nonspecific accumulation of the protein in this region of degenerative changes is uncertain. Similar accumulation of other proteins, such as ubiquitin, has also been described in association with autophagic vacuoles in IBM.^{30,31}

In summary, Bax immunostaining is prominently noted in muscle biopsies of patients with IBM; staining is localized to degenerating myofibers, regenerating myofibers, autophagic vacuoles, and the subsarcolemmal region (probably localized to mitochondria). There is no obvious difference between the expression of these proteins in degenerating and regenerating myofibers in IBM versus other inflammatory conditions of muscle. Unique to IBM is

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Figure 1. Moderate variation in muscle fiber size with endomysial fibrosis and autophagic (rimmed) vacuole (arrow) in inclusion body myositis (Gomori trichrome, original magnification ×400).

Figure 2. Ultrastructural appearance of tubulofilamentous intranuclear inclusions characteristic of inclusion body myositis (original magnification ×13 000).

Figure 3. Mitochondrial paracrystalline inclusions (arrow) in a patient with inclusion body myositis (original magnification ×10 000).

Figure 4. Inflammatory cells (lymphocytes) highlighted on Bcl-2 immunostaining in inclusion body myositis (original magnification ×240).

Figure 5. Increased staining with Bax antibody in a degenerating myofiber (arrow) (original magnification ×240).

Figure 6. Prominent subsarcolemmal staining pattern with Bax antibody (original magnification ×400).

Figure 7. Bax immunoreactivity associated with a rimmed (autophagic) vacuole (arrow) (original magnification ×400).

the localization of staining to the subsarcolemmal region and autophagic vacuoles. Subsarcolemmal localization may be related to mitochondrial DNA mutations and structural alterations of the organelle, which are commonly associated with this disorder. Application of the Bax immunostain in IBM may be a fairly useful way to confirm the presence of these mitochondrial abnormalities in this setting. Expression of apoptosis-associated proteins in regenerating muscle fibers suggests that regulation in this situation may be independent of apoptosis.

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