Early establishment of epithelial apoptosis in the developing human small intestine

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ABSTRACT In the adult small intestine, the dynamic renewal of the epithelium is characterized by a sequence of cell production in the crypts, cell maturation and cell migration to the tip of villi, where apoptosis is undertaken. Little is known about enterocytic apoptosis during development. In man, intestinal architectural features and functions are acquired largely by mid-gestation (18-20 wks); the question whether the establishment of enterocytic apoptotic processes parallels or not the acquisition of other intestinal functional features remains open. In the present study, we approached this question by examining enterocytic apoptosis during development of the human jejunum (9-20 wks gestation), using the ISEL (in situ terminal uridine deoxynucleotidyl nick-end labelling) method. Between 9 and 17 wks, apoptotic enterocytes were not evidenced. However, beginning at the 18 wks stage, ISEL-positive enterocytes were regularly observed at the tip of villi. Since the Bcl-2 family of proteins constitutes a critical checkpoint in apoptosis, acting upstream of the apoptotic machinery, we investigated the expression of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, Bad) and one non-homologous associated molecule (Bag-1). By immunofluorescence, we found that all homologs analyzed were expressed by enterocytes between 9 and 20 wks. However, Bcl-2 homologs underwent a gradual compartmentalization of epithelial expression along the maturing crypt-villus axis, to establish gradients of expression by 18-20 wks. Western blot analyses indicated that the expression levels of Bcl-2 homologs were modulated during morphogenesis of the crypt-villus axis, in parallel to their gradual compartmentalization of expression. Altogether, these data suggest that regulatory mechanisms of human enterocytic apoptosis become established by mid-gestation (18-20 wks) and coincide with the maturation of the crypt-villus axis of cell proliferation, differentiation and renewal.

KEY WORDS: Bcl-2 homologs, crypt-villus axis, enterocyte, gut, programmed cell death.
expression levels; however, post-transcriptional and post-translational modifications can also sway the balance in favor of either pro- or anti-apoptotic homologs (Gajewski and Thompson, 1996; Reed et al., 1996a; Adams and Cory, 1998). Furthermore, interactions with other types of molecules, such as the anti-apoptotic protein Bag-1 (Wang et al., 1994; Takayama et al., 1995; Wang et al., 1996), add another level of complexity to the regulation of Bcl-2 homolog functions (Gajewski and Thompson, 1996; Adams and Cory, 1998). Nonetheless, it is acknowledged that characterization of the expression profiles of Bcl-2 homologs in tissues constitutes a crucial step in the understanding of the regulation of apoptosis, in tissue-specific developmental and/or renewal systems (Hale et al., 1996; Moss and Holt, 1996; Reed et al., 1996a; Jacobson et al., 1997; Potten, 1997; Adams and Cory, 1998).

The small intestinal epithelium is a useful model for the in situ study of the establishment and working mechanics of tissue renewal processes, including programmed cell death. Its rapid, continuous cell renewal consists of spatially separated stem cells, proliferative and differentiated compartments, located respectively in the lower regions of the crypts and on the villi (Leblond, 1981; Jones and Gores, 1997; Potten, 1997). In human adults, the dynamic renewal of the intestinal epithelium is characterized by a sequence of cell production in the crypts, cell maturation and cell migration to the tip of villi, where apoptosis and shedding occurs (Jones and Gores, 1997; Potten, 1997). Spontaneous crypt cell apoptosis, a rarer (less frequent) process, serves to remove defective/injured progeny cells, as well as senescent Paneth cells (Potten, 1992; Moss and Holt, 1996; Jones and Gores, 1997; Potten, 1997). A role for some Bcl-2 homologs (namely Bcl-2, Bcl-XL, Mcl-1 and Bak) has been proposed in the regulation of apoptosis in the human adult small intestine, a potential function well illustrated by their differential patterns (or gradients) of epithelial expression along the crypt-villus axis (Hockenbery et al., 1991; Lu et al., 1993; Krajewski et al., 1994c, 1995, 1996).

However, little is known on enterocytic apoptosis during development. The morphogenesis of the small intestinal mucosa, and thus of the crypt-villus axis, has been the subject of many reviews (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). In man, intestinal architectural features and functions are acquired early during fetal life (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Villus formation by mesenchymal infiltration of the stratified epithelium begins around 8-9 wks of gestation, proceeding distally until the entire intestine is lined by short villi covered by a simple columnar epithelium. By 15 wks, crypt formation has begun with the invagination of the intervillus epithelium into the underlying mesenchyme. Finally, by mid-gestation (18-20 wks), the overall architectural and functional organization of the crypt-villus axis, including digestive capacities, are highly similar to those of the new-born/adult intestinal mucosa (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Therefore, the question whether the establishment of enterocytic apoptotic processes parallels or not the acquisition of other intestinal functional features remains open.

In the present study, we approached this question by examining epithelial apoptosis during development of the human small intestine between 9 and 20 wks of gestation. The epithelial expression and localization of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, Bad), and one non-homologous associated molecule (Bag-1), were investigated as well. Herein, we find that intestinal epithelial apoptosis is detected at the tip of villi at 18-20 wks, but not in the earlier developmental stages studied. We also show that the epithelial expression of Bcl-2 homologs undergo a gradual compartmentalization of expression during intestinal development, in order to establish differential patterns (or gradients) along the crypt-villus axis by 18-20 wks. This gradual compartmentalization of Bcl-2 homolog expression parallels mirror changes in their protein expression levels. Hence, these data altogether suggest that regulatory mechanisms of human enterocytic apoptosis become established by mid-gestation and coincide with the maturation of the crypt-villus axis.

Results

To ascertain whether the establishment of human enterocytic apoptotic processes parallels or not the morphogenesis and maturation of the small intestinal crypt-villus axis of cell proliferation and differentiation, we examined epithelial apoptosis during development of the human jejunum between 9 and 20 wks of gestation. In parallel, the epithelial expression and localization of Bcl-2 homologs, acknowledged as central regulators of programmed cell death, were investigated throughout the same developmental period.
Emergence of enterocytic apoptosis during morphogenesis of the crypt-villus axis

Villus-tip apoptosis, the normal fate of intestinal epithelial cells, is readily observed when using the ISEL method (Gavrieli et al., 1992; Hall et al., 1994; Moss and Holt, 1996, Aschoff et al., 1999). Using this approach, we examined the presence of epithelial apoptotic cells during the development of the human jejunum (9-20 wks). Between 9 wks and 14 wks of gestation, we failed to observe any enterocytic apoptosis either among villus cells (Fig. 1A) or intervillous cells (not shown). Absence of apoptotic enterocytes was likewise noted between 15 wks and 17 wks (not shown), although crypt formation had begun. However, beginning at 18 wks, villus-tip apoptotic enterocytes were consistently observed (Fig. 1B) and such consistent detection of villus-tip ISEL-positive enterocytes persisted at 19 and 20 wks (Fig. 1D). As shown with greater magnification in Figure 1D, typically 1-3 ISEL-positive cells were detected at the apex of villi. Interestingly, ‘spontaneous’ crypt cell apoptosis, a rarer (less frequent) process observed in the adult small intestine (Potten, 1992; Hall et al., 1994; Merritt et al., 1995; Moss and Holt, 1996; Jones and Gores, 1997; Potten, 1997), was not evidenced herein (Fig. 1C). Finally, it is of note that single ISEL-positive mesenchymal cells were occasionally observed throughout the developmental period studied (not shown).

Epithelial localization of Bcl-2 homologs during morphogenesis of the crypt-villus axis

Since our ISEL observations indicated that villus-tip enterocytic apoptosis emerges by mid-gestation (18-20 wks), and because the Bcl-2 family of proteins exert critical regulatory functions in apoptosis (Reed et al., 1996a,b; Adams and Cory, 1998), we then investigated the jejunal epithelial expression of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, Bad) and one non-homologous associated molecule (Bag-1). All molecules analyzed herein were readily detected in the jejunal epithelium beginning at 9 wks of gestation. Indeed, a cytoplasmic (non-nuclear) staining was observed in epithelial cells of both growing villi and intervillus regions for Bcl-2 (Fig. 2A), Bcl-XL (not shown), Mcl-1 (Fig. 2C), Bax (Fig. 3A), Bak (Fig. 3B) and Bad (Fig. 3C), as well as for the anti-apoptotic molecule Bag-1 (Fig. 2B). This rather homogenous staining along the intervillus-villus axis remained essentially unaltered between 9 and 14 wks of gestation (Fig. 2 A-C and Fig. 3 A-C). Between 15 and 17 wks, we observed that the epithelial expression of some of the Bcl-2 homologs underwent a compartmentalization process along the maturing crypt-villus axis. This gradual process was found to culminate by mid-gestation (Fig. 2 D-G and Fig. 3 D-E). Thus, beginning at 18 wks, staining for Mcl-1 (Fig. 2G) and Bak (Fig. 3D) were found concentrated in villus enterocytes, but poorly detectable or absent in crypt cells. Bcl-2 staining was found much weaker than in previous stages, and exhibited a decreasing crypt-to-villus gradient of staining (Fig. 2D). Likewise, Bag-1 exhibited a decreasing gradient of staining from the base of the crypts to the apex of villi (Fig. 2F). Bad also displayed a compartmentalized expression pattern along the crypt-villus axis around the 18 wks stage, staining being strong in the upper half of villi (with occasional strong staining at the base of villi as well), but weaker in the rest of the epithelium (Fig. 3E). However, Bcl-XL (Fig. 2E) and Bax (not shown) were still detected homogeneously throughout the intestinal epithelium. These differential crypt-villus patterns of Bcl-2 homolog expression remained unchanged from 18 to 20 wks (Fig. 2 D-G and Fig. 3 D-E).

Epithelial expression levels of Bcl-2 homologs during morphogenesis of the crypt-villus axis

To further characterize the crypt-villus compartmentalization process of epithelial expression of Bcl-2 homologs, we then investigated the developmental protein expression levels of these same homologs. Immunoblot analyses of lysates from jejunal mucosal scrappings demonstrated the protein expression of all molecules analyzed herein (Fig. 4). Thus, Bcl-2 (~26 kDa), Bcl-XL (~28-30 kDa), Bag-1 (~32-34 kDa), Mcl-1 (~39-42), Bax (~21
kDa), Bak (~25-28 kDa) and Bad (~28-32 kDa) were detected at all developmental stages studied as protein bands migrating at their previously reported relative molecular weights (Krajewski et al., 1994a-c, 1995; Takayama et al., 1995; Krajewska et al., 1996; Krajewski et al., 1996; Packham et al., 1997; Adams and Cory, 1998; Metcalfe et al., 1999).

To examine the developmental epithelial expression profiles of each molecule studied in relation to the morphogenesis of the crypt-villus axis, their relative epithelial expression levels were evaluated by comparison with a reference protein, cytokeratin 18 (K18). The densitometric data presented in Figures 5-7 show that the relative epithelial expression levels of all Bcl-2 homologs analyzed (including the Bag-1 protein) are differentially modulated in parallel to the morphogenesis of the crypt-villus axis, as well as in concomitance to the establishment of their differential expression patterns. In the case of anti-apoptotic proteins (Fig. 5), Bcl-2 levels increased slightly between 10-14 weeks, but decreased sharply around 15 wks (when cryptogenesis has begun) in order to stabilize at lower levels (Fig. 5A), thus resulting in a significant ~53% overall reduction (Fig. 7). Bag-1 expression levels gradually decreased between 10 and 20 wks (Fig. 5C), resulting in a significant ~30% overall reduction (Fig. 7). Although Bcl-X L decreased gradually between 10 and 15 wks, its levels returned to those of 10-12 wks in subsequent stages (Fig. 5B), thus resulting in a slight (and non-significant) ~10% overall increment (Fig. 7). Finally, Mcl-1 epithelial expression increased gradually between 10-20 wks (Fig. 5D), resulting in a significant ~20% overall increment (Fig. 7). In the case of pro-apoptotic Bcl-2 homologs (Fig. 6), Bax exhibited the most complex modulations of epithelial expression levels. Indeed, it increased between 10 and 14 wks, in order to decrease gradually between 14 and 18 wks, and then increase sharply again between 18 and 20 wks (Fig. 6A). This resulted in a significant ~30% overall increment (Fig. 7). On the other hand, Bak increased gradually and steadily between 10 and 20 wks (Fig. 6B), resulting in a significant ~100% overall increment (Fig. 7). Finally, Bad epithelial expression levels also increased gradually between 10 and 20 wks (Fig. 6C), resulting in a significant ~25% overall increment (Fig. 7).

**Discussion**

In this study, we examined the question whether the establishment of enterocytic apoptotic processes parallels or not the morphogenesis and maturation of the small intestinal crypt-villus axis of cell proliferation and differentiation. To do so, we investigated the presence of intestinal epithelial cell apoptosis, as well as the localization and expression of six Bcl-2 homologs (Bcl-2, Bcl-X L, Mcl-1, Bax, Bak, Bad) and one non-homologous associated molecule (Bag-1), in the developing human small intestine between 9 and 20 wks of gestation. We found that villus-tip epithelial cell apoptosis emerges only by the time of the 18 wks stage. We also observed a gradual compartmentalization of epithelial expression of Bcl-2 homologs which appears to culminate at 18 wks to establish differential patterns (or gradients) of expression along the crypt-villus axis, coinciding with the emergence of villus-tip apoptotic cells. Finally, we found that the expression levels of Bcl-2 homologs are modulated during morphogenesis of the crypt-villus axis, in

![Fig. 3. Epithelial localization of pro-apoptotic Bcl-2 homologs during development of the human jejunum.](image1)

![Fig. 4. Protein expression levels of Bcl-2 homologs during development of the human jejunum.](image2)
parallel to their gradual compartmentalization of epithelial expression and to the emergence of villus-tip apoptosis.

In contrast to laboratory animals with short gestational periods (e.g., rat and mouse), human intestinal architectural and functional features are acquired as early as by mid-gestation (18-20 wks) (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Such features include not only a well defined crypt-villus axis, but as well as enzymatic brush border membrane digestive activities that are similar to those measured in the newborn/adult intestinal mucosa (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Other mid-gestation intestinal characteristics such as absorption/transport of lipids, sugars and amino acids, as well as basement membrane composition and integrin expression, or hormonal/growth factor responses, are likewise highly comparable to those found in the newborn/adult (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Our observations, namely that villus-tip apoptosis and crypt-to-villus differential patterns of Bcl-2 homolog expression are already established by 18-20 wks, can be added to this seemingly growing list. Indeed, villus-tip apoptosis is the normal fate of enterocytes in the adult small intestine, and the epithelial stainings observed herein for Bcl-2, Bcl-XL, Bak and Mcl-1 at mid-gestation correlate with (or confirm) those previously observed in third-trimester and/or adult human small intestinal specimens (Hockenbery et al., 1991; LeBrun et al., 1993; Lu et al., 1993; Krajewski et al., 1994c, 1995, 1996) (to our knowledge, there has been no report of Bag-1, Bax or Bad expression in the human small intestine prior to the present study). In light of these considerations, and taking into account the acknowledged role of Bcl-2 homologs as decisional regulators of apoptosis, our data altogether strongly suggest that regulatory mechanisms of human enterocytic apoptosis become established by mid-gestation, as is the case for other intestinal epithelial cellular processes and functions.

Another aspect of our findings concerns the developmental establishment of differential patterns of epithelial expression of Bcl-2 homologs in the small intestine. The crypt-villus axis constitutes an elegant example of vectorial compartmentalization of proliferative/undifferentiated (crypts) and differentiated (villi) cell populations within the same tissue (Leblond, 1981; Jones and Gores, 1997; Potten, 1997). Such “gradient” of cell differentiation is further defined by functional properties of the fully differentiated villus cells, which distinguish them from the crypt cells (Leblond, 1981; Ménard and Beaulieu, 1994; Beaulieu, 1999). Although the predominant means to remove obsolete differentiated enterocytes is through apoptosis and shedding at the villus apex, “spontaneous” crypt cell apoptosis is a rarer (less frequent) process which serves to remove defective/injured progeny cells (Potten, 1992; Moss and Holt, 1996; Jones and Gores, 1997; Potten, 1997). This in turn suggests the possibility of compartmentalized cell survival properties along the crypt-
Fig. 7. Differential modulations of epithelial Bcl-2 homolog expression levels during development of the human jejunum. Total proteins (50 µg/well) from mucosal scrappings of human fetal jejunums at 10-12 and 19-20 weeks (wks) were separated by SDS-15% PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, probed with specific antibodies for the detection of Bcl-2, Bcl-X, Bag-1, Mcl-1, Bax, Bak, Bag, and Bad, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 10-12 and 19-20 wks of gestation (post-fertilization), in order to establish the relative increase or decrease of protein expression levels at 19-20 wks, in comparison to 10-12 wks. Values are expressed as % of levels at 10-12 wks”, where “%” indicates no differences in protein expression levels between 19-20 and 10-12 wks. Each column represents the mean ± SEM of at least six different specimens (i.e. n ≥3 at 10-12 wks, n ≥3 at 19-20 wks); statistically significant (0.001 ≤ p ≤ 0.05) differences are indicated by an asterisk (*).

In support of this, studies have consistently reported a dramatic increase of apoptosis in crypt cells, but little or no increase in villus cells, after irradiation or chemotherapeutic drug exposure (Potten, 1992; Hall et al., 1994; Merritt et al., 1995; Potten, 1997; Pritchard et al., 1999). In addition, some Bcl-2 homologs have been shown individually to exhibit gradients of expression along the crypt-villus axis, suggesting a role for these apoptotic regulators in intestinal epithelial programmed cell death (Hockenbery et al., 1991; LeBrun et al., 1993; Lu et al., 1993; Krajewski et al., 1994b,c, 1995; Merritt et al., 1995; Krajewski et al., 1996; Wilson and Potten, 1996; Aschoff et al., 1999). Our study, which analyzed six homologs at the same time, clearly illustrates a differential pattern of epithelial expression in the human small intestine, where proliferative/undifferentiated crypt cells exhibit a Bcl-2 homolog expression profile that differs from the one observed in differentiated villus cells. To this effect, analyses of enterocyteic apoptosis in bcl-2−/− and bax−/− knockout mice have reported differential consequences for crypt and villus cells with regards to apoptosis resistance and/or susceptibility after irradiation (Wilson and Potten, 1996; Potten, 1997; Pritchard et al., 1999). Finally, Bag-1, a protein known to associate with Bcl-2 and to participate in signal transduction pathways that promote cell survival (Wang et al., 1994; Takayama et al., 1995; Gajewski and Thompson, 1996; Reed et al., 1996a;b; Wang et al., 1996; Packham et al., 1997; Adams and Cory, 1998), was shown herein to exhibit a decreasing gradient of epithelial expression from the base of crypts to the apex of villi. Consequently, these data taken together strongly support the concept that intestinal epithelial cell survival and apoptosis may be regulated differentially according to the state of cell differentiation.

In conclusion, the present findings provide new insights into the developmental establishment of regulatory mechanisms of epithelial apoptosis in the human small intestine, whereby a compartmentalized epithelial expression of Bcl-2 homologs is established along the crypt-villus axis by mid-gestation, coincident with the emergence of apoptosis at the apex of villi. The developmental processes responsible for such early establishment of adult-like intestinal apoptotic features in man remain to be understood. For instance, are the modulations of Bcl-2 homolog expression levels observed herein simply consequent to the establishment of the differential crypt-villus patterns of epithelial expression, or could these be involved in the absence of apoptotic enterocytes until mid-gestation is reached? Further analyses, using in vitro model systems, will be required to dissect at the molecular level the mechanisms which influence the functions of Bcl-2 homologs in epithelial cells, as well as to identify the exact functions enacted by Bcl-2 homologs themselves in the regulation of epithelial programmed cell death.

**Materials and Methods**

**Tissue processing**

Human fetal jejunum specimens from 39 fetuses ranging in age from 9 to 20 weeks post-fertilization (fetal ages were estimated according to Streeter, 1920) were obtained from normal elective pregnancy terminations. Only specimens obtained rapidly (60 min or less) were used. The present study was in accordance with a protocol approved by the institutional Human Research Ethical Review Committee for the use of human biological materials. For immunolocalization and ISEL (in situ terminal uridine deoxynucleotidyl nick-end labelling) studies, tissues were washed in PBS (pH 7.4) and embedded in OCT (Optimum Cutting Temperature) compound (TissueTek, Miles Laboratories, Elkhart, IN), as previously described (Beaulieu et al., 1991). For analyses of protein expression levels, mucosal scrappings (Beaulieu et al., 1993; Beaulieu and Vachon, 1994) of samples were washed in PBS (pH 7.4) and homogenized in 20 mM Tris-HCl (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 µg/ml leupeptin, 50 µg/ml antipain, and 0.1 mg/ml aprotonin. Total proteins were measured using the BioRad (Hercules, CA) protein assay. Aliquots of homogenates were directly solubilized in 2x solubilization buffer (2.3% [w/v] SDS, 10% [v/v] glycerol, and 0.001% [w/v] bromophenol blue in 62.5 mM Tris-HCl [pH 6.8] containing 5% [v/v] β-mercaptoethanol), boiled (105°C, 5 min), cleared by centrifugation (13000g, 5 min, room temperature), and processed for storage as described (Beaulieu and Vachon, 1994; Vachon et al., 1995, 1996).

**Antibodies**

Primary rabbit polyclonal antibodies used in the present study were Ab 1682, directed against human Mcl-1 (Krajewski et al., 1994a, 1995); Ab 1695, directed to human/mouse Bcl-X (Krajewski et al., 1994c); Ab 1701 (Krajewski et al., 1994a) and Ab PC68 (Calbiochem, San Diego, CA), both directed against human Bcl-2; Ab 1712 (Krajewska et al., 1996; Krajewski et al., 1996); Ab I-19 (Santa Cruz Biotech., Santa Cruz, CA), directed to human/mouse Bak; Ab PC66 (Calbiochem), both directed to human Bax; Ab 1764 (Calbiochem), directed against human Bax (Krajewska et al., 1996; Krajewski et al., 1996); Ab I-19 (Santa Cruz Biotech., Santa Cruz, CA), directed to human/mouse Bak; Ab PC67 (Calbiochem), directed to human Bcl-X; Ab K-20 (Santa Cruz Biotech.), directed against human/mouse Mcl-1; and Ab 9292 (New England Biolabs, Beverly, MA) and Ab R-20 (Santa Cruz Biotech.), both directed to human Bad. Primary mouse monoclonal antibodies used were mAb K56C8 (Takayama et al., 1995; Wang et al., 1996), directed against human Bag-1; mAb 32 and mAb 48 (both from Transduction Labs./Biocanc Scientific, Mississauga, ON, Canada), directed to human Bad; and mAb CY-90 (Sigma-Aldrich Canada).
In situ detection of apoptosis-associated DNA strand breaks

In situ terminal deoxynucleotidyl transferase (TdT)-mediated aUTP nick-end labeling (TUNEL) (Gavrieli et al., 1992) was carried out as previously described (Vachon et al., 1996, 1997) on 4-6 µm thick cryosections of human fetal jejunal samples, using the ApopTag apoptosis detection kit (Oncair, Gaithersburg, MD). Preparations were then counterstained with Evans blue, mounted and viewed with a Reichart Polyvar 2 microscope (Leica, St-Laurent, QC, Canada) equipped for epifluorescence. In all cases, no specific immunofluorescent staining was observed when primary antibodies were omitted or replaced by non-immune (rabbit or mouse) serum (not shown). All immunofluorescent micrographs shown herein are representative of at least three (n≥3) different specimens for each developmental stage analyzed.

Analyses of protein expression levels

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% (w/v) acrylamide Tris-HCl gels (Bio-Rad) was performed as described previously (Vachon et al., 1996, 1997). Broad range molecular mass markers (6.8-209 kDa range; BioRad) were used as standards. Total proteins (50 µg/well) were separated by electrophoresis and then electrottransferred to nitrocellulose membranes (Support NitroCellulose-1; Life Technologies/Gibco-BRL, Burlington, ON, Canada) for subsequent immunoblotting (Vachon et al., 1996, 1997). Rabbit antisera were used at 1:100-1:1000 dilutions, and mouse monoclonals (Beaulieu et al., 1991; Beaulieu and Vachon, 1994; Vachon et al., 1996) were used as secondary antibodies. Sections were counterstained with 0.1% (w/v) Evans blue in PBS (pH 7.4), mounted in glycerol-PBS (9:1) containing 0.1% (w/v) paraphenylenediamine, and viewed with a Reichart Polyvar 2 microscope (Leica, St-Laurent, QC, Canada) equipped for epifluorescence. A representative of the at least three (n≥3) different specimens for each developmental age group; statistically significant (0.001 ≤ p ≤ 0.05) differences were determined with the Student t test.

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