Research report

The microglial reaction in spinal cords of jimpy mice is related to apoptotic oligodendrocytes

José M. Vela *, Ishar Dalmau, Berta González, Bernardo Castellano

Department of Cell Biology and Physiology, Unit of Histology, Faculty of Medicine, Autonomous University of Barcelona, Bellaterra 08193, Barcelona, Spain

Accepted 31 October 1995

Abstract

Jimpy is a shortened life-span murine mutant whose genetic disorder results in a severe hypomyelination in the central nervous system associated with a variety of glial abnormalities, including oligodendrocyte death. In this study, we report that oligodendrocyte death in jimpy occurs through an apoptotic mechanism, as demonstrated by in situ labeling of nuclear DNA fragmentation. Compared to those of normal littermates, the spinal cords of jimpy mice showed a significantly higher number of apoptotic cells. Our observations also corroborate that specific glial cell death in jimpy is restricted to oligodendrocytes, as evidenced by double labeling for DNA fragmentation and MBP immunocytochemistry. Cells labeled for DNA fragmentation were always negative for astroglial or microglial markers. Apoptotic oligodendrocytes were not aggregated into clusters and were ubiquitously distributed throughout the jimpy spinal cord, although were more numerous in white matter than in gray matter. We found no physical association between astrocytes and dying cells in jimpy. Microglial cells, however, were found closely attached to and even surrounding apoptotic cells. The possible role of microglial cells in relation to apoptosis is discussed.

Keywords: Apoptosis; DNA fragmentation; Oligodendroglial cell; Neuroglial cell; Terminal transferase; Lectin histochemistry; Hypomyelination

1. Introduction

Jimpy has a sex-linked recessive mutation [43] which produces a drastic myelin deficiency throughout the central nervous system (CNS) of affected hemizygous male mice [44,47,51]. In addition to hypomyelination, the neurological disorder in jimpy is associated with diverse abnormalities in glial cells. Astrocytes exhibit a pronounced hypertrophy [5,39,52], an increase in glial fibrillary acidic protein (GFAP) content [9,14,35] and abnormalities in their metabolism [25,27]. Microglial cells display morphological signs of reactivity and their number is largely increased [58]. The most severe alterations are, however, associated with the oligodendrogial cell lineage. Oligodendrocyte precursors show an abnormal cell cycle [30] and an increased proliferation rate [45,53], but the number of well-differentiated oligodendrocytes is severely reduced [18,21,52] as a consequence of the premature death of most oligodendrocytes as they begin to differentiate [29,59].

Dying oligodendrocytes recognized as pyknotic cells along the jimpy white matter have been described in different studies [31,44] and are ultrastructurally characterized by a progressive polarization of their chromatin and diminution of the integrity of their cytoplasmic constituents [29]. The morphological signs of apoptotic degeneration [28,63] are in agreement with the evolution and degenerative features of jimpy oligodendrocytes, but the possibility that chemical alterations triggered by the jimpy mutation could cause cell death through a necrotic mechanism cannot be discarded.

A hallmark of apoptosis, which is not observed in necrotic cell death, is the activation of a specific endogenous endonuclease that produces a double-strand cleavage of nuclear DNA at the linker regions between nucleosomes [3,11,61,63]. These DNA breaks can be detected in individual cells undergoing apoptosis by in situ labeling of nuclear DNA fragmentation utilizing tailing (terminal transferase) techniques [22]. This method, based on a terminal deoxynucleotidyltransferase-mediated incorporation of biotinylated nucleotides into the 3'-ends of DNA fragments (terminal dUTP nick end labeling; TUNEL),
enables the visualization and easy quantification of apoptotic cells on tissue sections [20]. In this paper, we report that jimpy oligodendroglial degeneration occurs through an apoptotic mechanism, as demonstrated by in situ labeling of nuclear DNA fragmentation by the TUNEL method. In addition, we have investigated the possible association of microglial cells and astrocytes with dying cells. To perform this study, we combined the TUNEL histoenzymatic technique either with lectin histochemistry to label microglial cells [1], with immunocytochemistry to glial fibrillary acidic protein (GFAP) to label astroglial cells [7], or with myelin basic protein (MBP) immunohistochemistry to label oligodendrocytes [55].

2. Materials and methods

2.1. Experimental animals

Male mice C57BL/6J used in this study were raised in the UAB animal center by mating jimpy carrier females (jp/+) which were obtained from the Institute Pasteur with normal males (+/Y). Jimpy males (jp/Y) were identified from normal control animals (+/Y) by their characteristic tremors and drastic myelin reduction. Five jimpy animals 10–12 days old and 5 jimpy animals 20–22 days old, together with 5 normal male littersmates of both ages, were used in these experiments.

2.2. Preparation of sections

The animals were anesthetized with sodium pentobarbital (100 mg/kg body weight) and sacrificed by intracardiac perfusion with a 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.2, for 5 min. The cervical segment of the spinal cord was carefully removed and immersed in the same fixative for 2 h at 4°C. After fixation, the spinal cord samples were rinsed in phosphate buffer for 4 h, dehydrated in graded ethanol, cleared in Xylene and embedded in paraffin. Coronal sections, 6 μm thick, were obtained in a microtome and mounted on glass slides pretreated with 1:10 poly-L-lysine solution (Sigma). Dewax and hydration was done by sequentially transferring the slides through Xylene, a degraded series of ethanols, and microwater in DDW. Blocking of endogenous peroxidase was carried out with 2.5% H₂O₂ in DDW for 10 min. Sections were then rinsed 4 × 2 min in DDW, transferred for 10 min to reaction buffer (30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM cobalt chloride), pH 7.2, and incubated for 25 min at 37°C in reaction buffer containing 0.3 U/μl terminal deoxynucleotidyl transferase (Boehringer Mannheim) and 20 μM biotinylated 16-dUTP (Boehringer Mannheim) in a moist chamber. The enzymatic reaction was stopped by rinsing twice for 10 min in saline sodium citrate buffer (300 mM sodium chloride, 30 mM sodium citrate) at RT. After rinsing twice for 2 min in DDW, sections were placed for 5 min in 0.05 M Tris-buffered saline (TBS), pH 7.4, and treated for 20 min at RT with 2% bovine serum albumin (BSA) diluted in TBS. The sections were then incubated for 60 min at RT with a 1:600 dilution of avidin–peroxidase (Sigma) in 2% BSA in TBS, and rinsed 3 × 10 min in TBS. The peroxidase reaction was visualized by incubating the sections for 5 min in 100 ml of 0.05 M Tris buffer (TB), pH 7.2, containing 50 mg 3,3′-diaminobenzidine (DAB) (Sigma), 4 ml of 1% ammonium nickel sulfate, 5 ml of 1% cobalt chloride and 66 μl H₂O₂. Finally, sections were rinsed in distilled water, dehydrated in graded ethanol, cleared in Xylene, and covarried in DPX. As positive control for the enzymatic reaction, sections of small intestine were identically processed. As negative control, some sections were incubated in medium lacking either the enzyme or the biotinylated substrate. Fresh solvents and sterile material were used to avoid DNase contamination and interference with the enzymatic reaction. Some sections were stained with hematoxylin for nuclear pyknosis analysis.

2.4. Double labeling technique

Simultaneous demonstration of apoptotic nuclei and oligodendroglia, astroglia or microglia was achieved through the sequential combination of the TUNEL technique and selective markers for these glial cells. Oligodendroglial and astroglial cells were demonstrated by immunocytochemical detection of myelin basic protein (MBP) [55] and glial fibrillar acidic protein (GFAP) [7], respectively, and microglial cells by tomato lectin histochemistry [1].

2.4.1. Immunocytochemical reaction for MBP and GFAP

Sections were rinsed 3 × 5 min in TBS, treated for 10 min with 2% H₂O₂ in 100% methanol to block peroxidase and rinsed again 3 × 5 min in TBS including 0.1% Triton X-100. Sections were placed in 10% fetal calf serum (FCS) diluted in TBS for 30 min to reduce unspecific staining, and then incubated overnight at 4°C with the primary rabbit anti-MBP or anti-GFAP antibody (Dako-patts) in a 1:400 TBS dilution with 10% FCS. After rinsing 3 × 5 min in TBS with 0.1% Triton X-100, the sections were incubated for 60 min at RT with a secondary anti-
Fig. 1. In situ labeling of DNA fragmentation by the TUNEL method in spinal cord sections from normal (A) and jimpy (B–D) mice. Cells undergoing apoptosis always appear as isolated cells showing nuclear staining. Note that the presence of a single positively labeled cell in the 10- to 12-day-old normal (A) contrasts with the increased numbers found in jimpy both at 10–12 (B) and 20–22 (C, D) days. GM, gray matter; WM, white matter; cc, central canal. Bar in A and B = 50 μm; in C and D = 25 μm.
rabbit IgG antibody (Dakopatts) in a 1:40 dilution in TBS with 10% FCS and rinsed again 3 × 5 min in TBS with 0.1% Triton X-100. Sections were then incubated for 60 min at RT with the peroxidase–rabbit anti-peroxidase complex (Dakopatts) in a 1:100 TBS dilution with 10% FCS. The peroxidase reaction was visualized by incubating the sections in 100 ml of TB containing 50 mg DAB (Sigma) and 33 μl H2O2 for 5 min. Finally, sections were rinsed, dehydrated in ethanol, cleared in xylene, and coverslipped in DPX. As a control for the immunocytochemical staining, the primary antibody was omitted.

2.4.2. Histochemical reaction for tomato lectin binding

Sections were rinsed 3 × 5 min in TBS and remaining peroxidase activity was blocked with 2% H2O2 in 100% methanol for 10 min at RT. Following rinsing 3 × 5 min in TBS containing 0.1% Triton X-100 the sections were incubated overnight at 4°C with the biotinylated tomato lectin (Lycopersicon esculentum; Sigma) diluted to 15 μg/ml in TBS. Sections were then rinsed 3 × 10 min in TBS, incubated for 60 min at RT with a 1:600 dilution of avidin–peroxidase (Sigma) in TBS, and rinsed again 3 × 5 min in TBS. Subsequent peroxidase demonstration was carried out using the same procedure described above for tomato lectin histochemistry. Finally, sections were dehydrated and coverslipped in DPX. As a control for the histochemical staining, lectin was omitted.

2.5. Cell count

The quantitative study was carried out in 10 complete spinal cord sections pertaining to 5 jimpy and 5 control animals per age. The apoptotic nuclei detected by the TUNEL method and the pyknotic nuclei detected in sections stained with hematoxylin were counted under a microscope by using a 40 × objective. ANOVA test was performed at the 95% significance level in order to contrast differences.

3. Results

3.1. Distribution and number of apoptotic cells

Microscopical examination of sections processed for TUNEL revealed the presence of a black staining in the nuclei of cells showing DNA fragmentation. The feasibility of nuclear labeling of cells undergoing apoptosis by the TUNEL method was tested in the small intestine as a positive control. Epithelial cells labeled by TUNEL in the intestine were mainly concentrated at the luminal extremity of the villus, but no labeling was found at the lower part of the crypt, in agreement with the study by Gravieli et al. [20] using the TUNEL method and studies describing the turnover and kinetics of the intestinal epithelium [62]. The omission of either the enzyme or its substrate gave completely negative results both in small intestine and spinal cord sections.

In the spinal cord, apoptotic cells were present in both the 10- to 12- and 20- to 22-day-old normal and jimpy animals (Fig. 1). Apoptotic cells were distributed throughout the gray and white matter areas of the jimpy and normal spinal cord and no laminae systematically devoid of apoptotic cells were found. However, the distribution and number of apoptotic cells in the mutant differed markedly from those observed in the normal animal at both ages.

The quantitative analysis indicated that the mean number of apoptotic cells in the spinal cord was significantly higher in mutants than in normal animals, this difference being more pronounced in the white than in the gray matter (Table 1). Only a few apoptotic cells were found in the spine and white matter of normal animals at both ages. In jimpy, apoptotic cells were numerous throughout the spinal cord, but the mean number of apoptotic cells in white matter was more than twice that found in gray matter. Apoptotic cells did not aggregate into clusters and they always appeared as isolated cells mostly showing a homogeneous dark nuclear staining. Isolated TUNEL-positive cells displaying a peripheral nuclear or punctate nuclear staining were also seen. Frequently, apoptotic nuclei were surrounded by a light ring. We were not able to establish significant differences in the number and distribution of apoptotic cells when comparing sections from 10- to 12- and 20- to 22-day-old jimpy animals.

The number of pyknotic cells was also estimated on paraffin sections stained with hematoxylin. The identification of pyknotic cells was ambiguous in some cases, especially in white matter due to the reduced size and darkness of normal oligodendroglial and microglial nuclei. The number of pyknotic nuclei clearly identified in hema-

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of TUNEL-positive cells/section</td>
</tr>
<tr>
<td>Age(days)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>10–12</td>
</tr>
<tr>
<td>20–22</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E.M. The data were obtained from the counts of 10 complete coronal sections of spinal cord pertaining to 5 jimpy and 5 normal animals per age. Significant differences were observed between jimpy and normal animals and between gray and white matter (ANOVA and Scheffé test; P < 0.0001). However, there was no significant difference (P > 0.05) between the two ages.
Fig. 2. Simultaneous visualization of apoptotic cells and oligodendrocytes in the white matter of the jimpy spinal cord after double TUNEL/MBP immunostaining. Double labeling was easily distinguished under the microscope since TUNEL staining was black and MBP immunostaining was brown. A shows an apoptotic cell in an early to moderate stage of degeneration characterized by the integrity of its cytoplasm and possesses a nucleus (asterisk) with peripheral chromatin fragmentation. Note that the MBP immunoreactivity is present in its perikaryal cytoplasm and cell processes (arrowheads). In B, a cell in a late stage of apoptotic degeneration, characterized by its inconsistent cytoplasm and its intense and homogeneous DNA fragmentation (asterisk), only shows MBP immunoreactivity in the plasma membrane around the cell body and a cell process (arrowheads). Bar in A and B = 5 μm.

toxylin-stained sections was negligible, if any, in normal animals, and in jimpy, only represented a 55% of the number of apoptotic cells detected by TUNEL. Significant differences in the number of dying cells/section were observed when compared these two methods.

3.2. Oligodendroglia, astroglia, microglia and apoptotic cells

The double-staining procedure combining TUNEL with markers for the different glial cell types enabled us to simultaneously visualize apoptotic nuclei (black staining) and oligodendrocytes, astrocytes or microglia (brownish staining). In normal mice, at both ages, the identification of individual MBP-positive apoptotic cells could not be carried out due to the strong staining of the densely packed myelinated fibers. In jimpy, there was a consistent correspondence between the pattern of TUNEL staining and the localization of MBP immunoreactivity. Most apoptotic cells present in the white and gray matter of the mutant spinal cord were immunocytochemically positive for MBP (Fig. 2). Only 8.5% (10-12 days) and 5.6% (20-22 days) of TUNEL-positive cells in jimpy were clearly recognized as MBP-negative cells. In apoptotic cells showing a dark and homogeneous nuclear TUNEL staining, the MBP antibody preferentially stained the plasma membrane around

Fig. 3. Simultaneous visualization of apoptotic cells and astrocytes in jimpy after double TUNEL/GFAP immunostaining. The two microphotographs, at different magnification, show astrocytes and apoptotic cells (arrowheads) in the same field. Note that there is no physical association or specific relation between astrocytes and dying cells. Bar in A = 25 μm; in B = 10 μm.
4. Discussion

Apoptosis is a selective process of cell deletion that is biochemically characterized and identified by internucleosomal DNA fragmentation via endogenous endonuclease activity [3,11,60,61,63]. In the present study, we report that a specific oligodendrocyte degeneration in jimpy occurs through an apoptotic mechanism, as demonstrated by the increased presence of nuclei labeled for DNA fragmentation by TUNEL in the mutant spinal cord. We found no significant differences in the number of apoptotic oligodendrocytes when comparing the 10- to 12- and the 20- to 22-day-old jimpy animals. At both ages, however, the mean number of dying cells in the white matter was higher than those observed in the gray. An increased number of dying cells, identified morphologically as pyknotic cells, have been described in different white matter areas of the jimpy mouse by several authors [29,31,44]. On the basis of ultrastructural observations, Knapp et al. [29] identified dying glial cells as oligodendrocytes. These authors quantified pyknotic death and found a 5-fold increase in the number of pyknotic cells in the white matter of the jimpy spinal cord both at 11–12 and 20–22 days of age. In our study, however, the jimpy mutants showed a 2.5-fold (10–12 days) and a 3.6-fold (20–22 days) increase in the levels of apoptotic cells present in the white matter of the cord. In addition, we found that pyknotic cells only represented a 55% of the number of TUNEL-positive cells. These differences could be explained in several ways. One possibility is that one fraction of the glial population undergoes necrotic cell death. On the basis of our study, we cannot discard the possibility that there could be necrotic cell death in addition to the apoptotic degeneration described here. Another possibility is that the concentration of DNA breaks in the first stages of apoptotic cell degeneration was so low that it was difficult to detect at the light microscope level by the TUNEL method. Yet another possibility is that methodological differences and/or differences in the personal criteria for classification of dying glia may account for the variability in the estimated numbers of pyknotic cells.

Apoptotic glial cell death in jimpy is restricted to oligodendrocytes, as evidenced both by the colocalization of the TUNEL and the MBP staining and by the absence of double labeling in TUNEL-positive cells when markers for microglia or astroglia were applied. Although MBP is expressed later than other myelin markers in the differentiation of oligodendrocytes [10], a large percentage of apoptotic cells in jimpy mice have synthesized detectable levels of MBP. Less than 10% of the apoptotic cell population was MBP-negative or had no detectable levels of MBP. In addition, cells in early phases of apoptotic degeneration, showing peripheral and punctate chromatination, had MBP immunostaining in the cell body and in some radiating processes; whereas cells in terminal stages of degeneration, showing an intense and homogeneous nu-
clear DNA fragmentation, preferentially displayed MBP immunostaining in the plasma membrane around the cell body and/or in a peripheral rim of the perikaryal cytoplasm. Transitional forms, showing both moderate MBP and TUNEL staining were also seen. In our opinion, these results indicate that jimpy oligodendrocytes begin to die after they have already differentiated far enough to express MBP, and that oligodendrocytes in terminal stages of apoptotic degeneration have already lost not only their main MBP content, but also the integrity of their cytoplasmic constituents (see [29]) and, therefore, the capability of synthesizing MBP.

In addition to pathological settings, apoptosis is a common phenomenon occurring in organ formation during normal development and in renovation of normal healthy tissues [60]. Large numbers of neurons die during the embryonic and early postnatal period [16,17,40] and the death of glial cells during normal CNS development has also been described [46,54,56]. In our study, we report the presence of some TUNEL-positive nuclei in the gray and white matter of normal animals, but the normal spinal cord at 10-12 days postnatum shows a mature lamination and the number of apoptotic cells at this age is substantially higher than those observed in the 20- to 22-day-old normal animals. This is suggestive of the involvement of a normal physiological process of control of cell numbers and deletion of defective cells, but we cannot discard the possibility that apoptotic cells observed by us in normal animals could be attributed to naturally occurring cell death during late development.

In addition to the cytoplasmic changes resulting in activation of a suicide pathway, it is known that apoptosis induces cell surface changes on degenerating cells that aid their recognition and engulfment by adjacent macrophages [15,37,48]. The microglia are the specialized resident macrophage in the CNS [41,42] and numerous studies demonstrate that resident microglial cells transform into phagocytic cells upon adequate stimulation [6,8,36]. In a previous work, we described a severe and extensive microglial cell reaction in the jimpy spinal cord and we observed reactive microglial cells engulfing or closely attached to nerve sheaths and pyknotic cells [58]. Electron microscopically, reactive microglial cells in jimpy accumulated lipid inclusions and heterogeneous material suggestive of being disintegrated cell debris. In the present study, we have confirmed the association of microglial cells with degenerative cells. Approximately 90% of the TUNEL-labeled degenerative cells were partially or completely surrounded by microglial cells or their processes. All these findings are strongly suggestive of the involvement of microglial cells in the removal of jimpy dying oligodendrocytes. In this way, it has been reported that astrocytes may become phagocytic under determinate circumstances [32,57], but our observations did not reveal any physical association between this cell type and dying cells in jimpy.

It is known that the genetic defect in jimpy is a point mutation in the proteolipid protein (PLP) gene [13,38] and it has also recently been reported that an apoptotic oligodendrocyte degeneration occurs in another PLP mutant mouse, the jimpy mtd [23], but the specific cause and cellular events leading from PLP mutation to oligodendrocyte apoptosis are unknown. On the other hand, different studies have related the apoptotic cell degeneration of a given cell population to the presence of cytotoxic agents [2,12,24]. As microglial cells are the main source of cytotoxins in the CNS [4], and as it is known that microglia in the nervous system and macrophages in other tissues are capable of recognizing and destroying cells carrying foreign or abnormal antigens [33], we would consider here the possibility that microglia recognize defective messages in the jimpy oligodendrocytes and mediate their destruction by means of the secretion of cytotoxins. Some cytotoxic products released by microglia have been described as directly influencing myelin integrity and oligodendrocyte survival. In particular, tumor necrosis factor-α, a product of secretion of activated microglial cells [19], produces demyelination and extensive fibrillar astrocytic reaction in the mouse optic nerve [26] and, when tested on glial cell cultures, induces myelin damage [49] and oligodendrocyte apoptosis in a time- and dose-dependent fashion [34,50]. The close contact between microglia and apparently normal oligodendrocytes described in jimpy at the electron microscope level [58] may be of importance for the recognition and subsequent stimulation of microglial cells to attack defective oligodendrocytes. At present, however, we do not know if cytotoxicity is an operative mechanism of the microglial reaction in jimpy.

Acknowledgements

We would like to thank Miguel Angel Martil and Anna Garrit for excellent technical assistance. This work was partially supported by DGICYT Grant PB92-0598.

References


