Summary. The purpose of this study is to evaluate, in an experimental model of spinal cord injury (SCI), the presence of apoptotic cell death after trauma and if early administration of a single bolus of methylprednisolone (MP) influences apoptosis in the zone of trauma and in adjacent spinal cord segments. For this study, a total of 96 adult female Wistar rats were subjected to spinal contusion at the T6-T8 level, producing immediate paraplegia. Forty-eight animals (treated group) received a single intraperitoneal injection of MP, at a dose of 30 mg/kg body weight, 10 minutes later. Cells undergoing apoptosis were detected by means of immunohistochemical labeling with the monoclonal antibody Apostain (anti-ssDNA MAb F7-26), in the injured spinal cord tissue, both in the zone of the lesion and in the adjacent spinal segments (rostral and caudal zones), 1, 4, 8, 24 and 72 hours and 1 week after injury. Apoptosis was detected in neurons and glial cells in the zone of the lesion 1 hour after trauma, with a pattern that showed no changes 4 hours later. Between 4 and 8 hours postinjury, the number of apoptotic cells increased, after which it decreased over the following days. In the adjacent spinal segments, apoptotic cells were detected 4 hours after trauma, and increased progressively over the remainder of the study, the number of apoptotic cells being similar in the lesion zone and in rostral and caudal zones one week after injury. When the group of MP-treated animals was considered, significant decreases in the number of apoptotic cells were detected in the lesion zone 24 hours after injury, and in the rostral and caudal zones, at 72 hours and at 1 week after trauma. These findings show that early administration of a single bolus of MP decreases apoptotic cell death after SCI, supporting the utility of MP in reducing secondary damage in injured spinal cord tissue.

Key words: Methylprednisolone, Spinal cord injury, Apoptosis, F7-26 marker

Introduction

It is a well-known fact that severe spinal injury triggers a series of biochemical and morphological changes that produce radial effects in the gray matter and white matter of the spinal cord, frequently resulting in the irreversible damage that can accompany the initial lesion (Allen, 1914; Tator and Fehlings, 1991; Isaac and Pejic, 1995). These alterations include changes in vascular permeability, edema, cell necrosis and apoptosis, excitatory amino release of calcium ions and of lactic acid, generation of free radicals and release of acids (Dohrmann et al., 1973; Green and Wagner, 1973; Kobrine et al., 1975; Vaquero et al., 1976; Young, 1985; Braughler and Hall, 1989; Lemke and Faden, 1990; Panter et al., 1990; Tator and Fehlings, 1991; Zurita et al., 1994, 2001; Hamada et al., 1996; Zhang et al., 1997; Springer et al., 1999).

Apoptosis is a physiological process of cell death that differs from necrosis and it is morphologically characterized by a decrease in cell size, nuclear condensation and chromatin fragmentation due to endonuclease activation (Kerr et al., 1972; Wyllie, 1980; Schwarch and Osborne, 1993). It is now known that apoptosis can be induced by a number of factors, such as hypoxia, ischemia and the presence of nitric oxide, excitatory amino acids or free radicals (Cummings et al., 1997; Brune et al., 1998; Beattie et al., 2000; Lu et al., 2000) and that it can be triggered in association with necrosis in damaged tissues such as that produced in the brain or in adult spinal cord after trauma (Linnik et al., 1993; Xu et al., 1998; Ng et al., 2000).

In 1995, Crowe et al. demonstrated the involvement of apoptosis in spinal cord injury (SCI), where it is responsible, at least in part, for tissue degeneration at the lesion site and chronic demyelination in distant zones, and at present there are a number of studies in the literature that describe the role played by apoptosis after severe SCI (Crowe et al., 1995; Li et al., 1995; Katoh et al., 1996; Li et al., 1996; Crowe et al., 1997; Liu et al., 1997; Emery et al., 1998; Lou et al., 1998), and its possible implication in the pathogenesis of posttrauma secondary damage has suggested the utility of...
antiapoptotic therapy in the early phases following SCI (Katoh et al., 1996; Liu et al., 1997; Emery et al., 1998; Lou et al., 1998).

On the other hand, the use of steroids in traumatic injury of the nervous system has been justified by several authors in view of its proven efficacy in the treatment of the vasogenic edema associated with tumors (Gallicich and French, 1961; Bekx et al., 1972), and its apparent utility in experimental injury models (Faden et al., 1983, 1984; Hoerlin et al., 1983, 1985).

Methylprednisolone (MP), a glucocorticoid, the plasma half-life of which ranges between 12 and 36 hours, has in the past been considered beneficial after SCI (Bracken et al., 1990, 1992, 2000; Bracken, 1991). According to the results of the National Acute Spinal Cord Injury Study (NASCIS) published by Bracken et al. in 1990 and 1992, MP appears to be capable of reducing the secondary damage that can develop after spinal cord trauma, provided it is administered within 8 hours postinjury. However, its true neuroprotective effects and possible mechanism of action after SCI in humans is presently being questioned (Simpson et al., 1989; Levy et al., 1996; Nesathurai, 1998; Hurlbert, 2000; Pointillart et al., 2000; Short et al., 2000) and from the experimental point of view, the findings concerning the mechanism of steroids and their utility in cases of SCI are also contradictory (Faden et al., 1983, 1984; Hoerlin et al., 1983, 1989; Hall, 1992; Behrmann et al., 1994; Constantini and Young, 1994; Chen et al., 1996; Kanellopoulos et al., 1997; Xu et al., 1998, 2001; Kaptanoglu et al., 1999, 2000; Oudega et al., 1999).

Taking into account previous evidence suggesting the role of apoptosis in the development of secondary lesions after SCI (Zurita et al., 2001), and that dexamethasone decreases the number of apoptotic cells in the injured spinal cord tissue (Zurita et al., 2002), we proposed to study the possible influence of a single bolus of MP on Apostain expression in the zone of trauma, and in adjacent spinal cord segments, in an experimental model of SCI. The monoclonal antibody Apostain (F7-26) detects apoptotic cells before internucleosomal fragmentation occurs and it has been found to be a highly specific marker for apoptosis (Tsai et al., 1989; Frankfurt et al., 1996; Zunino et al., 1996; Ferlini et al., 1997).

Material and methods

Experimental model

For this study, a total of 108 adult female Wistar rats (3 months old, 250 to 300 g body weight) were used. Following intraperitoneal (IP) anesthesia with diazepam (6 mg/kg body weight) and ketamine (60 mg/kg body weight), a laminectomy was performed at the T6-T8 level. In 96 animals, a traumatic injury was produced in exposed spinal cord by dropping a 12 mm² thick steel level. In 96 animals, a traumatic injury was produced in weight), a laminectomy was performed at the T6-T8 (6 mg/kg body weight) and ketamine (60 mg/kg body weight). Following intraperitoneal (IP) anesthesia with diazepam (3 months old, 250 to 300 g body weight) were used. 

For this study, a total of 108 adult female Wistar rats (3 months old, 250 to 300 g body weight) were used. Following intraperitoneal (IP) anesthesia with diazepam (6 mg/kg body weight) and ketamine (60 mg/kg body weight), a laminectomy was performed at the T6-T8 level. In 96 animals, a traumatic injury was produced in exposed spinal cord by dropping a 12 mm² thick steel bar weighing 25 g from a height of 10 cm. The paraspinal muscles and subcutaneous tissues were subsequently closed with absorbable suture (Surgilene, 3/0). Immediate total paraplegia, that remained unchanged throughout the entire study period, was observed in every case. Postoperative care included bladder expression two to three times a day, administration of lactated Ringer’s to prevent dehydration, and IP administration of gentamicin (0.8 mg/100 g body weight). Subcutaneous buprenorphine (0.02 mg/250 g body weight) was given as a postoperative analgesic.

After surgery, the animals were randomly divided into two experimental groups. The control group (n: 48) did not receive MP, while the animals in the MP group (n: 48) received an IP dose of 30 mg/kg body weight of MP 10 minutes postinjury, according to the approach suggested by Yoon et al. (1999). In addition to these two experimental groups, we studied another 12 adult female rats that underwent laminectomy and postoperative administration of lactated Ringer’s, gentamicin and buprenorphine, but were not subjected to injury (non-injured animals). In these rats, we observed no postoperative deficits. Six of the animals of this group did not receive MP after laminectomy, but the remaining 6 animals received the same dose as the animals of the injured MP group, 10 minutes after closure of the laminectomy.

All the injured animals were sacrificed, by means of IP anesthesia, in groups of 16 rats for each time point (8 rats from the control group and 8 from the MP group) at 1, 4, 8, 24 and 72 hours and 1 week after SCI. The 8 rats corresponding to each group and time point were randomly distributed for histological and immunohistochemical studies (4 rats), for analysis of DNA fragmentation (2 rats) or for electron microscopic study (2 rats). The 12 non-injured animals were sacrificed in groups of 2 rats each (one with MP and another without MP administration) at each of the same time points after laminectomy, and spinal cord samples from the T6-T8 level of these rats were subjected to histological studies using hematoxylin-eosin staining, to immunohistochemical studies to detect expression of Apostain (anti-ssDNA MAbs F7-26), to analysis of DNA fragmentation, and to electron microscopic studies.

In this study, care of the animals complied with that stipulated by the principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals issued by the American National Society for Medical Research and the National Academy of Sciences, respectively.

Histological and immunohistochemical studies

For histological and immunohistochemical studies, the rats were perfused through left trunk with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The zone subjected to laminectomy was removed in bloc and embedded in paraffin. Then, spinal cord sections were obtained from the zone of injury (lesion zone, 6 mm long) and from the adjacent segments (rostral zone, 3 mm above the lesion zone, and caudal zone, 3 mm below the lesion zone, Figure 1),
and stained with hematoxylin-eosin in order to evaluate tissue changes at different times after injury. Apoptotic cells were detected by means of immunostaining with Apostain. For this, histological sections from paraffin-embedded samples were mounted on glass slides and deparaffinized by treatment in xylene for 15 minutes. Then, sections were trypsinized for 15 minutes and rinsed in phosphate-buffered saline (PBS, pH 7.3-7.4). The slides were washed in citrate-buffered solution (pH 6.0) for 10 minutes under microwave heating, and placed in 3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. Sections were then immersed in PBS. The primary monoclonal antibody to Apostain (anti-ssDNA F7-26) (1:100, Alexis Biochemicals Corporation, San Diego, CA) was added and the slides were kept overnight at 4°C in a humidity chamber, after which they were again rinsed in PBS. A 60-minute incubation with biotinylated secondary antibody (1:100, Vector Labs. Burlingame, CA) at 37°C was followed by a standard PBS rinse. Another 60-minute incubation with streptavidin-peroxidase complex (Vector Labs. Burlingame, CA), at 37°C, was carried out and a chromogen solution was added (diaminobenzidine). Then, the slides were stained with hematoxylin, mounted, and examined microscopically. In all cases, negative controls were prepared using normal serum as primary antibody.

In order to better identify apoptotic cells, some sections were subjected to double immunohistochemical staining using the monoclonal antibody glial fibrillary acidic protein (GFAP, MAb 360, 1:100, Chemicon International, Inc., Temecula, CA) and the mouse anti-oligodendrocyte monoclonal antibody (MAb1580, 1:200, Chemicon International, Inc., Temecula, CA). For this, the sections were incubated with normal horse serum followed by monoclonal antibody anti-ssDNA MAb F7-26 overnight at 4°C. Biotinylated anti-mouse IgG and ABC reagent were applied as described above for the F7-26 immunohistochemistry. The antibody complex was visualized using 3,3’-diaminobenzidine (DAB) with nickel enhancement, which produced a black reaction product. The tissue was then immediately rinsed with PBS and incubated with the same blocking solution, followed by anti-GFAP or anti-oligodendrocyte monoclonal antibody, overnight at 4°C. The secondary antibody and ABC complex were used. The antibody complex was visualized using DAB (Vector Labs. Burlingame, CA) without nickel enhancement, which produced a brown reaction product. The tissue was then counterstained with hematoxylin.

Cell counts and statistical analysis

Twelve sections (separated from each other by approximately 1 to 2 mm) corresponding to the lesion zone (4 sections), rostral zone (4 sections) and caudal zone (4 sections) were selected at random from each animal and the number of Apostain-positive cells, based on the number of stained nuclei in each histological section, was recorded. These recordings were made by image analysis morphometry (Optimas, 6.2 software package, Optimas Corporation, Bothell, WA) using a macro application, conducted by two investigators trained in morphometric determinations, with no knowledge of the experimental group from which each sample had been obtained. Generally, there was a high degree of agreement between the observers but, in any case, the mean of the values recorded by these investigators were regarded as final values.

For each experimental group and for each time point, the total number of Apostain-positive cells per cross section in the lesion, rostral and caudal zones, were averaged and values were expressed as count means ± standard deviations. We used a simple ANOVA test for a comparison of apoptotic cells across time within each group, and the unpaired Student’s t test, for a comparison of the number of apoptotic cells in the two experimental groups, at different time points. This statistical analysis was performed by means of the InStat statistical system (v 1.01, GraphPad Software Inc., San Diego, CA), with p<0.05 considered as statistically significant.

Analysis of DNA fragmentation

For analysis of DNA fragmentation, samples of spinal cord obtained from the zone of the lesion and from rostral and caudal zones were immediately snap-frozen in liquid nitrogen and stored at −80°C. DNA from these samples was isolated using DNAzol (Molecular Research Center, Inc.) according to the method developed by Chomczynski et al. (1997). The tissue was lysed for 10 minutes at room temperature using 1 ml of DNAzol/50 mg of tissue. It was then centrifuged at 104 g for 10 minutes, the DNA was precipitated by 100% ethanol and several washes were carried out. The extracted DNA samples were resuspended in ethanol and, after quantification by spectrophotometry, the DNA was separated by 2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under ultraviolet illumination.

Electron microscopy studies

For ultrastructural studies, spinal cord tissue samples were fixed with 3% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in PBS for 3 hours, dehydrated in a graded series of alcohols and embedded in araldite. The semithin sections (1µ) were stained with toluidine blue. The ultrathin sections were stained with uranyl acetate and examined under a Philips EM-200 transmission electron microscope.

Results

Our present study shows that apoptotic cells appear in the injured spinal cord tissue in the course of the first hour after trauma. Apoptotic cells increase until reaching a maximum, 8 hours after SCI. After this time point, a progressive decrease in the number of apoptotic cells can
be seen. However, one week after SCI, the presence of apoptotic cells is still a constant finding in the injured spinal cord tissue. When MP-treated animals were considered, a significant decrease in the number of apoptotic cells was detected 24 hours after SCI, in comparison to controls. In the spinal cord tissue adjacent to the zone of SCI, apoptosis was first detected 4 hours after trauma, the number of apoptotic cells increased in the course of the first week, and it is not plateaued at this time point. When MP-treated animals were considered, a decrease in the number of apoptotic cells was detected in comparison to controls, starting 24 hours after SCI, and reaching a significant difference after 72 hours.

Histology and immunohistochemistry

In all the non-injured animals, histological studies showed the features of normal spinal cord, and immunohistochemical expression of Apostain was absent in all histological sections. In the injured rats, paraplegia was observed immediately after SCI and there were no signs of functional recovery throughout the entire follow-up period. In these animals, one hour after trauma, zones of microhemorrhages, tissue edema and early signs of neuronal degeneration were detected and remained a constant finding in the lesion zone. The immunohistochemical studies revealed a clear positivity to the marker Apostain in small neurons of dorsal horn. The motor neurons of ventral horn showed little immunopositivity for Apostain. Some Apostain-positive cells, co-expressing GFAP and, thus, identified as astroglial cells, were observed in the gray matter, here, too, among small hemorrhagic foci. In the white matter, a small number of Apostain-positive cells, mainly identified as oligodendrocytes due to their coexpression of MAb 1580, was recorded. At this time, morphometric studies showed that the mean number of Apostain-immunostained cells (± standard deviation) was 72±5.5 in spinal cord sections of non-treated animals and 69±5.6 in MP-treated animals. Statistical analysis revealed no significant differences between the two groups (p>0.05). At this time point, there were no signs
of tissue damage or Apostain-positive cells in the rostral and caudal zones.

Four hours after SCI, the histological changes observed in the lesion zone were similar to those described one hour postinjury, but hemorrhages in the gray matter and edema in the white matter were more evident. Morphometric studies at this time showed the mean number of Apostain-immunostained cells to be 76±4.1 in spinal cord sections of non-treated animals and 71.4±2.3 in MP-treated animals. Statistical analysis revealed no significant differences between the two groups (p>0.05). In the rostral and caudal zones, signs of tissue damage were beginning to appear at that time point. By then, the gray matter presented few Apostain-positive cells that coexpressed GFAP and, thus, were identified as astrocytes, but numerous Apostain-positive cells coexpressing MAb 1580 were observed. There were also a few Apostain-positive neurons. Most of the

Fig. 3. Examples of Apostain expression in neuronal and glial cells, at different time points after spinal cord injury. A. Non-treated animal. Ventral horn of the lesion zone, 8 hours after trauma. A motor neuron expressing Apostain can be seen (arrow). Oligodendrocytes, showing Apostain-positivity can be identified also. (Immunohistochemical stain for demonstration of Apostain, ABC technique, original magnification x 200). B. Non-treated animal. Gray matter of the rostral zone, 8 hours after trauma, showing Apostain-positive oligodendrocytes and neurons (arrow) without Apostain-positivity. (Immunohistochemical stain for demonstration of Apostain, ABC technique, original magnification x 200). C. Non-treated animal. Lesion zone. Astrocytes of the white matter showing coexpression of Apostain and GFAP, 72 hours after trauma. (Double immunostain for demonstration of Apostain and GFAP, ABC technique, original magnification x 1000). D. Non-treated animal. Lesion zone. Panoramic view of the gray matter, 24 hours after trauma, showing a number of Apostain-positive cells, mainly oligodendrocytes. (Double immunohistochemical stain for demonstration of Apostain and MAB-1580, ABC technique, original magnification x 40). E. Animal treated with methylprednisolone. Lesion zone. Panoramic view of the gray matter, 24 hours after trauma, showing a decrease in the number of Apostain-positive cells. (Double immunohistochemical stain for demonstration of Apostain and MAB-1580, ABC technique, x 40).
Apostain-positive cells identified in the white matter coexpressed MAb 1580 and, thus, were oligodendrocytes. Morphometric studies at this time showed the mean number of Apostain-immunostained cells to be 16.7±3 and 15±6.2 in the rostral and caudal zones, respectively, in spinal cord sections of non-treated animals and 13±1.4 and 11±8.2 for rostral and caudal zones, respectively, in MP-treated animals. Statistical analysis revealed no significant differences between the two groups (p>0.05) in any of the segments studied.

Eight hours after SCI, there were clearly visible areas of hemorrhagic necrosis in the spinal cord gray matter of the lesion zone. Histological studies identified apoptotic bodies in oligodendroglial cells and neurons, mainly located near areas of necrosis in the gray matter. Immunohistochemical staining detected a considerable increase in the number of Apostain-positive cells in the gray matter of both ventral and dorsal horns. Morphological and immunohistochemical studies identified these cells mainly as neurons and oligodendrocytes. On the other hand, a clear increase was observed in the number of apoptotic cells coexpressing MAb 1580. At this time point, morphometric studies showed mean numbers of Apostain-positive cells of 172±4.6 in MP-treated animals and of 186±5.8 in non-treated animals. Statistical analysis revealed no significant differences between the two groups (p>0.05).

These findings coincided with the observation of more severe tissue damage in the rostral and caudal zones, and immunohistochemical staining revealed that Apostain-positive cells were mainly glial cells coexpressing MAb 1580 and, to a lesser degree, GFAP. Apostain expression was practically absent in neurons. Morphometric studies showed mean numbers of Apostain-positive cells of 21±4.8 in the rostral zone and 19±3 in the caudal zone in non-treated animals and of 20±7 and 18±6.1, respectively, in MP-treated animals. Statistical analysis revealed no significant differences in the number of Apostain-positive cells in treated and non-treated animals (p>0.05).

Twenty-four hours after SCI, the lesion zone, only a very small number of neurons remained in the gray matter. There were clearly visible areas of necrosis with inflammatory cells and persistent microhemorrhagic foci. Images of the white matter also showed tissue damage with structural changes in axon morphology and the presence of reactive astrocytes. Positivity for Apostain was only observed in rounded cells having a dark, compact nucleus and a clearly visible perinuclear halo that were positive for MAb 1580 and expressed no positivity for GFAP; astrogial-like Apostain-positive cells coexpressing GFAP were present in lesser numbers. In the gray matter, the little positivity for Apostain observed in the two experimental groups was limited to oligodendrocytes. At this time, morphometric studies showed the mean number of Apostain-positive cells to be 153.1±2.1 in non-treated animals and 111±6.5 in MP-treated animals, the difference being statistically significant (p<0.05). At this time point, in the adjacent segments (rostral and caudal zones), neurons with signs of necrosis and considerable tissue edema predominated in the adjacent white matter. In the gray matter, immunohistochemical studies revealed oligodendrocytes coexpressing Apostain and MAb 1580, but in no case were Apostain-positive neurons identified. The white matter presented Apostain-positive cells coexpressing MAb 1580 or GFAP. Morphometric studies showed mean numbers of Apostain-positive cells of 35±8 in the rostral zone and 32±1.6 in the caudal zone in non-treated animals and of 22±6.8 and 21±3.4, respectively, in MP-treated animals. Statistical analysis revealed no significant differences in Apostain expression in the two experimental groups (p>0.05) in the spinal cord segments adjacent to the injured zone.

Fig. 4. Agarose gel electrophoresis of DNA extracted from normal and injured spinal cord tissue (zone of lesion) at 24 hours, 72 hours and 1 week after trauma. Lane 1 (L1): molecular weight markers (pUC19/HpaII digest, New England Biolabs, Beverly, MA, USA). Lanes 2 and 3 (L2 and L3): injured spinal cord tissue, 24 hours after trauma (C: non-treated; MP: treated with methylprednisolone). Lanes 4 and 5 (L4 and L5): injured spinal cord tissue, 72 hours after trauma (C: non-treated; MP: treated with methylprednisolone). Lanes 6 and 7 (L7 and L8): injured spinal cord tissue, 1 week after trauma (C: non-treated; MP: treated with methylprednisolone). Lane 8 (L8): spinal cord tissue from non-injured rats. DNA fragments of approximately 180 base pairs (bp) can be seen in injured spinal cord tissue.
and MAb 1580-positive) and in astrocytes (GFAP-positive). At this time, the finding of Apostain-positive neurons was exceptional. Morphometric studies showed the mean number of Apostain-positive cells to be 98±3.2 in non-treated animals and 89±4.5 in MP-treated animals, findings that were not significantly different (p>0.05). In the rostral and caudal zones, the histological lesions were similar to those previously described at 24 hours. Nevertheless, immunohistochemical staining showed a considerable increase in the number of Apostain-positive cells in the white matter of both spinal segments. These cells coexpressed MAb 1580 or GFAP and were, thus, identified as oligodendrocytes and astrocytes, respectively. Morphometric studies 72 hours after injury showed a mean number of Apostain-positive cells of 48±5 for the rostral zone and 51±3.3 for the caudal zone in non-treated animals, and of 21±3.6 and 24±1.9, respectively, in MP-treated animals. Statistical analysis revealed significant differences between the two experimental groups (p<0.05) in the two segments studied.

One week after SCI, there was very little Apostain-positivity in the lesion zone, as observed both in gray matter and white matter, and it was limited to glial cells. Morphometric studies showed a mean value for Apostain-positive cells of 51.6±4.1 for non-treated animals and of 47.2±3.4 for MP-treated animals, there being no statistically significant difference between the two experimental groups (p>0.05). At this time, Apostain-positive cells were observed in the rostral and caudal zones, mainly localized in the white matter. Most of these cells coexpressed MAb 1580 and, to a lesser extent, GFAP. Morphometric studies showed a mean number of Apostain-positive cells of 60±2.1 for the

**Fig. 5.** Electron microscopy showing an oligodendrocyte, in the gray matter of the lesion zone, with aggregation of chromatin into dense, sharply delineated masses, and preservation of cytoplasmic organelles. These findings identify an early phase of apoptosis. Non-treated rat, 8 hours after spinal cord injury. x 6,000
Dissociated neurons were transfected with 
DNA fragments.

bp DNA fragments, other intact high molecular weight which shows, together with the characteristic 180 to 200 DNA fragmentation studies are shown in Figure 4, and was similar one week after trauma. The results of postinjury (in both non-treated and MP-treated animals) in the segments adjacent to the lesion by 72 hours subsequent time points (at 72 hours and at 1 week after SCI). This pattern remained constant at the subsequent time points (at 72 hours and at 1 week after trauma). This pattern of DNA fragmentation was evident in the segments adjacent to the lesion by 72 hours postinjury (in both non-treated and MP-treated animals) and was similar one week after trauma. The results of DNA fragmentation studies are shown in Figure 4, which shows, together with the characteristic 180 to 200 bp DNA fragments, other intact high molecular weight DNA fragments.

DNA fragmentation

Agarose gel electrophoresis demonstrated that the DNA extracted from spinal cord samples of non-injured animals presented no sign of fragmentation. Between 1 and 8 hours postinjury, DNA isolated from the lesion zone and from the ventral and dorsal segments of the animals subjected to SCI (both MP-treated and non-treated) was also intact, there being no internucleosomal fragments. However, by 24 hours after injury, the spinal cord samples corresponding to the lesion zone presented DNA degradation (in both non-treated and MP-treated animals), showing a typical pattern of internucleosomal fragmentation of approximately 180 base pairs (bp) in length when compared with the molecular weight markers. This pattern remained constant at the subsequent time points (at 72 hours and at 1 week after trauma). This pattern of DNA fragmentation was evident in the segments adjacent to the lesion by 72 hours postinjury (in both non-treated and MP-treated animals) and was similar one week after trauma. The results of DNA fragmentation studies are shown in Figure 4, which shows, together with the characteristic 180 to 200 bp DNA fragments, other intact high molecular weight DNA fragments.

Electron microscopy studies

Electron microscopic studies carried out in spinal cord samples between one hour and one week after SCI showed coexistence of necrotic and apoptotic cells within the lesion zone. Early phases of apoptosis were identified by the aggregation of chromatin into dense, sharply delineated masses, with preservation of cytoplasmic organelles (Fig. 5). Further apoptotic changes were identified by cytoplasmic shrinkage, development of broad surface protuberances and breakdown of the nucleus into discrete, membrane-bound bodies. In non-injured animals, cells showing apoptosis were not found.

Discussion

In the past, the cell death that occurred after severe SCI was attributed to necrosis triggered by hemorrhagic and ischemic phenomena (hypoxia) and the inflammatory response that accompanies tissue damage (Young, 1985; Isaac and Pejic, 1995; Zunino et al., 1996; Chomcynski et al., 1997). However, there is now evidence suggesting the involvement of apoptosis, or programmed cell death, in the secondary lesions that develop after SCI, and that the role of the molecules that regulate the process of apoptosis is closely related to the nature and intensity of the stimulus inducing it, a circumstance that would explain the variability in studies of apoptosis in different tissues and situations (Wyllie, 1980; Crowe et al., 1995; Frankfurt et al., 1996; Crowe et al., 1997; Ferlini et al., 1997; Xu et al., 1998; Ng et al., 2000; Zurita et al., 2001). On the other hand, the use of techniques of varying degrees of sensitivity for detecting apoptosis and that detect it at different times after its onset may result in contradictory findings when apoptosis is studied in traumatic injuries of the nervous system. In the present study, we employed a new immunohistochemical procedure, specific for the detection of early phases of apoptosis in paraffin-embedded tissue samples even in the absence of DNA fragmentation (Tsai et al., 1989; Frankfurt et al., 1996; Zunino et al., 1996; Ferlini et al., 1997). Furthermore, keeping in mind that DNA fragmentation can occur in necrotic cells, leading to false positives (Hashimoto et al., 1995) we confirmed the presence of apoptosis by performing electron microscopy and by analysis of DNA fragmentation. Electron microscopy showed cell alterations attributed to apoptosis (Li et al., 1996; Cummings et al., 1997), and analysis of DNA fragmentation on injured spinal cord tissue showed, in a constant way, the presence of fragments of DNA with a size between 180 and 200 base pairs, a finding considered typical of the apoptotic phenomenon (Schwarch and Osborne, 1993; Cummings et al., 1997).

Our present study demonstrated the presence of Aposstain-positive cells in injured spinal cord tissue, one hour after trauma, and a peak of Aposstain expression 8 hours after SCI in both white matter and gray matter. Four hours after SCI, Aposstain expression was found in zones adjacent to the directly injured tissue and, in these zones, the number of immunostained cells increased between 8 hours and one week postinjury, in agreement with previous reports describing apoptosis in contused spinal cord (Liu et al., 1997). On the other hand, we have evidence to show that the administration of a single dose of MP of 30 mg/kg body weight 10 minutes postinjury was followed by a statistically significant decrease in the number of Aposstain-positive cells in injured spinal cord. This effect is observed 24 hours after trauma in the directly contused zone, and after 72 hours in the adjacent spinal cord segments, suggesting that MP decreases apoptosis in directly injured spinal cord tissue and, later, in the adjacent zones.

A number of studies support the efficacy of glucocorticoids in inhibiting morphological and functional alterations after SCI, a circumstance that has been related to a reduction in the accumulation of catecholamines (Osterholm and Mathews, 1972a,b) or of free radicals (Hall and Braughler, 1993) in the zone of...
the lesion. Other effects could include the modification of glutamic acid (Ogata et al., 1993), the stabilization of the cell membranes (Demopoulos et al., 1973), or actions involving lipid peroxidation (Osterholm and Mathews, 1972a,b; Demopoulos et al., 1973; Bracken et al., 1990; Ogata et al., 1993), arachidonic acid release (Anderson et al., 1985), oxygen free radical formation (Osterholm and Mathews, 1972a,b; Anderson et al., 1985; Koc et al., 1999; Benton et al., 2001) or a simple antiinflammatory effect (Bartholdi and Schwab, 1995; Slater et al., 1995; Xu et al., 1998).

We recently reported that dexamethasone decreases apoptotic cell death after SCI (Zurita et al., 2002), in agreement with previous studies (Brandoli et al., 2001). In view of the fact that MP is currently being used in early phases after SCI in humans (Bracken, 1991; Bracken et al., 1992), although this therapy has been widely questioned (Hurlbert, 2000), in the present study we examined the possible antiapoptotic effect of MP in injured spinal cord tissue. Nevertheless, there is no evidence of marked differences between the mechanisms of action of MP and dexamethasone or between their possible beneficial effects after trauma (Dohrmann et al., 1973; Yan et al., 1999).

In our present study, the doses and timing of MP administration were chosen on the basis of previous reports indicating a better dose-response relationship for MP after SCI (Hall et al., 1984; Yoon et al., 1999), and our results demonstrate the influence of a single bolus of MP on apoptotic cell death in injured spinal cord tissue. The comparison of these findings with those previously reported by us (Zurita et al., 2002) relative to the influence of dexamethasone on the expression of Aposain in the lesion zone after SCI shows that, between 8 and 72 hours after trauma, the decrease in the total number of Aposain-positive cells produced by dexamethasone, at a dose of 1 mg/kg body weight daily, starting after trauma, is more marked than that associated with MP at the dose utilized in the present study, suggesting that dexamethasone has a greater influence on apoptosis after SCI. In any case, it is a well-known fact that most of the actions of glucocorticoids take place through their binding to nuclear receptors and regulation of gene expression (Beato, 1989). Thus, they may attenuate the phenomena of apoptosis triggered after nervous system trauma by altering the expression of certain genes implicated in apoptosis such as bcl-2, caspases, bcl-x, bax, and p-53 (Beato, 1989; Katoh et al., 1996; Emery et al., 1998; Clark et al., 1999; Hara, 1999; Yoon et al., 1999; Li et al., 2000; Saito et al., 2000).

On the other hand, in our study, most of the cells expressing Aposain after SCI were oligodendrocytes and astrocytes, and our results show that administration of MP decreases the number of apoptotic glial cells. Although the benefit of preserving glial cells in the absence of neurons is questionable, it is possible that preservation of oligodendrocytes can play a role diminishing the effects of traumatic lesions, because it is known that demyelination occurs in the course of the first week after trauma (Vaquero et al., 1976; Zurita et al., 1994). In the same way, although astrocytes sometimes contribute to secondary injury after trauma, recent studies suggested an important role for these cells in Nervous System regeneration (Song et al., 2002; Lang et al., 2004). Thus, further studies are necessary to confirm if antiapoptotic therapies can achieve astrocyte preservation after SCI, and to know if this finding can be related to significant functional improvement after severe traumatic lesions.

Although at present, there is no evidence of a relationship between inhibition of apoptosis and long-term functional recovery after nervous system trauma, the results of our present study support the hypothesis that the effects of apoptosis on neuronal and glial cells play a role in cell death after SCI, and they show that apoptosis is reduced in injured spinal cord tissue after MP administration. It is obvious that these findings suggest the need for further studies to determine whether blocking apoptosis can modify the clinical outcome after severe SCI, and the possible beneficial effects of different steroids on apoptotic cell death after trauma. In conclusion, our present results confirm that apoptosis affecting neuronal and glial cells is a common finding after SCI, and that early administration of a single bolus of MP (at dose of 30 mg/kg body weight) can decrease apoptotic cell death, suggesting the possible utility of this therapy in reducing secondary damage within the injured spinal cord tissue.

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