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Melatonin ameliorates autoimmune encephalomyelitis through suppression of intercellular adhesion molecule-1

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Melatonin (N-acetyl-5-methoxytryptamine), a pineal neurohormone, is a hydroxyl radical scavenger and antioxidant, and plays an important role in the immune system. We studied the effect of exogenous melatonin on the pathogenesis of experimental autoimmune encephalomyelitis (EAE). EAE was induced in Lewis rats by immunization with rat spinal cord homogenates. Subsequent oral administration of melatonin at 5 mg/kg significantly reduced the clinical severity of EAE paralysis compared with administration of the vehicle alone ($p<0.01$). Infiltration of ED1+ macrophages and CD4+ T cells into spinal cords occurred both in the absence and presence of melatonin treatment, but melatonin-treated rats had less spinal cord infiltration of inflammatory cells than did the control group. ICAM-1 immunoreactivity in the blood vessels of EAE lesions was decreased in melatonin-treated rats compared to vehicle-treated rats. These findings suggest that exogenous melatonin ameliorates EAE via a mechanism involving reduced expression of ICAM-1 and lymphocyte function-associated antigen-1a in autoimmune target organs.

Key words: Melatonin, experimental autoimmune encephalomyelitis, intercellular adhesion molecule-1

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) that is a model for human demyelinating diseases, such as multiple sclerosis [24]. The clinical course of EAE is characterized by weight loss, ascending paralysis, and spontaneous recovery. EAE is characterized by T cell and macrophage infiltration and, at the peak stage of paralysis, increased expression of intracellular cell adhesion molecules [14]. EAE-induced paralysis is associated with inflammatory cytokines, including IL-1, and the effect of radicals (nitric oxide) on neurons, and thus it is ameliorated or prevented by the action of anti-inflammatory factors, including IL-4 [18], suppressor cells [2], and NK cells [17].

The neuroendocrine-immune system axis is one that has gained attention in recent years. Melatonin (N-acetyl-5-methoxytryptamine) is a neuro-modulator that is synthesized in the pineal gland [12]. Melatonin is known to play roles in many physiological processes. It is important in transmission of photoperiod information, control of reproduction [1], and through direct binding to melatonin receptors on T helper cells, modulation of the immune response [5,10]. Melatonin is also involved in inhibition of aging [19] and scavenging of free hydroxyl radicals [21]. Recent studies have shown that melatonin activates non-specific immunity by activating natural killer cells [4], stimulating IL-4 production [15], inhibiting nuclear factor kB (NF-κB) [3,9], and suppressing intercellular adhesion molecule (ICAM)-1 [6].

Since many of the melatonin effects described above are associated with modulation of the immune system, melatonin may be associated with suppression of autoimmune diseases, including EAE [13,16,20]. Despite this possible link, previous studies of the functional role of exogenous melatonin in the induction of EAE are limited. In this study, we sought to determine whether exogenous melatonin affects the pathogenesis of autoimmune encephalomyelitis, an animal model for human multiple sclerosis.

Materials and Methods

Induction of EAE and assessment of clinical signs

Lewis rats of both sexes (8-12 weeks old) were obtained from Harlan (Sprague-Dawley, Indianapolis, IN) and bred in our animal facility. EAE was induced in 8-12 week-old rats. Briefly, each rat was inoculated subcutaneously in

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both hind footpads with 100 μl of an emulsion containing 1 mg of fresh rat spinal cord homogenate in phosphate-buffered saline per ml of complete Freunds adjuvant (CFA; *Mycobacterium tuberculosis* H37Ra, Difco, Detroit, Michigan). Control animals received CFA only. Immunized rats were observed daily for signs of paralysis, which is the clinical manifestation of EAE. Paralysis was graded in five stages of severity (grade 0, no signs; grade 1, floppy tail; grade 2, mild paraparesis; grade 3, severe paraparesis; grade 4, tetraparesis or moribund condition). Duration was also noted for paralysis grades>2, as described in a previous paper [23].

Administration of melatonin to rats

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in ethanol (5% w/v) and administered orally to each rat at a dose of 5 mg/kg. The melatonin-treated group consisted of 9 rats. As controls, the mice in the vehicle-treated group (n = 8) received 5% ethanol orally. Melatonin was administered from the day of immunization (day 0) to day 14 post-immunization (PI).

Antisera

The following antisera were used in this study: Monoclonal antibodies against ICAM-1 and lymphocyte function-associated antigen (LFA)-1a were obtained from Seikagaku Corp (Tokyo). Monoclonal antibodies OX-22 (anti-leukocyte common antigen), ED1 (for macrophages), and 3.2.3 (anti-NKR-P1 on rat NK cells) [5] were obtained from Serotec (London, UK). Rabbit antibody against glial fibrillary acidic protein (GFAP), which was used for staining astrocytes, was from Dakopatte (Copenhagen, Denmark).

Tissue sampling

Three rats from each group were sacrificed with ether on days 15 and 21 PI, *i.e*., during the peak and recovery stages of EAE, respectively. Tissue samples were taken from each rat. The spinal cords were removed and several segments of the spinal cords were flash-frozen in OCT compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan). Ten μm thick sections were cut and stored at −80°C until use.

Immunohistochemistry

Frozen sections of the spinal cord were air-dried and fixed in ether for 10 min. After three washes with PBS, the sections were exposed to normal goat serum for 30 min and then incubated in optimally-diluted mouse primary antisera (anti-ICAM-1 at 1 : 100, anti-LFA-1α at 1 : 100, OX-22 at 1 : 800, ED1 at 1 : 3200, or anti-NK cell at 1 : 400) for 60 min at room temperature. To identify cell types, rabbit anti-GFAP (1 : 800) (for astrocytes) and ED1 (1 : 800) (for macrophages) were applied to adjacent sections. After three washes in PBS, the sections were incubated with biotinylated anti-rabbit or anti-mouse antibody and then with an avidin-biotin reagent (Vector, Burlingame, CA) and the chromogen diaminobenzidine. Slides were counter-stained with hematoxylin, dehydrated, and mounted in balsam (Sigma).

Statistical analysis

Statistical comparisons among groups were made using the Student-Newman-Keuls Multiple Comparisons test. Differences with a p-value<0.05 were considered to be significant.

Results

Effect of melatonin on the course of EAE

All animals, with or without melatonin treatment, developed some degree of paralysis, signifying EAE. Oral administration of melatonin significantly reduced the severity and duration of paralysis compared with the control, vehicle-treated group (Table 1).

Immunohistochemistry

The histological lesions in melatonin-treated animals were nearly identical to those of control animals at the peak stage of EAE (day 15 PI) (Fig. 1). The inflammatory cells consisted primarily of ED1+ macrophages, CD4+ T cells, and CD8+ T cells (data not shown). NK+ cells and OX-22+ cells were also present. The inflammatory cells were less abundant in melatonin-treated rats than in vehicle-treated rats. The proportion of NK+ cells/total inflammatory cells was higher (about 2-fold) in melatonin-treated animals than in the vehicle-treated control group (Fig. 2).

Adhesion molecules, including LFA-1α and ICAM-1, were examined in both groups. The intensity of ICAM-1 immunoreactivity in the blood vessels of EAE lesions was

Table 1. Effect of melatonin administered orally from day 0 to day 14 after immunization on the expression of clinical signs during EAE

Treatment	Incidence	Clinical score≥2	Mean peak clinical score[a] (scale from 0 to 5)	Duration of paralysis[a] (days)	Suppression of EAE (%)	
					Incidence	Clinical score≥2
Vehicle	7/8	3/8	1.63±0.42	3.63±0.89		
Melatonin (5 mg/kg)	1/9	1/9	0.33±0.33*	0.44±0.44**	92	70

[a]Data are expressed as mean±s.e.m. *, P<0.05 and **, P<0.01, melatonin-treated compared with vehicle-treated, Students unpaired, two-tailed t-test.

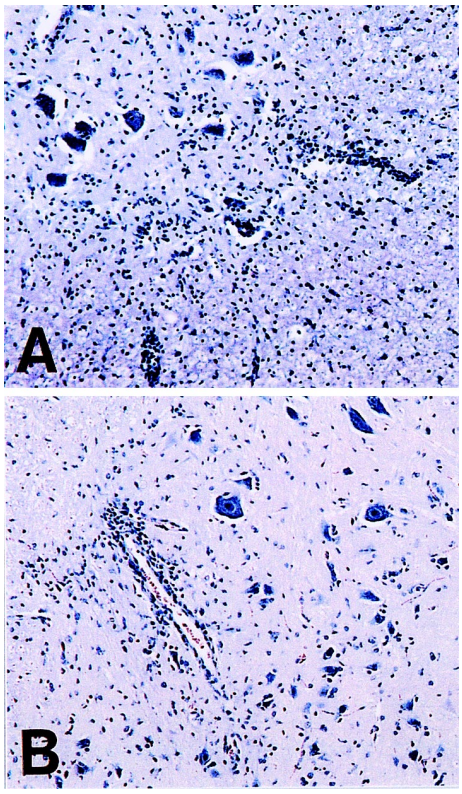

Fig. 1. Histological findings in spinal cords from vehicle-treated control (A) and melatonin-treated Lewis rats (B) with EAE (day 15 PI). A and B show inflammatory lesions in the spinal cord that are typical of EAE, but a comparison of A with B shows that many more inflammatory cells infiltrate the parenchyma in A than in B. H & E stain, Magnification: 200×.

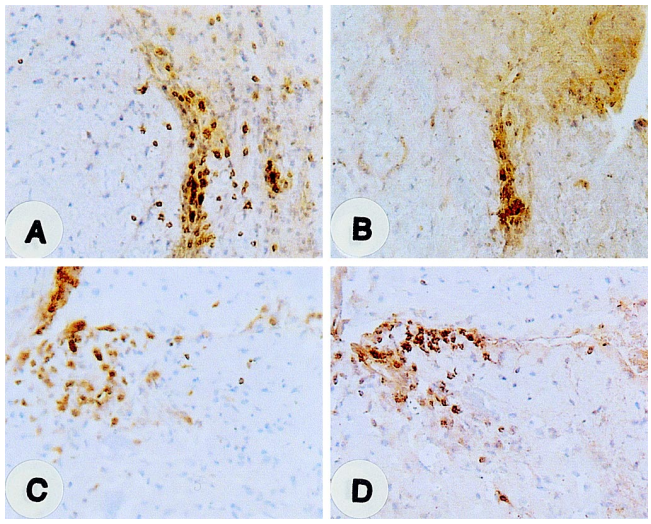

Fig. 2. Immunohistochemical detection of OX-22+ and NK+ cells in vehicle-treated (A and B) and melatonin-treated (C and D) groups at the peak stage (day 14 PI) of EAE. OX-22+ (A and C) and NK+ (B and D) cells were mainly located in the perivascular region but some were in the parenchyma. Counterstained with hematoxylin. Magnification: 132×.

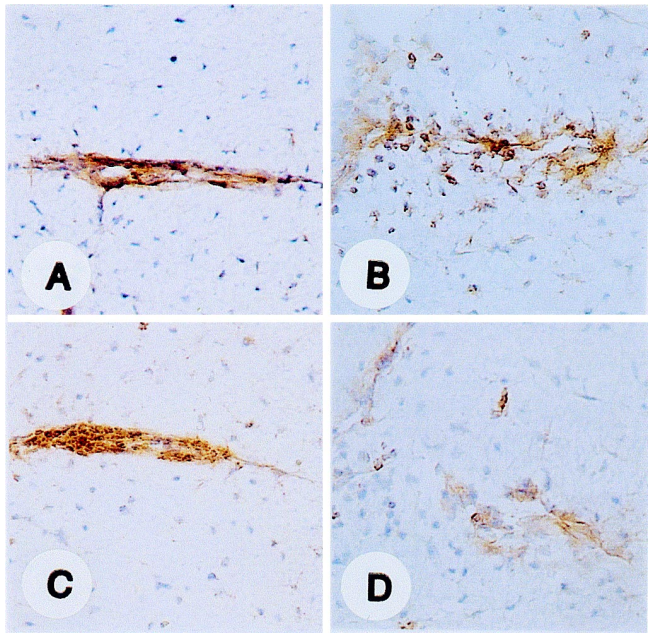

Fig. 3. Immunohistochemical detection of LFA-1a and ICAM-1 in melatonin-treated (B and D) and vehicle-treated (A and C) rats at the peak stage (day 14 PI) of EAE. LFA-1α positive cells were mainly located in the perivascular region, but some were in the parenchyma (A and B). ICAM-1 immunoreactivity was mainly detected in the blood vessels of the vehicle-treated (C) rats, but it was rarely detected in spinal cords of the melatonin-treated rats (D). Counterstained with hematoxylin. Magnification: 400×.

weaker for melatonin-treated rats than for vehicle-treated rats, whereas the intensity of LFA-1α immunoreactivity (indicating inflammatory cells) was similar in both groups (Fig. 3) (Table 2).

Discussion

This study constitutes the first clinical demonstration that exogenous melatonin affects the progression of the autoimmune disease model EAE. The underlying mode of melatonin action in preventing EAE paralysis remains controversial. A recent study has suggested that melatonin causes non-specific immunity by inducing production of NK cells [5] and IL-4 [20], which are important mediators in the amelioration or prevention of EAE-induced paralysis [4]. Melatonin is also implicated in reducing expression of NF-κb [9] and ICAM-1 [13], and thus may ameliorate the progression of EAE by blocking these factors.

By demonstrating that melatonin induces repression of ICAM-1 expression, this study suggests another possible mechanism for EAE amelioration by melatonin. We postulate that suppression of cell adhesion molecules is an important factor in reducing cell infiltration into CNS tissues. Adhesion molecules are important in the

Table 2. Immunohistochemical localization of W3/25, R73, OX22, ED1, LFA-1a and ICAM-1 positive cells, and NK cells in the spinal cords of vehicle control and melatonin treated EAE rats at the peak stage (D14PI).

Antibody	Vehicle control[a]		Melatonin treatment[a]	
	Perivascular	Parenchyma	Perivascular	Parenchyma
W3/25	++[b]	++	++	+
R73 (anti-TCRαβ)	++	+	++	±
OX22	++	+	+	±
ED1	++	++	++	±
NK cell (anti-NKR-P1)	++	+	++	+
LFA-1α	++	+	++	±
ICAM-1	+	±	±	-

[a]Three to five animals were examined in each group.
[b]Stained sections were scored on the number of cells per field that were positive. The number of positive cells was defined in the average of 5 randomly chosen 100× fields: -, no positive cells; ±, <10 cells per field; +, <30 cells; ++, ≥30 cells.

trafficking of peripheral leukocytes into the CNS, which is a major event in the pathogenesis of the inflammatory demyelinating disease multiple sclerosis [7,8,22]. In our previous study [24], we found that ICAM-1 significantly increased in the early stage of EAE.

This study confirms the high frequency of NK cells present in EAE lesions that we found in our previous study [17]. These cells suppress the progression of EAE paralysis. Although this study does not quantify the increase in NK cells, the trend in our immunohistochemical data shows that NK cells are present in higher amounts in melatonin-treated rats than in vehicle-treated rats. This aspect of NK cell biology needs further quantitative study to confirm this phenomenon.

We did not expect that melatonin treatment would completely block the onset of EAE, since this disease is caused by the homing of autoreactive T cells to the target tissue (spinal cord), and melatonin does not prevent the generation of autoreactive T cells, even though it reduces their homing ability. However, the findings of this study suggest that melatonin has an anti-inflammatory effect on EAE, caused by suppression of ICAM-1 and possibly by induction of NK cells production.

Although we found amelioration of EAE in melatonin-treated rats, Constantinescu *et al*. (1997) reported that melatonin receptor antagonists ameliorate EAE paralysis in rats, suggesting that melatonin is in fact detrimental in EAE, and that it may play a role in increasing inflammation. We speculate that these seemingly contradictory results may arise from experimental differences in the state of EAE severity. Whereas Constantinescu *et al*. used myelin basic protein as an immunogen, we used rat spinal cord homogenates as an immunogen. Myelin basic protein may cause more severe inflammation than rat spinal cord homogenate, which caused mild EAE in our system. This idea gains support from the observation that melatonin stimulates the production of inflammatory cytokines in human CD4+ cells [10]. The exact function of melatonin in modulating the immune system response needs further study.

Taking all of these observations into consideration, we postulate that melatonin suppresses autoimmune diseases, including mild EAE, by suppressing production of ICAM-1 in the target organ, the spinal cord.

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