Trabajo realizado por el equipo de la Biblioteca Digital de la Fundación Universitaria San Pablo-CEU

Me comprometo a utilizar esta copia privada sin finalidad lucrativa, para fines de investigación y docencia, de acuerdo con el art. 37 del T.R.L.P.I. (Texto Refundido de la Ley de Propiedad Intelectual del 12 abril 1996)
Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro


Abstract: The pineal secretory product melatonin reportedly regulates release of growth hormone in humans and prevents phototherapy-induced hypocalcemia in newborn rats, suggesting that melatonin affects bone metabolism. Little is known about the effects of melatonin on bone in vitro or in vivo. The present study was undertaken to examine whether melatonin acts directly on normal human bone cells (HOB-M cells) and human osteoblastic cell line (SV-HFO cells) to affect osteogenic action in vitro. The effect of melatonin on bone cell proliferation was determined using the 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay after a 24 hr incubation with melatonin. Melatonin significantly and dose-dependently increased the proliferation in HOB-M cells and SV-HFO cells by 215 ± 22.1%, and 193 ± 6.4%, respectively, with a maximal effect at a concentration of 50 μM. To evaluate the effect of melatonin on bone cell differentiation, alkaline phosphatase (ALP) activity, osteocalcin secretion and procollagen type I C-propeptide (PICP) production (a measure of type I collagen synthesis) were measured after a 48 hr treatment. While melatonin at micromolar concentrations did not significantly affect either the ALP activity or the osteocalcin secretion, it significantly and dose-dependently increased the PICP production in HOB-M cells and SV-HFO cells by 983 ± 42.2%, and 139 ± 4.2%, respectively, with the maximal stimulatory doses between 50 and 100 μM. These results provide new evidence that melatonin stimulates the proliferation and type I collagen synthesis in human bone cells in vitro, suggesting that melatonin may act to stimulate bone formation.

Introduction

Melatonin, a secretory product synthesized nocturnally by the pineal gland, plays a major role in the coordination of seasonal reproduction [Reiter, 1991] and pubertal development in some mammals [Silman et al., 1979]. Melatonin is a potent antioxidant agent [Marshall et al., 1996; Reiter et al., 1997] and may possess anti-aging [Sandyk, 1990; Armstrong and Redman, 1991] and oncostatic properties [Blask et al., 1988; Lissoni et al., 1994; Kojima et al., 1997; Panzer and Viljoen, 1997]. Melatonin also influences release of growth hormone in humans [Smythe and Lazarus, 1974; Valcavi et al., 1993], serum levels of corticosterone in rats [Oxenkrug et al., 1984] and the circadian rhythm of osteoblast metabolism in rats [Cane et al., 1991]. In addition, melatonin prevents phototheraphy-induced hypocalcemia in newborn rats [Hakanson and Bergstrom, 1981]. These findings suggest that melatonin affects bone and mineral metabolism. Furthermore, considerable indirect evidence links melatonin to osteoporosis [Sandyk et al., 1992]. For example, melatonin secretion decreases sharply during menopause [Sack et al., 1986], declines with immobility [Vaughan et al., 1978; Yocca and Freidman, 1984] and increases with exercise [Carr et al., 1981]. Osteoporosis and pinel calcification are uncommon among black populations [Adeloye and Felson, 1974]. While melatonin inhibited proliferation of human breast cancer cells [Hill and Blask, 1988; Molis et al., 1994], human benign prostate epithelial cells [Gilad et al., 1997] and rat hepatocytes [Kojima et al., 1997], its effect on bone cells have not been
addressed. The present study was to examine whether melatonin affects proliferation and differentiation in human bone cells in vitro. In this report, we provide new evidence that melatonin at micromolar concentrations stimulates proliferation and type I collagen synthesis in human bone cells in vitro.

Materials and methods

Materials

Tissue culture supplies were obtained from Iwaki Glass (Funabashi, Japan) or Falcon (Oxnard, CA). Dulbecco's modified Eagle's medium (DMEM), 0.5% trypsin–5.3 mM EDTA solution and collagenase were products of Gibco/BRL Life Technologies (Grand Island, NY). Iron-supplemented bovine calf serum was purchased from JRH Bioscience (Lenexa, KS). Melatonin, bovine serum albumin (BSA), 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT), Triton X-100, dimethyl sulfoxide (DMSO) and p-nitrophenyl phosphate (pNPP) were products of Sigma Chemical Co. (St. Louis, MO). 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) was obtained from Biomol Research (Plymouth Meeting, PA). Gla-type osteocalcin EIA and procollagen type I c-peptide (PICP) EIA kits were purchased from Takara (Ohtsu, Japan). All other chemicals were reagent grade, either from Sigma Chemical Co. or from Kanto Chemical Co. (Tokyo, Japan).

Cell cultures

Normal human bone cells were isolated from mandibular bone samples by collagenase digestion as previously described [Wegedal and Baylink, 1984]. The resulting bone cells (HOB-M cells) were shown to be of osteogenic nature, based on their responsiveness to parathyroid hormone (PTH) for increased cAMP production and to 1,25(OH)₂D₃ for increased alkaline phosphatase (ALP) activity and osteocalcin secretion [Wegedal and Baylink, 1984; Nakade et al., 1995]. The cells were maintained in DMEM supplemented with 10% bovine calf serum andpassaged every week at a 1:4 dilution ratio. Cells from passages 3–7 were examined in this study. Simian virus 40-immortalized human osteoblastic cell line (SV-HFO cells) were kindly provided by Dr. Hideki Chiba (Department of Pathology, Sapporo Medical University, Sapporo, Japan). Two kinds of human bone cells (HOB-M cells and HFO cells) were respectively plated at a density of 7,500 cells/cm² (for proliferation assay) or 10,000 cells/cm² (for ALP, osteocalcin and PICP assays) in DMEM supplemented with 10% bovine calf serum. After the cells attached to the plates for 24 hr, the culture medium was changed to fresh serum-free DMEM-containing 0.01% BSA. Twenty-four hours later, effectors (e.g., melatonin) were added, and the cells were incubated for either 24 hr for the proliferation assay or 48 hr for ALP, osteocalcin and PICP assays. Melatonin stock solutions were dissolved in DMSO and diluted in DMEM-containing 0.01% BSA immediately before use. The final DMSO concentration was 0.1%. The same DMSO concentration was included in each assay as vehicle control.

Effect on cell proliferation

The effect of melatonin on the bone cell proliferation was assessed by XTT assay [Roehm et al., 1991], after a 24 hr incubation with melatonin in serum-free DMEM containing 0.01% BSA. The assay is dependent on a cellular reduction of XTT by the mitochondrial dehydrogenase of viable cells to a red formazan product which represents the cell proliferative activity [Roehm et al., 1991]. The mitogenic effect was compared with that of ascorbic acid which is also an antioxidant agent [Glasscott et al., 1996]. Each treatment group consisted of six replicates.

Effect on cell differentiation

Cellular ALP activity, osteocalcin and type I collagen are generally regarded as osteoblastic differentiation markers [Farley and Baylink, 1986; Owen et al., 1990; Pockwinse et al., 1992; Termine and Robey, 1996]. Thus, we evaluated the effect of melatonin on differentiation of human bone cells by monitoring cellular ALP activity, osteocalcin secretion and type I collagen synthesis. Cellular ALP activity was assayed as the rate of hydrolysis of pNPP at pH 10.3 per mg protein [Farley and Jorch, 1983]. Cell extracts were prepared with 0.1% Triton X-100 and the enzyme activity was normalized against cellular protein [Wichelman et al., 1988]. Osteocalcin secretion in conditioned medium was assayed using a specific Gla-type osteocalcin EIA kit after a 48 hr treatment [Koyama et al., 1991]. The effect was tested in the presence of 10⁻⁸ M 1,25(OH)₂D₃, because both HOB-M cells and SV-HFO cells did not secrete detectable amounts of osteocalcin under basal conditions [Nakade et al., 1995]. The amount of osteocalcin in the conditioned medium was normalized against cellular protein content. PICP is a cleaved peptide before fibril formation during the
extracellular processing of type I collagen and represents cellular type I collagen synthesis. Thus, the effect on the type I collagen synthesis was indirectly assessed by PICP production in conditioned medium using a specific EIA kit after a 48 hr treatment [Suzuki et al., 1995]. The amount of PICP in the conditioned medium was normalized against cell number [Nakade et al., 1995].

Statistical methods

Results are shown as mean ± S.E.M. (n = 6). Statistical significance of the difference from the vehicle controls was analyzed with a two tailed Student’s t-test. Statistical significance of the difference between the groups was determined by one-way analysis of variance (ANOVA). The difference was considered significant when P < 0.05.

Results

Stimulation of the proliferation of human bone cells by melatonin in vitro

Fig. 1 shows that melatonin at micromolar concentrations significantly and dose-dependently increased the proliferation in HOB-M cells (215 ± 9.0%, P < 0.001 by ANOVA) and SV-HFO cells (192 ± 6.4%, P < 0.005 by ANOVA), with a maximal effect at 50 μM. In the same experiment, 10–50 μM ascorbic acid failed to significantly influence it in either HOB-M or SV-HFO cells (data not shown). These findings indicate melatonin at micromolar concentrations can act as a mitogen in human bone cells in vitro.

Effect of melatonin on differentiation of normal human bone cells in vitro

As shown in Tables 1 and 2, melatonin at micromolar concentrations failed to significantly affect either the ALP activity or the osteocalcin secretion in HOB-M and SV-HFO cells. On the other hand, as shown in Table 3, melatonin at micromolar concentrations significantly and dose-dependently influenced it in either HOB-M or SV-HFO cells (data not shown). These findings indicate melatonin at micromolar concentrations can act as a mitogen in human bone cells in vitro.

Table 1. Effect of mitogenic concentrations of melatonin on ALP activity in human bone cells in vitro

<table>
<thead>
<tr>
<th>Melatonin (μM)</th>
<th>HOB-M</th>
<th>SV-HFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (vehicle control)</td>
<td>14.7 ± 0.7</td>
<td>3,618 ± 263</td>
</tr>
<tr>
<td>5</td>
<td>12.9 ± 0.5*</td>
<td>3,854 ± 420*</td>
</tr>
<tr>
<td>10</td>
<td>14.5 ± 1.2*</td>
<td>3,426 ± 318*</td>
</tr>
<tr>
<td>50</td>
<td>12.4 ± 0.8*</td>
<td>4,002 ± 257*</td>
</tr>
<tr>
<td>100</td>
<td>12.6 ± 0.8*</td>
<td>3,595 ± 347*</td>
</tr>
</tbody>
</table>

Cellular ALP activity was determined after a 48 hr treatment of mitogenic doses of melatonin. Enzyme activity was normalized against cellular protein. Melatonin treatment did not significantly influence the ALP activity in either HOB-M nor SV-HFO cells (by ANOVA). Results are shown as mean ± S.E.M. (n = 6 for each group).

Table 2. Effect of melatonin on osteocalcin secretion of human bone cells

<table>
<thead>
<tr>
<th>Melatonin (μM)</th>
<th>HOB-M</th>
<th>SV-HFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (vehicle control)</td>
<td>11.1 ± 1.2</td>
<td>17.6 ± 1.8</td>
</tr>
<tr>
<td>5</td>
<td>13.3 ± 0.9*</td>
<td>17.7 ± 2.3*</td>
</tr>
<tr>
<td>10</td>
<td>13.5 ± 0.6*</td>
<td>19.1 ± 2.6*</td>
</tr>
<tr>
<td>50</td>
<td>14.4 ± 0.9*</td>
<td>19.0 ± 2.6*</td>
</tr>
<tr>
<td>100</td>
<td>12.1 ± 2.1*</td>
<td>17.2 ± 2.4*</td>
</tr>
</tbody>
</table>

The cells were treated with 0–100 μM of melatonin in the presence of 10⁻⁶ M of 1,25(OH)_2D₃ for 48 hr. The amount of osteocalcin in the conditioned media was normalized against cellular protein content. Melatonin did not significantly affect the osteocalcin secretion in either HOB-M or SV-HFO cells (by ANOVA). Results are shown as mean ± S.E.M (n = 6 for each group).

Fig. 1. Effect of melatonin on cell proliferation in human bone cells in vitro. Formazan absorbance values reflect proliferative activity of the cells in the XTT assay. Melatonin at micromolar concentrations significantly stimulated the proliferation in both HOB-M cells (215 ± 9.0%, P < 0.001 by ANOVA) and SV-HFO cells (192 ± 6.4%, P < 0.005 by ANOVA), in a dose-dependent manner with an optimal concentration of 50 μM. Results are shown as relative percentage (mean ± S.E.M.) of vehicle-treated control (n = 6 for each group). Dotted line represents 100% of vehicle control.
Table 3. Effect of melatonin on type I collagen synthesis in human bone cells

<table>
<thead>
<tr>
<th>PICP levels (ng/10,000 cells ± S.E.M.)</th>
<th>HOB-M (% control)</th>
<th>SV-HFO (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (vehicle control)</td>
<td>31.4 ± 4.2 (—)</td>
<td>187 ± 13.3 (—)</td>
</tr>
<tr>
<td>5</td>
<td>70.2 ± 5.7</td>
<td>235 ± 6.3</td>
</tr>
<tr>
<td>(223 ± 18.1)*</td>
<td>(126 ± 3.4)**</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>71.9 ± 7.6</td>
<td>234 ± 5.1</td>
</tr>
<tr>
<td>(229 ± 24.2)*</td>
<td>(125 ± 2.7)**</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>309 ± 13.3</td>
<td>249 ± 7.0</td>
</tr>
<tr>
<td>(983 ± 42.2)*</td>
<td>(133 ± 3.7)**</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>192 ± 6.4</td>
<td>260 ± 7.8</td>
</tr>
<tr>
<td>(612 ± 20.4)*</td>
<td>(139 ± 4.2)*</td>
<td></td>
</tr>
</tbody>
</table>

The effects of type I collagen synthesis was indirectly estimated by the amount of PICP in conditioned medium using a specific EIA kit, after a 48 hr treatment. The amount of PICP was normalized against cell number. Melatonin at micromolar concentrations significantly increased the PICP production, in either HOB-M cells (P < 0.001 by ANOVA) or SV-HFO cells (P < 0.001 by ANOVA). Results are shown as mean ± S.E.M (n = 6 for each group).

*P < 0.001, **P < 0.005, ***P < 0.01 compared with vehicle control by Student’s t-test.

increased the PICP production in either HOB-M or SV-HFO cells by 983 ± 42.2% (P < 0.001 by ANOVA) and 139 ± 4.2% (P < 0.001 by ANOVA), respectively, with the maximal stimulatory doses between 50 and 100 µM. These findings indicate that micromolar concentrations of melatonin can stimulate type I collagen synthesis, but neither ALP activity nor osteocalcin secretion in human bone cells.

Discussion

The present study demonstrates that melatonin at micromolar concentrations stimulates the proliferation and type I collagen synthesis in human bone cells (HOB-M cells) and human osteoblastic cell line (SV-HFO cells) in vitro. Although melatonin significantly increased the type I collagen synthesis in both human osteoblastic cells, the magnitude of the stimulation was different between HOB-M cells (983 ± 42.2%) and SV-HFO cells (139 ± 4.2%), indicating that it varies depending on cell type. The concentrations of melatonin that stimulated proliferation and type I collagen synthesis (from 10⁻⁵ to 10⁻⁴ M) in human bone cells in the study are higher than either circulating serum (from 10⁻¹¹ to 10⁻⁹ M) or pharmacological levels (from 10⁻⁷ to 10⁻⁵ M) applied to breast cancers [Vaughan et al., 1978; Panzer and Viljoen, 1997], preliminarily suggesting that our in vitro observations may not be clinically relevant. However, our recent preliminary work suggested that the daily injection of melatonin at 5–50 mg/kg/day increased the tibial bone mineral density and bone volume in both growing young mice and ovariectomized old rats (unpublished data). Thus, the observed in vitro osteogenic effects of melatonin may be clinically relevant, and melatonin might be a potential useful therapeutic agents in situations where increased bone formation is desired such as fracture healing and osteoporosis.

Little is known about the mechanisms regarding the mitogenic action of melatonin on bone cells, whereas melatonin exerts the inhibitory effect on the proliferation of human breast cancer cells through regulation of the estrogen receptor [Molis et al., 1994]. It is also unclear why melatonin selectively increases type I collagen synthesis in the three kinds of osteoblastic differentiation markers. In this regard, the gene expression of type I collagen peaks earlier, compared with that of ALP or osteocalcin during osteoblastic maturation [Owen et al., 1990; Pockwinse et al., 1992]. Therefore, we suppose that melatonin may act on bone cells to stimulate the proliferation and relatively early stage of the differentiation. Further studies are, however, needed to elucidate it.

In conclusion, we provide new evidence in this paper that melatonin stimulates proliferation and type I collagen synthesis of human bone cells in vitro, suggesting that melatonin may act as a potent stimulator of bone formation.

Acknowledgments

The authors wish to thank Dr. K-HW. Lau, Loma Linda University, for helpful discussions. The work was presented in part at the 19th Annual Meeting of the American Society of Bone and Mineral Research, Cincinnati, OH, September 1997 (abstract S232).

Literature cited


Nakade et al.


