Serotonin and Melatonin Do Not Play a Prominent Role in the Growth of Prostate Cancer Cell Lines

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Introduction

Hormone-refractory prostate cancer (PCa) represents the end stage of a progressive and fatal disease for which only palliative treatment options exist. Numerous concepts have been proposed to explain the biological progression of hormone-sensitive PCa to hormone-resistant PCa including stromal-epithelial interactions [1, 2], changes in androgen receptors [3], cell cycle machinery impairment [4], and abnormal signaling pathways [1, 5–8]. Currently, serotonin is considered to be of importance in the progression of PCa towards the hormone-refractory state [1, 9, 10]. The basis for this hypothesis is the reported expression of 5-HT receptors in LNCaP and PC3 cell lines [5, 6]. Serotonin-mediated mitogenic activity in vitro [11] and in vivo [12] as well as effects of 5-HT agonists/antagonists have been reported [5, 6, 13, 14]. 5-HT was thus described as a potent mitogen in vitro acting in the nM to µM concentration range and stimulating proliferation of PCa cell lines [11]. The serotonin receptor subtypes 5HTR1a, -1b, -2b and -4 were recently identified and characterized in PCa cell lines [5, 6]. Serotonin-mediated mitogenic activity in vitro and in vivo as well as effects of 5-HT agonists/antagonists have been reported [5, 6, 13, 14]. 5-HT was thus described as a potent mitogen in vitro acting in the µM concentration range and stimulating proliferation of PCa cell lines [11]. The serotonin receptor subtypes 5HTR1a, -1b, -2b and -4 were recently identified and characterized in PCa cell lines [5, 6]. These studies, involving the administration of 5-HT agonists/antagonists in vitro reported that stimulation at low concentrations (nM to µM) leads to increased proliferative activity, while inhibition leads to decreased proliferation in a concentration-dependent manner [5, 6, 13]. For such experiments, hormone-dependent (LNCaP), hormone-independent (PC3, DU145) and androgen-responding (22RV1)
human PCa cell lines are used as convenient in vitro models [15, 16].

The pineal hormone melatonin (MLT) may also be involved in the molecular interactions in different malignancies including PCa [2, 17–23]. Studies in LNCaP cell lines in vivo have shown an inhibition of tumor growth by MLT at nM concentrations with decreased expression of cell cycle markers [20]. It was concluded that the antiproliferative effects of MLT in LNCaP line were partly mediated by MT1 receptors and influenced the cell cycle with the possible induction of apoptotic changes [20]. The data obtained from other studies [19] on androgen-deprived LNCaP lines also showed a direct antiproliferative effect of MLT by impairment of the cell cycle (G0/G1 arrest) [21]. Results obtained from studies on MLT activity in DU145 cell lines have shown cell cycle arrest in the G0/G1 phase due to direct stimulation of nuclear MT receptors [23]. Membrane-associated receptors are suggestible to be different sites of action of MLT in PC3 cell lines [22].

The aims of our work were to investigate the expression of two serotonin receptors (5HTR1a and 5HTR1b) and the effects of serotonin, several serotonin agonists and antagonists and MLT in the regulation of malignant growth in several PCa cell lines (LNCaP, PC3, DU145, 22Rv1).

Materials and Methods

Cell Culture and Reagents

The human PCa cell lines LNCaP (hormone-dependent), 22Rv1 (hormone-responding), PC3 and DU145 (hormone-independent) were used for in vitro experiments (ATCC). As reference, human fibroblasts and BPH-1 cell line were used in experiments (DSMZ). The cell lines were cultivated in RPMI 1640 medium supplemented with 10% FBS and 1% MEM (Invitrogen, Karlsruhe, Germany) without antibiotics in 75-cm² flasks (Greiner, Frickenhausen, Germany). Cell culture activity was evaluated as low (LD; <50% visual density) and high (HD; 50–75% visual density). Cell counting was performed after trypsinization with DMSO). For this, 10⁶/well WST-1 was added, incubated with DMSO. For this, 10 µl/well WST-1 was added, followed by 1–1.5 h of incubation (37°C, 5% CO2). Cell viability was determined by absorbance measurement at 450 nm (reference wavelength 620 nm) using a microplate reader (Anthos Labtec, Krefeld, Germany). Viability after incubation with agonists/antagonists was expressed relative to control (incubated with DMSO).

Cell Cycle, Apoptosis Analysis by Flow Cytometry

Changes in cell cycle of 2 × 10⁴ nuclei were analyzed using Cycle Test Plus DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany) and FACScan (Becton, Dickinson). The early (annexin+/propidium iodide−) and late (annexin+/propidium iodide+) apoptosis was studied by Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Results

Basal mRNA Expression Levels of 5HTR1a and 5HTR1b in PCa Cell Lines

The results of the quantification of the relative mRNA expression levels of the 5HT receptor subtypes HTR1a and HTR1b corresponding to the growth density are shown in figure 1. Transcripts were detectable in all cell
**Fig. 1.** Relative mRNA expression of 5HTR1a (a) and 5HTR1b (b) normalized to the reference gene HPRT in PCa cell lines corresponding to the growth density of the cells.

**Fig. 2.** Relative viability of 22RV1, PC3 and DU145 cell lines (normalized to control incubated with DMSO) 24 h after incubation with 5-HT.
The comparison with growth density showed a higher expression of both 5HTR receptor subtypes in HD (50–75%) compared to LD (<50%) in all cell lines (fig. 1), with the exception of 5HTR1a expression in 22RV1 and human fibroblasts.

**Effects of 5-HT and 5HTR1a/-1b Agonists and Antagonists on Cellular Viability**

The cellular viability of 22RV1, PC3 and DU145 cells after 24 h of incubation with 5-HT (10^{-8} to 10^{-4} M) is shown in figure 2. The pronounced stimulatory effect
with increased cellular growth was observed in PC3 cells (+21.2, +31.8 and +59.5% at 10^{-8}, 10^{-6} and 10^{-4} M, respectively). In DU145 cells, a slight stimulatory effect was observed only at 10^{-4} M (+28.2%). LNCaP and 22RV1 cells did not respond at all to 5-HT stimulation (data not shown). Figure 3 gives the results of cell viability assessment without 5-HT stimulation in the presence of the 5-HT1a antagonist NAN-190 hydrobromide or the 5-HT1b antagonist methiothepin mesylate. After 48 h of incubation, cellular viability was not affected by the antagonist in the concentration range of 10^{-8} to 10^{-5} M. At a concentration of 10^{-4} M, no changes were observed after 5HTR1a inhibition. A concentration of 10^{-4} M of methiothepin mesylate was cytotoxic and caused total necrosis after 12 h of incubation. A 24-hour incubation of the cell lines PC3 and DU145 after 24 h with NAN-190 and methiothepine mesylate at a concentration of >10^{-5} M induced dramatic cell death. In the same experiments using the 5HTR1a and -1b agonists (±)-8-OH-DPAT and CGS-12066A, maleate salt did not affect cellular viability (data not shown).

**Effects of 5HTR1a/-1b Antagonists on Apoptosis**

In DU145 cells, significant apoptotic changes were observed after 24-hour incubation with NAN-190 at a concentration of 10^{-4} M. The changes were significant for early and late apoptosis. Incubation with NAN-190 did not induce apoptosis, but necrosis in PC3 cell culture was seen at a concentration of 10^{-4} M (fig. 5).

**Changes in Cell Cycle Phases after Incubation with 5HTR1a/-1b Antagonists**

Figure 6 summarizes the effects seen on cell cycle. In PC3 and DU145 cell lines, the highest proportion of cells which underwent G_0–G_1 phase arrest were detected after
24 h of incubation with the 5HTR1a antagonist (NAN-190) at a concentration of $10^{-4}$ M (fig. 6). Methiothepin caused no significant changes in cell cycle phases (data not shown).

**Effects of MLT on Cellular Viability**

Treatment of PC3, DU145 and 22RV1 cells with MLT showed time- and dose-dependent effects on cellular viability in supraphysiologic concentrations only (fig. 7). After 72 h of incubation with MLT in the concentration range $10^{-8}$ to $10^{-3}$ M, no inhibitory effects were observed (data not shown), while at supraphysiologic concentrations of MLT ($10^{-3}$ to $10^{-2}$ M) a marked concentration-dependent decrease in cellular viability was seen (fig. 7).

**Discussion**

PCa progression remains a complex process which still evades complete understanding. Many biologic factors capable of influencing or regulating cell growth have been implicated and probably are involved [24, 25]. MLT has been shown to have an influence on cancer cell growth in several cancer entities including PCa cells growing in vitro [26, 27]. There may even be potential therapeutic implications for MLT [28]. All these reasons make MLT a substance of interest in PCa research, and we therefore undertook these experiments on MLT receptors in PCa cell lines. We found an expression of both 5HTR subtypes 5HTR1a and 5HTR1b in the four PCa cell lines used (PC3, DU145, DU145, PC3, and 22RV1).

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**Fig. 5.** Effects of NAN-190 on apoptosis after 24-hour incubation in DU145 (a) and PC3 (b).
Fig. 6. Cell cycle arrest in G_0/G_1 in DU145 (a) and PC3 (b) cells after 24-hour incubation with NAN-190. Percentages of cells in the different phases of the cell cycle (G_0/G_1, S, G_2/M) are shown. Control refers to incubation with DMSO.

Fig. 7. Cellular viability test in PC3, DU145 and 22RV1 after 72-hour incubation with MLT in a concentration range of $10^{-3}$ to $10^{-2}$ M.
Role in PCa Cell Line Growth

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LNCaP and 22RV1) as well as in the BPH-1 line and human fibroblasts. The relatively low expression of 5HTR1a and 5HTR1b receptors observed, however, may signify that in nonstimulated PCa cells the 5HTR1a/-1b expression at mRNA level does not play a significant role in regulation of growth. Reports in the literature [5] described the qualitative (detected by PCR) and immunohistochemical features of 5HTR1a/-1b, but their functional activity may well depend on the quantitative properties.

In experiments with 5-HT stimulation, we found that the stimulatory effect was dose-dependent at 10^{-8} to 10^{-4} M during the first 24 h and highest at 10^{-4} M. The data from the Malmö group described a stimulatory effect of 5-HT on proliferation in DU145 and PC3 cells at 10^{-8} M with no significant effect on LNCaP cells [5]. These and other authors describe 5-HT as a mitogen in vitro [5, 11, 12]. It is possible that with low 5HTR1a/-1b expression and high concentrations of 5-HT, the viability and proliferation effects may be mediated by other types of serotonin receptors as other types of serotonin receptors in PCa cell lines have been identified (5HTR2b, 5HTR4) with a putative role in proliferation and differentiation [6].

Our results with 5HTR1a/-1b agonists did not show any effect of selective 5HTR1a/-1b stimulation on physiological concentrations. This fact may also support the low functional activity of 5HTR1a/-1b receptor subtypes. The inhibition experiments showed physiological effects on cellular viability in the concentration range 10^{-8} to 10^{-4} M, and only cytotoxic effects of methiothepin at 10^{-4} M. Our data suggest that inhibition of 5HTR1a/-1b in physiologic concentrations does not affect cellular viability. In contrast to our findings, other authors [5] have reported that 5HTR1a inhibition in DU145, PC3 and LNCaP cells was concentration-dependent and maximal at 10^{-6} M. However, neither those nor our results support a prominent functional role of 5HTR1a/-1b or 5-HT stimulation in cell viability in PCa cell lines.

In agreement with others, we did find a time- and dose-dependent effect of MLT on cellular viability in PC3, DU145 and 22RV1 [22, 23]. In contrast to a hypothesis reported in the literature, this effect in our observation did not seem to be related to hormone dependency of the cell lines [19, 20, 26]. Reported maximum MLT inhibitory activity was at high concentrations of 10^{-3} to 10^{-2} M after 72 h of continuous incubation [26], making MLT a potent inhibitor in vitro only in mM concentrations with effects only after prolonged incubation. Our data correspond to these reported findings concerning concentration range and time effect as well as more effective inhibition in moderate cellular density (3,000 cells/well) [26]. The findings thus somewhat characterize MLT as an antiproliferative agent in PCa cell lines, but may cast doubt on the hypothesis that this effect is highly specific and of great biological significance in PCa.

Similarly, while a putative role of MLT in apoptosis is still controversial, we have observed an apoptotic effect of MLT in DU145 and PC3 only at concentrations of 10^{-4} M. Since MLT as a pineal hormone exerts its biological effect in vivo at nM concentrations [17, 18], the observed apoptotic effect in cell culture is highly unlikely to be specific.

Changes in cell cycle due to G_0/G_1 phase arrest were described as one of several possible mechanisms in the regulation of proliferation [4]. We observed a G_0/G_1 phase arrest induced by the 5HTR1a receptor antagonist NAN-190 after 24 h of incubation at 10^{-4} M in PC3 and DU145 cells. Again, the concentration at which this effect was observed does not support a biological significance.

Conclusions

Our results do not support the hypothesis that activation or inhibition of the serotonin receptor subtypes 5HTR1a or 5HTR1b plays a prominent role in the control of growth and viability of PCa cell lines. Inhibitory effects of MLT in PCa cell lines in vitro occur at supraphysiological concentrations only. Thus, neither MLT nor the serotonin receptor subtypes 5HTR1a and 5HTR1b seem to have an important role in the in vitro growth regulation of human PCa cell lines.

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