Serotonin Regulates Macrophage-Mediated Angiogenesis in a Mouse Model of Colon Cancer Allografts

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Abstract

Serotonin, a neurotransmitter with numerous functions in the central nervous system (CNS), is emerging as an important signaling molecule in biological processes outside of the CNS. Recent advances have implicated serotonin as a regulator of inflammation, proliferation, regeneration, and repair. The role of serotonin in tumor biology in vivo has not been elucidated. Using a genetic model of serotonin deficiency (Tph1+/−) in mice, we show serotonin to be crucial for the growth of s.c. colon cancer allografts in vivo. Serotonin does not enhance tumor cell proliferation but acts as a regulator of angiogenesis by reducing the expression of matrix metalloproteinase 12 (MMP-12) in tumor-infiltrating macrophages, entailing lower levels of angiostatin—an endogenous inhibitor of angiogenesis. Accordingly, serotonin deficiency causes slower growth of s.c. tumors by reducing vascularity, thus increasing hypoxia and spontaneous necrosis. The biological relevance of these effects is underscored by the reconstitution of serotonin synthesis in Tph1+/− mice, which restores allograft phenotype in all aspects. In conclusion, we show how inflammation, proliferation, regeneration, and repair. The role of serotonin in tumor biology in vivo has not been elucidated. Using a genetic model of serotonin deficiency (Tph1+/−) in mice, we show serotonin to be crucial for the growth of s.c. colon cancer allografts in vivo. Serotonin does not enhance tumor cell proliferation but acts as a regulator of angiogenesis by reducing the expression of matrix metalloproteinase 12 (MMP-12) in tumor-infiltrating macrophages, entailing lower levels of angiostatin—an endogenous inhibitor of angiogenesis. Accordingly, serotonin deficiency causes slower growth of s.c. tumors by reducing vascularity, thus increasing hypoxia and spontaneous necrosis. The biological relevance of these effects is underscored by the reconstitution of serotonin synthesis in Tph1+/− mice, which restores allograft phenotype in all aspects. In conclusion, we show how serotonin regulates angiogenesis in s.c. colon cancer allografts by influencing MMP-12 expression in tumor-infiltrating macrophages, thereby affecting the production of circulating angiostatin. [Cancer Res 2008;68(13):5152–8]

Introduction

Serotonin is a biogenic amine widely appreciated as a neurotransmitter with numerous functions in the central nervous system (CNS). In addition to its classic functions in intestinal motility, fluid secretion, and blood flow (1), serotonin is emerging as a key mediator of different biological processes in peripheral organ systems. Serotonin confers its biological functions in a receptor-dependent (2) or receptor-independent manner (3). To date, 7 classes including 14 subtypes of serotonin receptors (HTR) have been identified (4). Depending on the receptor involved, serotonin can act as a cellular mitogen, e.g., in fibroblasts (5), smooth muscle (6), and endothelial cells (7). Recent advances have implicated serotonin as a regulator of inflammation (8), proliferation (9), regeneration (2), and repair (10).

Colorectal cancer remains the third most common malignancy in Western societies, predicted to cause 50,000 deaths during 2006 in the US (11). Although chemotherapeutic compounds and protocols have seen steady improvements over the last decades, chemotherapy is still fraught with side effects and long-term treatment failure. This inadequacy drives continuing research to identify new classes of antineoplastic compounds, such as inhibitors of angiogenesis (12).

The few experimental studies to have analyzed the role of serotonin in tumor biology were limited to in vitro analyses of mitogenic effects on malignant cells, such as lung (13) or head and neck cancer (14). Accordingly, antagonists for serotonin receptors 1A and 1B reduced the proliferation rate of bladder cancer cells in vitro (15). However, in vivo studies investigating the function of serotonin in cancer growth and progression are lacking.

Interestingly, a recent population-based study showed a high intake of selective serotonin reuptake inhibitors (SSRI) to correlate with a reduced incidence of colorectal cancer (16). This observation suggests a biological relevance of serotonin for colorectal cancer growth in vivo. On these grounds, we decided to investigate the function of serotonin in the growth of colon cancer, using a unique genetic model of peripheral serotonin deficiency in mice.

Materials and Methods

Cell culture and in vitro experiments. The mouse colon cancer cell line MC-38, generated in C57BL/6 mice (17), was a kind gift of Carole Bourquin (Ludwig-Maximilian University of Munich, Germany). The CT-26 (mouse colon carcinoma) and LLC1 (mouse Lewis lung carcinoma) cell lines were purchased from American Type Culture Collection. Mouse peritoneal macrophages were isolated by peritoneal lavage with 0.9% NaCl and subsequent centrifugation at 200 g. MC-38, LLC1, and peritoneal macrophages were cultured in Dulbecco’s Minimal Essential Medium (Invitrogen), whereas CT-26 were cultured in RPMI Medium (Invitrogen). Culture medium were supplemented with 10% fetal bovine serum (PAA Laboratories), with the addition of 100 units/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen) for MC-38, CT-26, and LLC1. Cells were maintained at 37°C in a 5% CO2 atmosphere. Cells were seeded into 12-well plates at a density of ~50% corresponding to 5 × 105 cells per well and allowed to adhere overnight, before the medium was changed to the specified conditions, containing different concentrations of serotonin creatinine complex (Sigma Aldrich). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed as previously described (18).

Animal experiments. All animal experiments were in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Mice ages 8 to 12 wk were kept on a 12-h day/night cycle with free access to food and water. Strains used were C57BL/6 (Harlan) and Tph1−/− on a C57BL/6 background (own breeding). As previously described (19), these mice have a disrupted gene for tryptophan hydroxylase 1 and therefore lack serotonin outside of the CNS. For tumor cell inoculations, MC-38 and LLC1 cultured at exponential growth rate were trypsinized (Invitrogen), washed in PBS, and resuspended in serum-free medium at a concentration of 2.5 × 106 × mL−1, before injecting 500,000 cells s.c.
growth was monitored daily with a sliding caliper and the volume was calculated according to the ellipsoid formula \( \frac{4}{3} \pi \times \frac{1}{2} \times w/2 \times w/2 \), where \( l \) is the length and \( w \) the width. Serotonin receptor agonists and antagonists were obtained from Sigma Aldrich and administered once daily by s.c. injection in 200 \( \mu \)l NaCl commencing 2 d before tumor inoculation, whereas controls received NaCl. Substances used were 2.5-Dimethoxy-4-iodoamphetamine (DOI; 1 and 10 mg/kg), methysergide (3 and 30 mg/kg), quipazine (5 and 30 mg/kg), and ketanserine (3 and 30 mg/kg). Reloading with the serotonin precursor 5-hydroxytryptophan (Sigma Aldrich) was performed by twice daily s.c. injections of 50 mg/kg in 200 \( \mu \)l NaCl commencing 2 d before tumor inoculation, whereas controls received NaCl.

**Histologic examination.** Tumor tissue was immersion-fixed in 4% PBS-buffered formalin, embedded in paraffin, sectioned, and stained with H&E using standard histologic techniques. In addition, slides were immunostained for Ki-67 (monoclonal rabbit clone SP6; NeoMarkers), CD31 (goat polyclonal; M20; Santa Cruz Biotechnology), GLUT-1 (rabbit polyclonal; MVM AB 1351; Chemicon International), and F4/80 (rat monoclonal; T-2006; BMA Biomedicals). Ki-67 and GLUT-1 (20) staining procedures were performed on a Benchmark immunohistochemistry staining system (Ventana Medical Systems). For CD31 staining, detection of primary antibody was performed with a Histofine staining kit (Nichirei Corporation) and 3',3'-diaminobenzidine as a chromogen. All immunostains were counterstained with hematoxylin. Necrotic tumor area was assessed on one transection per tumor (magnification, \( \times 40 \)) by two investigators blinded with respect to the experimental group. Mitotic figures and Ki-67 positive nuclei were quantified as previously described (21).

**Quantitative real-time PCR.** Total RNA was extracted from 50 mg of tumor tissue using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA extraction from peritoneal macrophages was performed using the RNasey MinElute Cleanup kit (Qiagen) following the manufacturer's instructions. Five micrograms of RNA were reverse transcribed using Thermoscript reverse transcription-PCR (RT-PCR) System (Invitrogen) yielding the cDNA template. Quantitative real-time PCR amplification and data analysis were performed using an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems). Taq Man gene expression assays (PE Applied Biosystems) for Htr1a (Mm00434106_s1), Htr1b (Mm00439577_s1), Htr2a (Mm00555764_m1), and Htr2c (Mm00434127_s1) were used to quantify mRNA expression of the respective genes. Messenger RNA expression levels of each sample were normalized to 18S RNA (Taq Man RNA control reagents; PE Applied Biosystems). Results of in vitro experiments represent fold induction of mRNA compared with tumors of wild-type animals at 7 d, whereas results of in vivo experiments represent fold induction of mRNA compared with untreated macrophages.

**Western blotting.** Tumor protein extracts or serum samples were diluted in sample buffer [187.5 mmol/L Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mmol/L DTT, and 0.3% bromophenol blue] and boiled for 10 min at 90°C. One hundred micrograms of protein were loaded, SDS-PAGE was performed, and samples were blotted onto a polyvinylide difluoride membrane. Primary antibodies were rabbit polyclonal anti-matrix metalloproteinase (MMP) 12 (ab39876; Abcam), rabbit polyclonal anti-angiostatin (NB 300-544; Novus Biologicals), and rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9485; Abcam). Secondary staining and detection was performed according to standard protocols with the ECL detection reagent (GE Healthcare Ltd).

**Statistical analysis.** Data are given as mean \( \pm \) SD or mean \( \pm \) 95% confidence interval (CI) as indicated. Student’s t test was used to compare means in experiments with two groups. ANOVA with Tukey’s HSD for post hoc comparisons was used to compare means in experiments with three groups. The level of statistical significance was set at below 0.05. Statistical analyses were performed with SPSS 14.0 (SPSS, Inc.).

**Results**

Serotonin deficiency decreases the growth of colon cancer allografts *in vivo*. We first investigated the effect of serotonin on the proliferation of the two different mouse colon cancer cell lines CT-26 and MC-38 (Supplementary Fig. S1). Dose escalation over six log scales showed a marked increase of proliferative activity at serotonin concentrations of 0.01 and 0.1 mmol/L. To establish whether serotonin affects colon cancer cell proliferation *in vivo*, we used Tph1/−/− mice, which have a disrupted gene for tryptophan hydroxylase 1 and, therefore, lack serotonin outside the CNS (19), and the MC-38 colon cancer cell line, which is syngeneic with the C57BL/6 background (17). In mice lacking peripheral serotonin, the establishment and early growth of s.c. tumor allografts up to 6 days after injection was the same as in controls. However, allograft growth from day 7 onwards was significantly slower in Tph1/−/− mice, leading to significantly lower tumor volume after 14 d (Fig. 1A). This implicates serotonin as a key mediator of the growth of colon carcinoma allografts *in vivo*.

**Reconstitution of serotonin synthesis restores tumor allograft growth.** To substantiate whether the impaired tumor allograft growth in Tph1/−/− mice was in fact caused by the lack of serotonin, we restored serotonin synthesis in Tph1/−/− mice by the administration of 5-hydroxy-tryptophan, the product of TPH1 (reloading). As shown in Fig. 1B, reloading of serotonin-deficient animals restored tumor allograft growth to the levels observed in wild-type animals. We then assessed whether the growth-stimulating effect of serotonin is mediated by a receptor-dependent pathway. Serotonin receptors 1A, 1B, 2A, 2B, and 2C have been shown to influence proliferative activity outside the CNS (2, 9). Therefore we first analyzed the role of serotonin receptors *in vivo* with well-described serotonin receptor agonists (methysergide for HTR1; DOI for HTR2), with the aim to “rescue” tumor growth in Tph1/−/− animals. As shown in Fig. 1C, neither methysergide nor DOI increased tumor growth. As a next step, serotonin receptor antagonists targeting the same receptors were evaluated for their potential to decrease tumor growth in wild-type mice (quipazine for SHT1; ketanserine for HTR2). Again, neither substance showed a significant effect on tumor allograft growth (Fig. 1D). Because quipazine and ketanserine seemed to slightly decrease tumor allograft growth, we repeated all antagonist and agonist experiments with a 10-fold dosage increase. Even at this dose, neither substance significantly affected tumor allograft growth (data not shown). Our results implicate serotonin as a mediator of tumor growth in s.c. colon cancer grafts via a receptor-independent mechanism.

**Serotonin deficiency reduces angiogenesis in s.c. colon cancer allografts.** As serotonin increased tumor cell proliferation *in vitro*, proliferative activity was assessed in our model of colon cancer. Neither the mitotic index (data not shown) nor Ki67 labeling index (Fig. 2A) showed any significant difference between tumors allografts growing in wild-type, Tph1/−/−, and reloaded animals. Interestingly, on H&E-stained sections, we observed a prominent degree of tumor necroses in Tph1/−/− animals. Quantification of necrotic tumor area revealed significantly larger areas of necroses in Tph1/−/− mice, which was fully reversible when serotonin synthesis was reconstituted (Fig. 2B). To test whether the higher level of spontaneous tumor necroses in Tph1/−/− mice was associated with tumor hypoxia, we performed GLUT1 immunostaining. In line with increased necrosis, tumors growing in serotonin-deficient mice displayed strikingly more hypoxic tumor area (Fig. 2D) than tumors growing in wild-type animals (Fig. 2C).
The reduced oxygen status in these tumors prompted us to investigate whether this was related to insufficient tumor vascularization in mice lacking peripheral serotonin. Indeed, serotonin deficiency entailed a significant reduction of microvascular density as assessed by CD31 immunostaining compared with wild-type animals (Fig. 3). Of note, reloading ameliorated tumor vascularization. These experiments provide evidence that serotonin modulates tumor allograft growth in vivo by regulating tumor vascularization.

Serotonin deficiency reduces angiogenesis by MMP-12 up-regulation and consecutive angiostatin activation. To further elucidate the mechanism of differential growth between allografts growing with and without serotonin, we stained for possible infiltrating inflammatory cells. Although neutrophils as well as B and T lymphocytes were scant (data not shown), macrophages were found to be the predominant infiltrating cell population (Fig. 4). Tumor-infiltrating macrophages are known to secrete MMP-12 (22), a key enzyme for the conversion of plasminogen to angiostatin, which acts as an endogenous inhibitor of angiogenesis. Allograft transcript levels of all MMPs able to cleave plasminogen into angiostatin (MMP-2, MMP-7, MMP-9, and MMP-12) were analyzed by RT-PCR. Transcripts for MMP-2, MMP-7, and MMP-9 were barely detectable in allografts, whereas MMP-12 expression was well-detectable in all tumor-bearing mice, in line with the presence of tumor-infiltrating macrophages. To rule out an epiphenomenon, we also investigated transcript levels of vascular endothelial growth factor (VEGF) and VEGF receptor, which were not different. Transcript levels of MMP-12 were significantly higher in tumors allografts growing in serotonin-deficient mice at 7 and 14 days after tumor cell injection, whereas serotonin reloading reduced MMP-12 expression to wild-type levels (Fig. 4B). Because this effect was not due to a difference in the quantity of tumor infiltrating macrophages (Fig. 4C), MMP-12 expression was analyzed in peritoneal macrophages in vitro in response to serotonin exposure. Interestingly, serotonin suppressed the expression of MMP-12 by >25% at nanomolar concentrations compared with baseline (Fig. 4D).

To test whether serotonin would also affect protein levels of MMP-12 in vivo, we performed Western blot analyses of tumor allografts growing in Tph1−/− and wild-type mice using a commercially available antibody directed against proMMP-12. As indicated in Fig. 5A, protein levels of proMMP-12 were significantly lower in allografts of serotonin-deficient animals, pointing to a higher conversion into the active form. Again, reloading restituted the wild-type phenotype. Higher MMP-12 transcripts paired with lower protein proform levels point to the presence of more active MMP-12. MMP-12 mediates the cleavage of circulating plasminogen into angiostatin. As illustrated in Fig. 5B, higher MMP-12 activity in tumor-bearing Tph1−/− animals led to higher levels of circulating angiostatin, which could be restored to wild-type levels by reloading. Of note, baseline levels of naïve wild-type and Tph1−/− were not different. These results suggest serotonin to
confer its effects on colon cancer growth through a regulation of MMP-12/angiostatin pathway.

Serotonin deficiency decreases Lewis lung cancer allograft growth in vivo by reducing angiogenesis. The previous in vivo experiments were conducted in a model of s.c. colon cancer allografts. To rule out a cell line–specific effect of serotonin on tumor allograft growth, we repeated the growth experiments using the Lewis lung carcinoma. This cell line was chosen because its growth in vivo is strongly regulated by macrophage-derived MMP12 signaling (23). As shown in Fig. 6A, Lewis lung carcinoma allografts grew significantly slower in mice lacking peripheral serotonin. In line with the observed effects in colon cancer, Lewis lung cancer allografts growing in Tph1−/− mice showed significantly higher degree of tumor necrosis as well as significantly lower microvascular density (Fig. 6B and C). Taken together, serotonin emerges as a positive regulator of angiogenesis in models of s.c. tumor growth by suppressing MMP-12 expression in macrophages, thus inhibiting the production of angiostatin.

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Serotonin Regulates Angiogenesis in Colon Cancer

Figure 2. Tumor proliferation, necrosis, and hypoxia. MC-38 cells were injected s.c. and harvested after 14 d. A, Ki67 labeling index showed no significant difference between tumors allografts growing in wild-type (open column), Tph1−/− (black column), and reloaded Tph1−/− mice (gray column). B, necrotic tumor surface area was significantly higher in tumor allografts growing in Tph1−/− mice (black column) compared with wild-type (open column); *, P = 0.020. Reloading of Tph1−/− animals with 5-hydroxy-tryptophan (gray column) restored tumor necrosis to wild-type levels. Columns, represent mean of 10 animals; bars, SD. Hypoxic tumor area was illustrated by GLUT-1 immunostaining of tumor allografts growing in wild-type (C) and Tph1−/− animal (D).

Figure 3. Microvascular density in s.c. colon cancer allografts. Microvascular density was assessed by CD31 immunostaining. A, representative microphotograph of a tumor allograft growing in a wild-type animal. B, representative microphotograph of a tumor allograft growing in a Tph1−/− mouse. C, microvascular density was significantly lower in tumor allografts growing in Tph1−/− animals (black column) compared with wild-type (open column); *, P < 0.001. Reloading of Tph1−/− animals with 5-hydroxy-tryptophan (gray column) ameliorated microvascular density. Columns, mean of 10 animals; bars, SD.
Discussion

This study unveils a novel function of serotonin as a mediator of angiogenesis in colon cancer allografts. Using a unique genetic model of peripheral serotonin deficiency, we provide evidence that the lack of serotonin causes a reduction of tumor growth by the up-regulation of MMP-12 in macrophages, leading to higher levels of circulating angiostatin, an established endogenous inhibitor of angiogenesis.

Serotonin was previously shown to be mitogenic in different cancer cell lines (13–15). In line with these reports, colon cancer cell lines CT-26 and MC-38 showed increased proliferative activity when exposed to serotonin in vitro. We therefore hypothesized that tumors growing in mice lacking peripheral serotonin would display reduced growth due to a lack of serotonin-induced proliferation. Although tumor growth was significantly impaired in Tph1−/− animals, this effect could not be explained by a reduction of proliferative activity. Of note, tumor engraftment and early growth (up to 6 days) were identical, speaking against an influence of minor strain differences or immunologic factors. Autocrine stimulation was excluded by negative serotonin immunostaining of tumors (data not shown). Additionally, specific effects related to the heterotopic tumor localization are unlikely as serotonin is predominantly stored and distributed to all organ systems in platelets (2, 10). However, the widely used model of s.c. colon cancer allografts does not reproduce all aspects of tumor biology.

Figure 4. Tumor-infiltrating macrophages and MMP-12 expression. A, representative microphotograph of a tumor allograft growing in a wild-type animal. Tumor-infiltrating macrophages were stained by F4/80 immunostaining. B, MMP-12 transcript levels in s.c. tumor allografts assessed by RT-PCR. MMP-12 expression was significantly higher in tumors allografts growing in Tph1−/− mice at both time points compared with wild-type (*, P = 0.032; **, P = 0.023). C, quantification of F4/80-positive cells in s.c. tumor allografts. Columns, mean of 10 animals; bars, SD. D, expression of MMP-12 in peritoneal macrophages exposed to serotonin in vitro. Points, mean of triplicate experiments; bars, SD.

Figure 5. Protein levels of proMMP-12 and angiostatin. A, Western analysis of proMMP-12 in s.c. tumor allografts. Extract of JEG3 cells was used as positive control (pos. ctrl), whereas levels of GAPDH served as loading control. B, Western analysis of angiostatin K1–3 (top) and K1–4 (lower) are indicated in plasma of tumor-bearing mice. Recombinant human angiostatin, containing kringle regions 1 to 3, was used as positive control.
In contrast to our previous work in liver regeneration (2), serotonin-derived effects on tumor growth could not be attributed to the activation of serotonin receptors 5HT1R or 5HT2R. It is conceivable but unlikely that another member of the serotonin receptor family is involved in this process because those receptors have almost exclusively been described in the CNS. A receptor-independent pathway (3) is the most likely mechanism. This might also offer an explanation for the reduction of human colorectal cancer incidence by SSRIs (16), as these reduce the uptake of serotonin into colonic enterocytes.

Decreased tumor growth in Tph1−/− mice could not be explained by reduced tumor cell proliferation. Histologic analyses revealed dramatically larger areas of spontaneous necroses and tissue hypoxia in mice lacking peripheral serotonin, which could be attributed to diminished tumor vascularity pointing to a disturbed angiogenesis in these animals. This explains the apparently divergent effects of serotonin in vitro and in vivo because tumor growth in vivo is not only affected by tumor cell proliferation but also by stroma-related factors such as angiogenesis. In our model, these stroma-related effects of serotonin seem to be more important than the direct mitogenic actions.

Tumor-infiltrating macrophages are associated with tumor progression and metastasis. They were reported to regulate angiogenesis in breast (24) as well as lung cancer and melanoma (23). Macrophages are known to specifically secrete MMP-12, which leads to enhanced generation of angiostatin (22) and thereby suppresses tumor growth by halting angiogenesis (23, 25). Interestingly, transfection of the Mmp-12 gene into the colon cancer cell line CT-26 suppressed tumor angiogenesis and tumor growth (26). As macrophages were the predominant infiltrating cells in our experiments and their number did not differ between groups, we asked whether there might be functional differences. Indeed, mRNA levels of MMP-12 were higher in serotonin-deficient tumor allografts, whereas protein levels of proMMP-12 were lower. Both alterations could be restored by reloading. These data suggest serotonin to suppress MMP-12 production in macrophages, which could be confirmed by exposing peritoneal macrophages to different concentrations of serotonin.

MMP-12 cleaves plasminogen into angiostatin, which suppresses angiogenesis in solid tumors. In line with previous reports (23, 27), enhanced transcription and activation of MMP-12 observed in tumors of serotonin-deficient mice entailed higher levels of circulating angiostatin, causing a reduction of tumor vascularity, enhanced hypoxia, and consequently, tumor necrosis. Serotonin-dependent effects were reproducible in Lewis lung cancer, in accordance with previous studies showing supranormal serotonin levels to enhance lung cancer proliferation (13, 28) and macrophage-derived MMP-12 to regulate Lewis lung cancer growth (23, 27).

In conclusion, we show how serotonin regulates angiogenesis in s.c. colon cancer allografts by influencing MMP-12 expression in tumor-infiltrating macrophages, thereby affecting the production of circulating angiostatin. Hence, serotonin might represent a novel target for the prevention and treatment of colon cancer, especially as numerous safe and effective serotonin-targeting drugs are in clinical use today.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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