SPANDIDOS PUBLICATIONS

Incubation with somatostatin, 5-aza decitabine and trichostatin up-regulates somatostatin receptor expression in prostate cancer cells

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Abstract. Somatostatin (SMS), binds to its specific receptors (SSTRs) and transduces growth inhibitory, anti-secretory and apoptotic signals. Several human cancers express SSTRs, including prostate cancer, and therefore SMS is of interest for anti-cancer therapy. DNA methylation and histone modifications are involved in normal cell development, gene imprinting and human carcinogenesis. Reversing DNA methylation is an attractive therapeutic possibility, since epigenetic modifications change gene expression without changing the gene function. DNA methylation inhibitors such as 5-aza-2'-deoxycytidine (5'-aza, decitabine) have been used to treat several types of haematological malignancies. Histone deacetylase inhibitors such as trichostatin (TSA), are a new class of 'targeted anti-cancer agents'. TSA and decitabine can induce growth arrest, apoptosis or terminal differentiation in a variety of solid and haematological cancers in advanced disease patients. In the present study, the LNCaP cell line (prostate cancer) was incubated with SMS or Somadex (an SMS polymer conjugate) for three days, 1 nM per day, and the untreated cells were the negative control. For DNA demethylation, cells were grown in the presence of 2.5 μM 5-aza for 120 h, and re-fed with 5-aza-containing fresh medium at day 3. The total incubation time with 5-aza was 120 h. TSA at 1.0 μM was added into the cultured cells for 24 h. The combined treatment of 5-aza and TSA was performed by incubating the cells with 5-aza for 120 h followed by a 24-h exposure to TSA. Using cDNA obtained from these cell lines, the difference in the expression level of SSTR mRNA transcripts before and after 5-aza and TSA treatments was analyzed by RT-PCR. An increased induction of mRNA expression of the five SSTR subtypes was observed in the LNCaP cells when incubated with SMS/Somadex (dose-dependent). The inhibition of DNA methylation and histone acetylation resulted in the up-regulation of SSTR5 mRNA expression. The results demonstrate a positive feedback loop between SMS and its receptors. This regulation pathway may enhance the anti-tumor activity of somatostatin. To benefit from this effect in a clinical setting, the dose, dose frequency and pan affinity of the SMS derivative are important factors. The epigenetic manipulation with DNA methylation or histone deacetylase inhibitors, combined with SMS, may offer a novel alternative for the treatment of advanced prostate cancer.

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Introduction

Somatostatin (SMS), originally identified as a neuropeptide inhibiting growth hormone release >30 years ago, is widely present in central and peripheral human cells/tissues including prostate (1-5). Biologically active forms of SMS include sms14 and sms28, both of which are derived from a common precursor through tissue-specific proteolytic processing. In addition to being a neuropeptide with multiple physiological activities, SMS has been shown to exert a potent anti-tumor action by affecting tumor cell proliferation, apoptosis, angiogenesis and the host's immune response (1-6).

Cellular functions of SMS are induced through binding to G-protein-coupled plasma membrane receptors SSTR1, 2A, 2B, 3, 4 and 5 (1-5). These six somatostatin receptors (SSTRs), although encoded by five different genes located on different chromosomes, exhibit significant sequence homology (40-60%). It has been well characterized that most carcinomas express SSTRs with variable abundance of subtypes. For instance, prostate cancer cells have SSTR1-3 expression while lacking SSTR4 and 5 transcripts (5,7). Similarly, the inhibitory effect of SMS analogs on prostate cancer has been observed *in vivo* and *in vitro* (1,2,8,9). In addition, given the observation that most prostate tumors express SSTRs, radiolabeled octreotide (the SMS analog) scintigraphy has been applied to localize prostate cancer metastases (2,10).

A more abundant expression of SSTRs on prostate cancer cells is likely to lead to a better response to SMS treatment. Therefore, knowledge on SSTR regulation/control can have important clinical implications on 'how to use' SMS as a treatment. To this end, we investigated SSTR expression on

the prostate cancer cell line LNCaP and its hormone-resistant variant LNCaP-r, looking for methods to up-regulate the SSTR expression.

The present study shows that SMS and its polymer conjugate, Somadex, significantly induce increased SSTR mRNA expression. Additionally, DNA demethylation and histone acetylation transactivated the SSTR5 gene.

Materials and methods

Cell lines, cell culture and reagents. The prostate cancer cell line, LNCaP, and its hormone-resistant variant (LNCaP-r), were maintained at 37˚C/5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and streptomycin). The reagents used in the study were: SMS (Ferring, Kiel, Germany); Somadex (Sigma, St. Louis, MO) was prepared as described previously (1); the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5'-aza) and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (Sigma). Prior to experiments, cells in the fresh medium were grown overnight and were ~40% confluent when the different chemicals/reagents were added. SMS and Somadex-treated cells were harvested at various time points. For DNA demethylation, cells were grown in the presence of 2.5 μM 5'-aza (Sigma), as described (11), and re-fed with 5'-aza-containing fresh medium at day 3. The total incubation time with 5'-aza was 120 h. TSA at 1.0 μM was added into the cultured cells for 24 h. The combined treatment of 5'-aza and TSA was performed by incubating the cells with 5'-aza for 120 h, followed by a 24-h exposure to TSA.

RNA extraction and RT-PCR. Total cellular RNA was extracted using TRIzol™ (Invitrogen, Carlsbad, CA) according to the manufacturers' instruction. cDNA was synthesized using random primers (N6) and MMLV-reverse transcriptase.

The PCR for SSTR mRNA was performed by using the following primer pair (Zatelli *et al*): SSTR1, forward: 5'-AGC CGG TTG ACT ATT ACG CC-3' and reverse: 5'-GCT CTC ACT TCT ACC ATT GTC-3'; SSTR2, forward: 5'-GGT GAA GTC CTC TGG AAT CC-3' and reverse: 5'-CCA TTG CCA GTA GAC AGA GC-3'; SSTR3, forward: 5'-TCA TCT GCC TCT GCT ACC TG-3' and reverse: 5'-GAG CCC AAA GAA GGC AGG CT-3'; SSTR4, forward: 5'-CGG CAG TCT TCG TGG TCT AC-3' and reverse: 5'-GCA TCA AGG TCG GTC ACG AC-3' and SSTR5, forward: 5'-AAC ACG CTG GTC ATC TAC GTG GT-3' and reverse: 5'-AGA CAC TGG TGA ACT GGT TGA C-3'. PCR conditions were as described by Zatelli *et al* (12) with minor modifications. In the present study, we used 36, 33, 35, 36 and 36 PCR cycles for SSTR1, 2, 3, 4 and 5, respectively. Moreover, ß-actin expression was used as a control for RNA loading and RT efficiency and amplified with its specific primers using 25 cycles. PCR products were resolved in 2% agarose gels, stained with ethidium bromide and visualized in UV light.

Results

SSTR mRNA expression in prostate cancer cell lines. A number of observations have demonstrated that primary and cultured prostate cancer cells express SSTRs with variable abundance

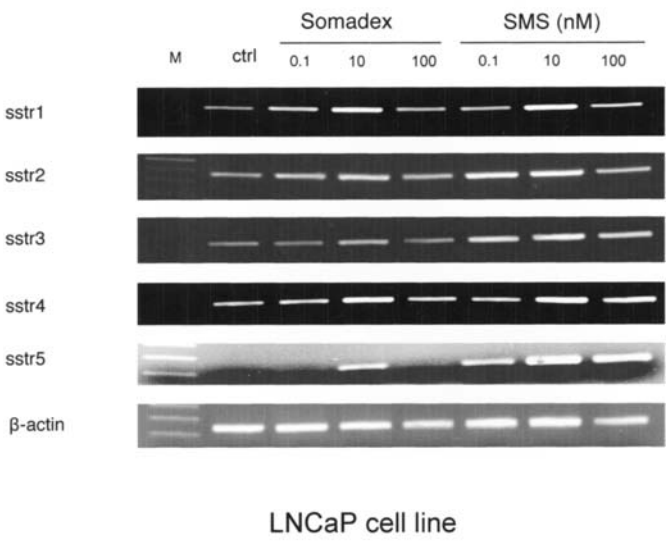

Figure 1. SMS and Somadex induce the up-regulation of SSTR mRNA expression in LNCaP cells. The cells were treated with either SMS or Somadex for 72 h and then harvested for the analyses of SSTR mRNA by using RT-PCR. M, molecular marker and C, control cells without SMS or Somadex treatment.

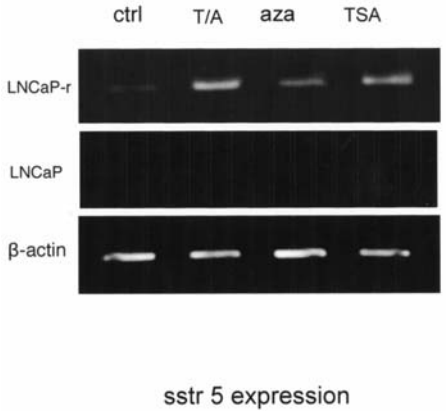

Figure 2. DNA methylation and histone deacetylase inhibitors stimulate SSTR5 mRNA expression in LNCaP-r cells. The cells were treated with 5'-aza (A), TSA (T) and A plus T, as indicated and then harvested for the analyses of SSTR5 mRNA expression by using RT-PCR.

of the subtypes (5,7,13,14). Consistent with these studies, we detected the presence of SSTR1-4 mRNA expression but no SSTR5 transcripts in LNCaP cells (Fig. 1). In DU145 cells, SSTR1-3 mRNA, but not SSTR4-5 mRNA, was detectable (data not shown). PC3 cells expressed SSTR1-3 and 5 (data not shown).

Up-regulation of SSTR mRNA expression by SMS and Somadex in prostate cancer LNCaP cells. Since the three cell lines have a similar expression profile of SSTR mRNA, we focused on the LNCaP cells and treated them with SMS and its analogue Somadex to see whether they affected their own receptor expression. The exposure of LNCaP cells to SMS or Somadex for 3 days significantly up-regulated SSTR1-4 mRNA expression (Fig. 1). Notably, SSTR5 mRNA, absent

ed cells, appeared after 3 days of incubation with somadex. Compared to other concentrations, SMS and Somadex at 10 nM induced the highest levels of SSTR mRNA, while SMS was more efficient (Fig. 1).

Transactivation of the SSTR5 gene by DNA methylation and histone deacetylase inhibitors in LNCaP cells resistant to hormone. As SSTR5 mRNA was undetectable in LNCaP cells, and epigenetic alterations frequently occur during tumor genesis and cancer progression (15-19), we explored whether the silent SSTR5 gene was due to abnormal epigenetic changes. To this end, the cells including LNCaP and its hormone-resistant sub-line LNCaP-r cells were incubated with the DNA methyltransferase inhibitor 5-aza at 2.5 μM, and histone deacetylase inhibitor TSA at 1 μM. Although 5-aza and TSA failed to stimulate SSTR5 expression in LNCaP cells (data not shown), treatment either with 5-aza or TSA led to a significant induction of SSTR5 mRNA in LNCaP-r cells, a hormone-resistant sub-line. The combination of the two compounds had an additive effect on the transcriptional activation of the gene (Fig. 2).

Discussion

SMS binds to SSTRs, inducing biological responses (1,3-5). The expression of SSTRs is a condition for direct cellular response to SMS. The direct cellular treatment effect of SMS on human malignancies, including prostate cancer, depends on the presence of functional SSTRs on the tumor cells (3-5). Primary and *in vitro* cultured prostate cancer cells have been shown to express SSTR1, 2 and 3 but lack SSTR4 and 5 (5,7,13,14). According to earlier observations, we found that SSTR1-4 were predominately expressed by LNCaP cells.

SMS has a high affinity to the five SSTRs and the biological response depends on which SSTR is activated (3-5). The activation of SSTR1, 2 and 5 results in cell cycle arrest while SSTR2 and 3 induce apoptosis (3,4). Thus, the subtypes of SSTRs present on the given cells determine their response to SMS, i.e. growth arrest, apoptosis or both. Given the expression of SSTR1 and 4 on LNCaP cells, SMS or Somadex treatment will theoretically lead to cell growth arrest and apoptosis. The major objective of the present study is to search for mechanisms that potentially up-regulate SSTR expression.

We found that there is a positive feedback loop between SMS/Somadex and SSTRs. Treatment with SMS/Somadex led to significant increases in SSTR 1-4 mRNA levels. The effect was clearly dose-dependent within 0.1-10 nM, and the strongest induction was seen at 10 nM for SMS and Somadex. Notably, SSTR5 mRNA, undetectable in untreated cells, was induced following cellular exposure to SMS/Somadex at 10 nM. It is reasonable to assume that the up-regulation of SSTR expression would increase the biological response to SMS therapy. It is currently unclear how SMS/Somadex induces increased receptor expression. It may be the result of alterations in SSTR biosynthesis. Presumably, such changes involve the promoter regions of SSTR genes, as well as transcriptional factors. In the clinical setting, enhanced treatment sensitivity as a result of increased SSTR expression, will depend on the dose, dose frequency and affinity profile of the SMS, as well as the duration of SMS treatment.

Epigenetic elements have been indicated in the regulation of gene expression. Dynamic DNA methylation and histone acetylation, are fundamental factors in controlling gene transactivation and repression (1,16-19). In general, DNA methylation and demethylation is associated with repressively and actively transcribed genes, respectively. Histone hypo-acetylation and hyperacetylation contribute to gene silence and activation, respectively. During tumorigenesis, aberrant DNA methylation frequently occurs, leading to the transcriptional inactivation of certain tumor suppression genes as exemplified by p16 (1,11,16-19). Moreover, a number of oncogenic proteins may directly or indirectly recruit histone deacetylases (HDACs) to specific genes, thereby silencing them by changing the histone acetylation status on the gene promoters. Herein we observed a strong induction of SSTR5 mRNA in LNCaP-r cells following their exposure to the DNA methylation and histone acetylation inhibitors, indicating the involvement of DNA methylation and histone acetylation in controlling SSTR5 expression.

In conclusion, the present study demonstrates that SSTR expression can be up-regulated by SMS/Somadex and by DNA methylation and HDAC inhibitors. It is reasonable to assume that this may increase the direct tumor cell sensitivity to SMS treatment, in addition to the effects of the DNA methylation/HDAC inhibitors (20-22). Therefore, epigenetic manipulation combined with SMS treatment, may offer a novel alternative for the treatment of advanced prostate cancer. The exact protocol for such combination therapy remains to be determined.

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