Human Somatostatin Receptor Mediated Antiproliferative Action Evokes Subtype Selective Cytotoxic and Cytostatic Signaling

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Somatostatin (SST)[b] exerts diverse biological actions through a family of five cell surface SST receptors (SSTR) subtypes. These can be categorized into two subgroups, one consisting of SSTRs 2,3 and 5 and the other made up of SSTRs 1 and 4 on the basis of their ability or inability to bind octa- and hexa- peptide SST analogs [1, 2]. SST influences multiple second messenger systems including adenylyl cyclase-cAMP, Ca^{2+}, K^+, protein tyrosine phosphatase (PTP), protein serine phosphatase, mitogen activated protein kinase and Na^+/H^+ antiporter, and exerts an inhibitory effect on hormone secretion, cell growth and proliferation (reviewed in [1-3]). SST-induced inhibition of cell proliferation can elicit either cytostatic (growth arrest) as well as cytotoxic (apoptosis) in tumor cells which express multiple SSTR subtypes [4-9]. We have shown that SSTR mediated cytotoxic signaling in MCF-7 breast cancer cells involves rapid recruitment of the src homology 2 domain containing tyrosine phosphatase SHP-1, is associated with the induction of the wild type (*wt*) tumor suppressor protein p53, the proapoptotic protein Bax and a cation-insensitive acidic endonuclease [9, 10]. The mechanism underlying its cytostatic signaling reported to occur in other tumor cells such as GH_3 cells has not been elucidated.

Molecular mediators that regulate cytotoxic and cytostatic events include the tumor suppressor protein p53, the cyclin dependent kinase inhibitor p21[WAF1/CIP1], the retinoblastoma protein pRB and the protooncogene product c-Myc. p53 can induce G_1 cell cycle arrest via p21[WAF1/CIP1] in the presence of growth factors or trigger apoptosis by inducing Bax in the absence of mitogenic signals [11-13]. Both these actions of p53 are dependent on its ability to acquire *wt* conformation in a manner requiring dephosphorylation of phosphoserine residues [14]. c-Myc, like p53, can also induce either G_1 arrest or apoptosis and moreover, can abrogate p21[WAF1/CIP1] induced G_1 arrest [15]. pRB in its hypophosphorylated form induces G_1 cell cycle arrest independent of p53 [16]. In order to determine if SST influences these mediators in a SSTR subtype selective manner to differentially regulate cell cycle progression and apoptosis we set out to elucidate human (h) SSTR subtype selectivity for cytotoxic and cytostatic signaling and demonstrated that SST-induced apoptosis is signaled uniquely via hSSTR3 whereas the other hSSTR subtypes signal G_1 cell cycle arrest without inducing apoptosis [17, 18]. Here I summarize current evidence demonstrating that SST elicits cytotoxic action uniquely via hSSTR3 in a p53 dependent manner whereas its cytostatic action is exerted through other subtypes involving induction of pRB in a p53-independent manner.

We first assessed the subtype selectivity for regulating cell cycle in CHO-K1 cells stably expressing individual hSSTRs. Cells were treated with 100 nM OCT (hSSTRs 2,3,5)

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[b] *Abbreviations:* SST, somatostatin; SST receptor, SSTR; *wt*, wild type; PTP, protein tyrosine phosphatase; h, human; PI, propidium iodide.

or D-Trp[8] SST-14 (hSSTRs 1,4) for 24 hr, the DNA was stained with the intercalating dye propidium iodide (PI) and analyzed by flow cytometry. The proliferative index was estimated from the ratio of populations of cells in G_1 and S phases (Figure 1). In cells expressing hSSTR3, OCT induced a decrease in G_1 and an increase in S phase indicating that it triggered cell cycle progression via this subtype. By contrast, cells expressing other SSTR subtypes responded with decreased cell proliferation, with the percentage of cells increasing in G_1 relative to S demonstrating that hSSTRs 1,2,4 and 5 signal G_1 cell cycle arrest. Such cytostatic signaling was associated with the induction of pRB (hSSTR5>hSSTR2>hSSTTR4>hSSTR1) (Figure 2). The cytotoxic signaling via hSSTR3 as well as the cytostatic signaling through the other hSSTRs was pertussis toxin sensitive G protein dependent and was mediated by orthovanadate sensitive PTP [17]

In CHO-K1 cells expressing hSSTR3 the antiproliferative action of OCT led to the induction of *wt* p53 and apoptosis. The activity of tumor suppressor protein p53 is regulated by Ser phosphorylation. p53 is believed to be in an inactive conformation in its phosphorylated form and assumes its active conformation upon dephosphorylation of Ser residues [14]. These forms can be distinguished by using epitope-specific anti-p53 antibodies pAb 240 and pAb 1801: only the inactive form of (or mutant) p53 is recognized by pAb 240, whereas both the inactive and active forms bind to pAb 1801. The results summarized in Figure 3 show that OCT induces a 10-fold increase in *wt* p53 in its active conformation detected with pAb 1801 in CHO-K1 cells expressing hSSTR3, but not other
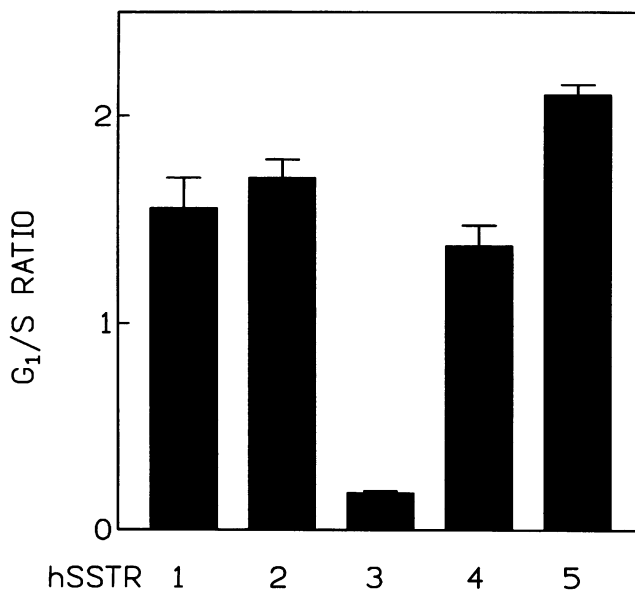

Figure 1. hSSTR subtype selective regulation of cell cycle progression in CHO-K1 cells. Proliferative index was calculated from ratio of cells in G1 and S phase in following treatment with 100 nM OCT (hSSTRs 2,3,5) or D-Trp[8] SST-14 (hSSTRs 1,4) for 24 hr. The change in G1/S ratio in the treated cells is expressed relative to the G1/S ratios of the corresponding untreated cells taken as 1. OCT treatment elicited selective proliferative response in hSSTR3 expressing cells as evidenced by the striking decrease in G1/ S ratio. By contrast, the increase in G1/S ratios in cells expressing other four hSSTRs indicates that peptide treatment elicits cytostatic response through these subtypes.
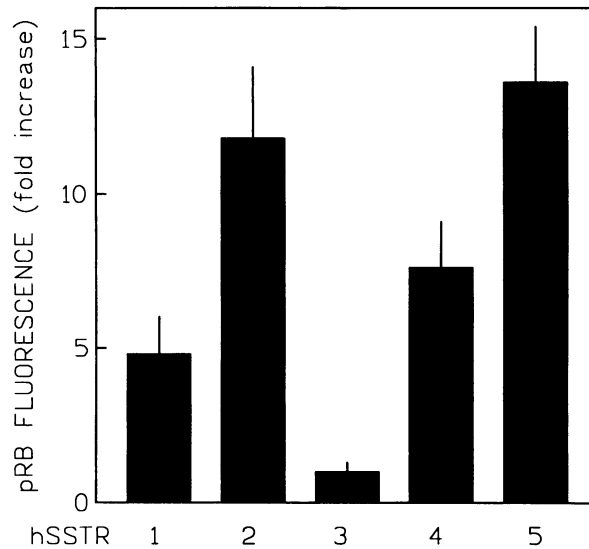
Figure 2. G1 cell cycle arrest signaled via hSSTRs 1,2,4 and 5 in CHO-K1 cells is associated with induction of pRB. Values represent increase in fluorescence intensity of pRB labeled with anti pRB antibody and FITC conjugated second antibody. Results are expressed as increase in fluorescence relative to that in the respective untreated cells taken as 1 (mean ± SE, n = 4).
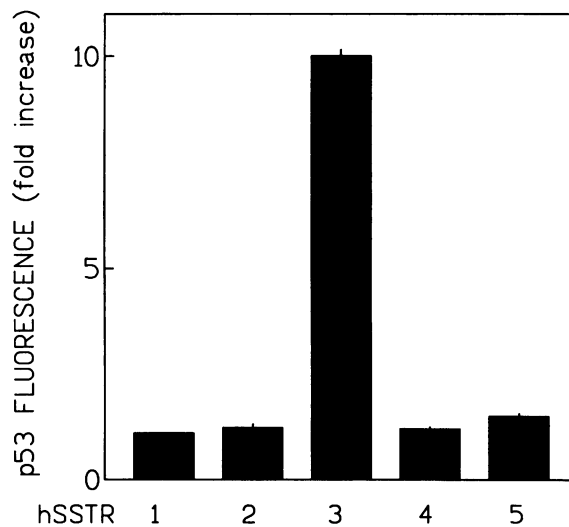

Figure 3. hSSTR subtype selective induction of *wt* p53. Increase in p53 detected by *wt* specific anti- p53 antibody pAb 1801 is selectively signaled via hSSTR3 in CHO-K1 cells. pAb 1801 immunoreactive p53 was counterstained with FITC conjugated second antibody and quantitated by flow cytometry. Results are expressed as increase in fluorescence relative to that in the respective untreated cells taken as 1 (mean ± SE, n = 4).
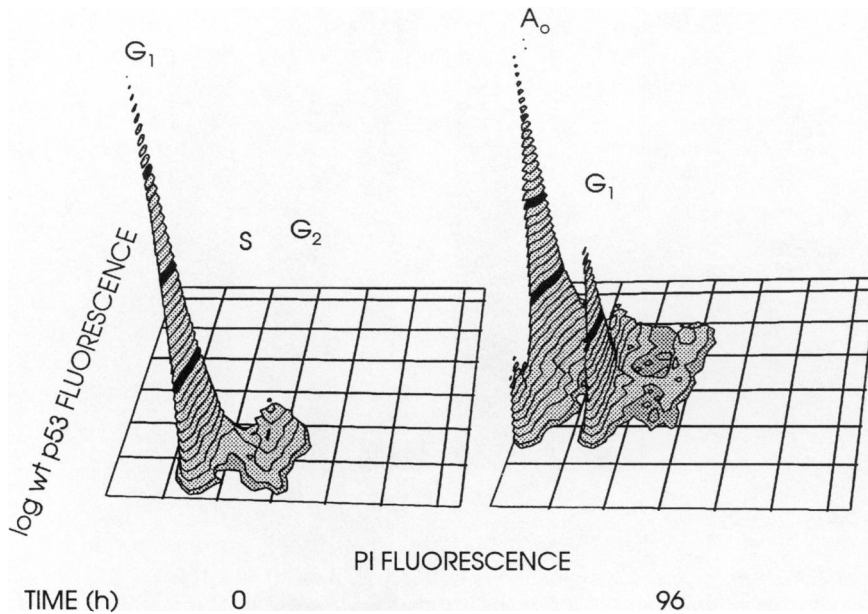
Figure 4. Bivariate analysis of hSSTR3 expressing CHO-K1 cells dual labeled with pAb 1801 (*wt* p53) and propidium iodide (DNA). OCT (100 nM) induced increase in *wt* p53 occurs in all phases of the cell cycle. Additionally, the correlation between induction of *wt* p53 and apoptosis is seen from the increase in this tumor suppressor protein in cells with hypodiploid DNA content in the A0 region.

hSSTRs. By contrast, no change was observed in p53 recognized by the mutant-specific anti-p53 antibody pAb 240 as described in detail previously by us [17].

Analysis of cells dual labeled with pAb 1801 and PI revealed that hSSTR3 signaled increase in *wt* p53 occurs in all phases of the cell cycle during treatment with OCT for 96 hr (Figure 4). Bivariate analysis of dual labeled cells depicted as 3-D Isocontour plots illustrate the decrease in G_1 with concomitant accumulation of cells with hypodiploid DNA content in the region marked A_0. The presence of subdiploid DNA peak is a hallmark for apoptosis. That OCT induces apoptosis in hSSTR3 expressing cells in a time-dependent manner was confirmed by the detection of oligonucleosome sized DNA fragments by inversion field gel electrophoresis [17].

wt p53 is known to trigger either G_1 arrest or apoptosis. hSSTR3 initiated increase in p53 did not cause G_1 cell cycle arrest. In support of this is our finding that OCT did not influence either p21$^{WAF1/CIP1}$ (required for p53-induced G1 arrest) or c-Myc (needed for abrogating p21$^{WAF1/CIP}$1 mediated G1 arrest) in these cells [17]. We have shown that hSSTR3 mediated induction of *wt* p53 occurs within minutes, increases in a time-dependent manner and precedes the induction of Bax and the onset of apoptosis [17]. Furthermore, p53 exhibits perinuclear localization in untreated cells but display preferential intranuclear accumulation concomitant with nuclear shrinkage, a characteristic feature of cells undergoing apoptosis [17].

Based on these findings, I propose the following mechanistic model of hSSTR3 initiated cytotoxic signaling (Figure 5). The first event in this signal cascade is the regulation of a SST-sensitive PTP in a G protein dependent manner. The principal mediator of cytotoxic signaling initiated through hSSTR3 is *wt* p53 in its active conformation. A rapid, time-dependent increase in *wt* p53 and its nuclear translocation occurs in OCT-treated cells and precedes the onset of apoptosis. This, in turn, leads to the activation of Bax and acidic endonuclease, thereby causing oligonucleosomal DNA fragmentation and apoptosis. The nuclear accumulation of *wt* p53 parallels nuclear shrinking, a characteristic feature of apoptosis. The increased pAb 1801 immunoreactive *wt* p53 as well as its activation are dependent on dephosphorylation of Ser residues suggesting that by hSSTR3 linked PTP may activate Ser/Thr phosphatase(s) [14, 17]. SST-sensitive PTP has been implicated in attenuating growth factor receptor kinase induced mitogenic signaling [19, 20]. Inhibition of mitogenic signaling via hSSTR3 may, therefore, be conducive for induction of *wt* p53 dependent apoptosis. PTP dependent antiproliferative signaling through other SSTR subtypes may lead to G_1 arrest since these do not induce either *wt* p53 or
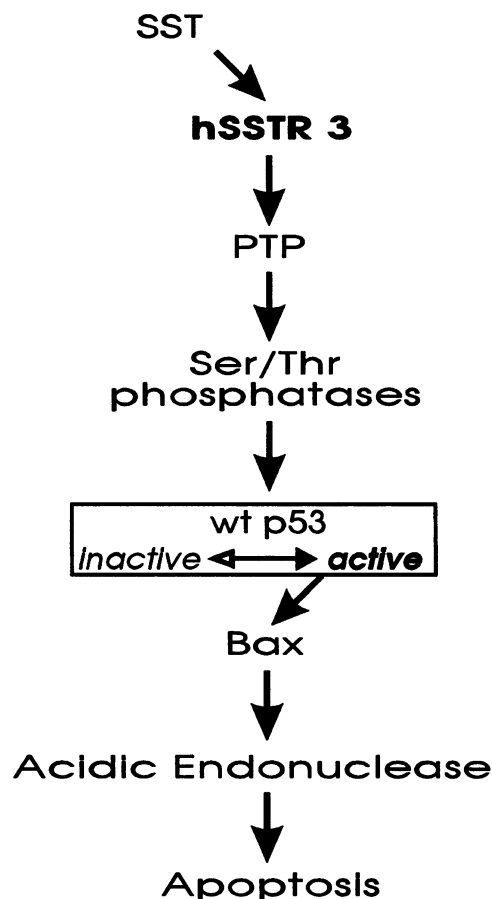

Figure 5. Proposed mechanism of hSSTR3 selective cytotoxic signaling.

apoptosis. This raises the question of whether hSSTR3 and other SSTR subtypes signal through different PTPs. We have recently reported that membrane associated PTP is not directly activated in tumor cells, contradicting previous claims [10, 19, 20]. We demonstrated that a net increase in membrane-associated PTP occurs in MCF-7 breast cancer cells preincubated with OCT and, additionally, have identified SHP-1 (PTP1C/SHPTP1/HCP/ SHP) as the intracellular PTP recruited by OCT in a time- and concentration-dependent manner [10]. Studies are in progress to delineate the role of SHP-1 in subtype-selective hSSTR-mediated cytotoxic and cytostatic signaling.

We have shown that OCT induced cytotoxic signaling in MCF-7 cells occurs in a p53 dependent manner, requiring the downstream induction of Bax and cation-insensitive acidic endonuclease [9]. Surprisingly, the rate of cytotoxic signaling was faster in MCF-7 cells compared to CHO-K1 cells expressing hSSTR3 [9]. The rapidity of OCT induced cytotoxic signaling in MCF-7 cells may be due to higher levels of SHP-1 and/or wt p53 compared to CHO-K1 cells. Our findings suggest, but does not prove, that the cytotoxic signaling of OCT in this cell line is signaled via hSSTR3 subtype. This contradicts the reported absence of SSTR3 transcripts in MCF-7 cells by RT-PCR analysis [21]. Studies are currently in progress to resolve this issue. If this is confirmed at the protein level, then the potential existence of a novel SSTR subtype that is functionally similar to hSSTR3 should be considered, since the other known hSSTR subtypes do not activate the apoptotic pathway. In contrast to the cytotoxic effects documented in MCF-7 and AtT-20 cells, SST is reported to elicit only cytostatic effect in GH_3 cells which do express SSTR3 [7-9, 22]. This may be due to functional differences between human and rat SSTR3 or, alternatively, due to mutation in the p53 molecule may render it incapable of assuming the active conformation in GH_3 cells.

Overexpression of Bax has been shown to sensitize MCF-7 cells to radiation [23]. Agonist-dependent SSTR internalization has been observed in tumor cells and is reported to occur maximally via hSSTR3 [24, 25]. The selectivity of hSSTR3 for inducing Bax in wt p53-dependent manner, combined with its ability to sequester the ligand within the cell, provides a rational basis for exploiting the therapeutic potential of α- and β- emitting OCT tagged radionuclides such as ^{111}In, ^{99}Tc, [67/68]Ga and [161]Tb in the treatment of tumors expressing hSSTR3 and wt p53. Such compounds are already in use for scintigraphic visualization of SSTR-positive tumors [26-29].

Tamoxifen potentiates the cytotoxic activity of OCT in two estrogen-responsive cell lines MCF-7, T47-D and ZR-75-1 (Sharma and Srikant, unpublished observations). A positive influence of OCT enhancing the antineoplastic effect of tamoxifen and ovariectomy has also been reported *in vivo* on 7,12-dimethylbenzanthracene-induced mammary carcinoma [30]. Its cytotoxic and/or cytostatic activity *in vivo* is difficult to assess since SST exerts growth inhibitory action indirectly also by decreasing growth factor (e.g., IGF-1) bioavailability, [31, 32].

In summary, cytotoxic signaling of SST resulting in apoptosis is selectively transduced through hSSTR3. Such cytotoxic signaling requires dephosphorylation dependent conformational change and activation of wt p53, and its rapid nuclear translocation followed by induction of Bax and acidic endonuclease. These events occur in cells actively traversing the cell cycle. Since hSSTR3 bound agonist is maximally internalized, I predict that α- and β- emitting OCT-tagged radionuclides will be most useful in treating tumors expressing hSSTR3 and wt p53, not only because of receptor mediated cytotoxic signaling, but also due to greater sensitization by hormonally induced Bax to radiation damage by internalized radioactivity as a consequence of receptor-mediated endocytosis.

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