Increased expression of the mRNA encoding the somatostatin receptor subtype five in human colorectal adenocarcinoma

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ABSTRACT

Numerous studies have suggested that the anti-proliferative potency of somatostatin (SS) analogues may be an efficient tool to improve the prognosis of colorectal cancer. In order to facilitate current efforts to design potent antitumour SS analogues, we studied the distribution of human SS receptors (hsst1–5) mRNAs in a large set of tumoural and normal colonic tissues. Localisation of hsst1–5 mRNAs in normal and tumoural tissues was performed by *in situ* hybridisation using radioactive antisense or sense riboprobes. Semi-quantitative analysis of hsst5 mRNA was performed using a computerised image analysis system. Hsst binding sites were characterised by studying the relative potency of SS14, SS28 or SS analogues in displacing [¹²⁵I]Tyr^o-D-Trp⁸-SS14 bound to HT29-D4 cells. Hsst5 mRNA was by far the most expressed subtype in both normal and transformed epithelial cells as well as in the HT29-D4 cell line.

An increased expression of hsst5 mRNA was found in tumours. Hsst1 mRNA was expressed preferentially as clusters in immune cells in lamina propria and in stroma close to the tumour. A low expression of hsst4, hsst3 and hsst2 was seen in normal and tumoural tissue. In HT29-D4, binding experiments with SS14 demonstrated the existence of one SS binding class ($K_d = 524$ nM, $B_{max} = 1$ fmol/10⁶ cells). In competition binding studies, SS28 and BIM23268 (an analogue that shows preferential specificity towards hsst5) effectively inhibited binding of [¹²⁵I]Tyr^o-D-Trp⁸-SS14 ($IC_{50} = 15$ and 157 nM respectively), while BIM23197 (an analogue that shows preferential affinity for hsst2) was ineffective. Our results show a high expression of hsst5 mRNA in human tumoural colonic tissue, while hsst5 protein is the predominant hsst protein subtype in a tumoural colonic cell line.

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INTRODUCTION

Colorectal cancer is one of the most common cancers in the Western world. The prognosis of patients with advanced colorectal cancer remains poor and new treatment modalities are needed. One of these treatments may involve the use of

somatostatin (SS) analogues. SS is a pan-inhibitory peptide widely distributed in the central nervous system and in peripheral tissues. In the gastrointestinal tract it inhibits secretin, gastrin, histamine and gastric acid secretion and decreases gastric and intestinal motility, mesenteric blood flow and intestinal absorption (Lewin 1992). In addition, SS

inhibits cell proliferation by an inhibition of the release of various hormones and growth factors, an inhibition of angiogenesis, a direct inactivation of growth factors or an activation of programmed cell death (Lamberts *et al.* 1991, Patel & Srikant 1997). SS and its analogues such as octreotide or RC160 inhibit *in vitro* tumoural colon cell proliferation (Palmer Smith & Solomon 1988, Qin *et al.* 1991, Dy *et al.* 1992). *In vivo*, in murine colon cancer models and in human tumours grafted to nude mice, these substances reduce tumour volume and metastatic development and decrease tumoural markers (Dy *et al.* 1992, Qin *et al.* 1992, Dy & Morris 1993, Ruzsiewicz *et al.* 1993, Davies *et al.* 1995). However, clinical studies with SS analogues in patients with advanced colorectal cancer failed to demonstrate a significant benefit although a decrease of tumoural markers could be seen (Goldberg *et al.* 1995, Cascinu *et al.* 1997); a 2-week treatment with octreotide decreased the Ki67 kinetic index and the carcinoembryonic antigen level in some patients with primary rectal cancer (Iftikhar *et al.* 1991). Similarly, octreotide treatment reduced the proliferating cell nuclear antigen-maximum proliferative index (Stewart *et al.* 1995).

Five human SS receptors (*hsst*) of seven transmembrane G protein-coupled types have been cloned (Patel & Srikant 1997). They are all negatively coupled to adenylate cyclase and can also modulate guanylate cyclase and calcium or potassium channels, activate protein phosphatase, transmodulate tyrosine kinase type receptors, act on the mitogen-activated protein (MAP) kinase signalling pathway, and even induce apoptosis (Florio & Schettini 1996, Patel & Srikant 1997, Pollak & Schally 1998).

Binding assays and autoradiographic studies have shown the presence of *hsst* in normal and tumoural colon mucosa, nerve plexus, lymphoid tissue and the peritumoural veins (Iftikhar *et al.* 1992, Radulovic *et al.* 1992, Reubi *et al.* 1992, 1994, 1996). Few recent studies have analysed the expression of the different *hsst* subtypes in colonic tissue, giving controversial results. Buscail *et al.* (1996) found by RT-PCR a heterogeneous expression of *hsst1*–*5* in colorectal cancer with a characteristic loss of *hsst2* expression in the advanced stages; Laws *et al.* (1997), using RT-PCR and *in situ* hybridisation, found a retained expression of *hsst2* but a decreased expression of *hsst5* in late-stage tumours.

In order to get more insight into *hsst* in colorectal cancer, we studied, using semi-quantitative *in situ* hybridisation, the expression of *hsst1*–*5* mRNAs in normal and tumoural colonic tissues. We also analysed on a human tumoural cell line (HT29-D4) the binding of [¹²⁵I]Tyr^o-D-Trp⁸-SS14 and its

displacement by SS28 or by the newly described SS analogues BIM23268 and BIM23197 that show preferential specificity towards *hsst5* and *hsst2* respectively.

MATERIALS AND METHODS

Materials

Samples were obtained during surgery for colorectal cancer. All patients gave their written consent to participate in the study. The study was approved by the local Ethics Committee (Marseille I). One part of each specimen was used for histopathological classification and individual colon carcinomas were staged according to the Dukes' classification system (Dukes & Bussey 1958). The other part was immediately frozen on dry ice and stored at -70°C . The results of histopathological studies are summarised in Table 1.

Peptides

SS14 and SS28 were from Bachem (Voisins-le-Bretonneux, France). BIM23197 (N-hydroxyethylpiperazinyl-acetyl-D-Phe-c[Cys-Tyr-D-Trp-Lys-Abu-Cys]-Thr-NH₂) and BIM23268 (c[Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys]-NH₂) were provided by Biomeasure Inc. (Milford, MA, USA).

Culture of HT29 cell line

HT29-D4 human colon adenocarcinoma cell line was routinely cultured in Dulbecco's Modified Eagle's medium (Life Technologies, Cergy Pontoise, France) containing 25 mM glucose, 5 mM glutamine, antibiotics (streptomycin, penicillin) and 10% heat-inactivated foetal calf serum in 150 cm² tissue culture flasks for binding assays. For *in situ* hybridisation studies, cells were cultured for 3 days on Labtek chamber slides (Nalge Nunc International, Roskilde, Denmark). The medium was then removed and cells were rinsed twice with PBS and processed immediately for *in situ* hybridisation assays.

In situ hybridisation

Consecutive sections (12 µm) were cut on a cryostat microtome at -20°C . The sections were thaw-mounted onto twice gelatine-coated slides, dried on a slide warmer and kept at -70°C . Adjacent sections were hybridised with probes recognising the five mRNAs encoding the different *hsst* subtypes. *In situ* hybridisation was performed as

TABLE 1. Histopathological studies of the tumours

Patient no.	Age (years)	Sex	Localisation	Dukes' stage	Differentiation
1	84	F	R	A	WD
2	60	M	R	A	WD
3	60	M	R	B	WD
4	86	F	R	B	WD
5	64	F	R	C	WD
6	71	F	R	D	MD
7	76	M	R	D	MD
8	75	M	R	D	WD
9	72	M	LC	A	WD
10	66	F	LC	A	WD
11	65	F	LC	B	WD
12	59	F	LC	C	WD
13	77	F	LC	C	WD
14	77	F	LC	C	WD
15	59	F	LC	C	MD
16	45	F	LC	D	WD
17	71	F	LC	D	MD
18	60	F	RC	A	WD
19	62	F	RC	B	WD
20	84	F	RC	C	UD
21	71	M	RC	C	WD
22	62	F	RC	C	WD

R: rectal; LC: left colon; RC: right colon. WD: well differentiated; MD: moderately differentiated; UD: undifferentiated.

previously described (Grino & Zamora 1998). Briefly, the sections were warmed at room temperature and fixed with 4% formaldehyde in PBS, pH 7.2. After two washes in PBS, they were placed in 0.25% acetic anhydride in 0.1 M triethanolamine–0.9% NaCl, pH 8, for 10 min and delipidated in ethanol and chloroform. They were hybridised with 50 µl buffer containing 50% formamide, 600 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 10% (w/v) dextran sulphate, 0.2 mg/ml tRNA, 100 mM dithiothreitol, and 2×10^7 d.p.m./ml of antisense or sense (control) probe under a glass coverslip. After 20 h incubation in moist sealed chamber at 56 °C, coverslips were removed in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). Slides were subsequently treated for 30 min at 30 °C with 10 µg/ml Rnase A in $2 \times$ SSC and washed at room temperature with decreasing concentrations of SSC ($2 \times$ to $0.1 \times$) containing 10 mM β-mercaptoethanol (β-ME) and finally incubated for 1 h at 65 °C in $0.1 \times$ SSC, 10 mM β-ME. Sections were exposed to X-ray films (Biomax-MR; Kodak, Rochester, NY, USA) for 24 h and subsequently dipped in nuclear emulsion (1:1 in water, K5; Ilford, Saint-Priest, France). In order to get a sufficient but not overexposed signal, antisense and sense slides were exposed for 3 days for *hsst5*, 9 days for *hsst1*, *hsst3* or *hsst4*, and 3 weeks for *hsst2*, based on the intensity of the signal on the

X-ray film. After development, sections were counterstained with eosin and haematoxylin.

Labelled riboprobes were prepared using [³⁵S]UTP (New England Nuclear, Paris, France, SA: 1300 Ci/mmol) and T3 or T7 RNA polymerase (Stratagene, Paris, France) to synthesise sense or antisense transcripts from the *hsst1*–*5* cDNAs cloned into Bluescript. The incorporation of [³⁵S]UTP was comparable for all the antisense or sense probes. The *hsst* constructs used to generate the ³⁵S-labelled cRNA probes were as follows: 228–1217 for *hsst1*, 320–1020 for *hsst2*, 232–1176 for *hsst3*, 201–1245 for *hsst4* and 81–1016 for *hsst5*. The specificity of these probes has been described previously (Dutour *et al.* 1998).

Quantification of hybridisation signal

Hsst5 mRNA hybridisation signal was quantified on emulsion-dipped tissue sections and on monolayers of HT29-D4 cultured cells. Silver grain density was measured under brightfield microscopy (magnification $\times 650$) using a computerised image analysis system (Unilog and Ware, Grenoble, France). For each case, three different areas were analysed: the tumoural tissue, morphologically normal tissue adjacent to the tumours and morphologically normal tissue taken at distance (>3 cm) from the

tumour. Silver grain density (grains/0.1 mm²) was measured on ten randomly chosen fields for each area analysed. The mean background level (measured for each tissue on serial sections hybridised with the *hsst5* sense probe) was subtracted from each value.

Statistical analysis

Statistical analysis was performed by the Kolmogorov–Smirnov test, using a computerised program (Stat View 512; Brain Power, Inc., Calabasas, CA, USA).

[¹²⁵I]Tyr^o-D-Trp⁸-SS14 binding assay

¹²⁵I-Labelled Tyr^o-D-Trp⁸-SS14 (specific activity: 2200 Ci/mmol) was prepared in our laboratory by lactoperoxidase iodination and purified by reverse phase HPLC (Magnan *et al.* 1993). The iodinated peptide was diluted 1:2 in binding buffer, stored at 4 °C and used within 3 days. For binding experiments, 10⁶ cells/tube were used. Tissue culture flasks were washed twice with 5 ml PBS followed by PBS containing 5 mM EDTA to detach the cells. Binding was performed for 2 h at 4 °C in 20 mM Hepes pH 7.4, 5 mM MgCl₂, 100 µg/ml bacitracin, 230 µM phenylmethylsulphonyl fluoride, 100 µg/ml aprotinin, 0.1% BSA in a total volume of 0.1 ml containing 0.25 nM [¹²⁵I]Tyr^o-D-Trp⁸-SS14 without (total binding) or with increasing amounts (10⁻¹¹ to 10⁻⁵ M) of SS14, SS28, BIM23268 or BIM23197. To test the specificity of the binding, gastrin was also used. The incubation was stopped by diluting the mixture with 5 ml ice-cold 50 mM Tris–HCl buffer containing 1% BSA. Separation of free and bound radioligand was obtained by rapid filtration *in vacuo* through Whatman GF/C glass-filter circles (2.5 cm diameter), pre-soaked in 50 mM Tris–HCl containing 0.3% polyethyleneimine and 0.1% BSA. Filters were washed with 10 ml ice-cold buffer. All assays were performed in triplicate. Radioactivity was counted in a Cobra II (Packard, Warrenville, IL, USA) (99% efficiency for ¹²⁵I).

Data analysis

Analysis of binding isotherms was performed by linear regression methods using the LIGAND program (Munson & Rodbard 1980). Curve fitting and IC₅₀ estimation were performed using the Allfit program (Delean *et al.* 1978).

RESULTS

After hybridisation of normal or tumoural tissues with the sense probes, silver grains were sparse and

homogeneously distributed, demonstrating the specificity of the probes (Figs 1 and 2B, D, F, H and J).

Moderate levels (i.e. detected after 9 days of exposure) of *hsst1* mRNA were found in the mucosa, mainly in lamina propria. The labelling was heterogeneous and distributed preferentially as foci in immune cells facing Lieberkühn glands. However, epithelial cells were also clearly labelled (Fig. 1A). Interestingly, no signal was detectable in lymphoid follicles as compared with immune cells in the mucosa. No signal was detected in endothelial, fibroblastic, nervous and muscle cells of the submucosae and in the three muscle layers (muscularis, longitudinal and circular). In tumour, *hsst1* mRNA was also expressed by tumoural cells and its expression was heterogeneous within each tumour and between tumours. In many cases, immune cells of stroma close to epithelial cell clusters showed a strong signal (Fig. 2A).

The expression of *hsst2* mRNA was very low (i.e. detected after 21 days of exposure) and sometimes barely detectable (Fig. 1C). *Hsst2* mRNA was found in immune cells and in epithelial cells of the mucosa. In tumours, *hsst2* subtype was also distributed homogeneously at a very low level in both tumoural epithelial and stromal cells (Fig. 2C).

Hsst3 mRNA was expressed at moderate levels (i.e. detected after 9 days of exposure) in the mucosa both in epithelial and immune cells (Fig. 1E). The lamina propria was heterogeneously labelled, the density of silver grains being marked in foci located below the cryptic epithelial cells. *Hsst3* mRNA was also expressed in the submucosa. There was a quite marked expression in the three muscle layers. The tumoural distribution pattern of *hsst3* mRNA was more diffuse without any foci (Fig. 2E). *Hsst3* mRNA was homogeneously expressed in tumoural cells and immune cells of the stroma.

In the mucosa, *hsst4* mRNA had the same distribution pattern as *hsst1*, the signal being preferentially distributed in immune cells (Fig. 1G). A weak signal was detected in the three muscle layers. In tumours, *hsst4* expression was weak and heterogeneous and localised preferentially in immune cells (Fig. 2G).

In normal tissue, *hsst5* mRNA was homogeneously expressed at a high level in epithelial cells, already highly detected after 3 days of exposure (Fig. 1I). Moderate levels of *hsst5* mRNA were also detected in the three muscle layers, at the interface of the circular and longitudinal layers of the muscle and in endothelial cells. Interestingly, an increased labelling for *hsst5* mRNA was found in all the tumours (Fig. 2I, Fig. 3A and B). In all patients analysed, silver grain density of the *hsst5* mRNA

FIGURE 1. Localisation of *hsst1-5* mRNA in normal colonic mucosa (patient no. 2) by *in situ* hybridisation. (A, C, E, G, I) Tissue sections were hybridised with *hsst1-5* antisense riboprobes; (B, D, F, H, J) tissue sections were hybridised with *hsst1-5* sense riboprobes (control). Tissue sections were exposed for 3 days for *hsst5*, 9 days for *hsst1*, *hsst3* or *hsst4* and 21 days for *hsst2*. *Hsst1* mRNA is expressed in immune cells of the lamina propria as foci facing glandular epithelial cells and at low levels in epithelial cells (A). *Hsst2* mRNA is expressed at very low levels in both immune and epithelial cells (C). *Hsst3* mRNA is expressed at moderate levels in epithelial cells and in the lamina propria (E). *Hsst4* mRNA expression pattern is identical to that of *hsst1* (G). *Hsst5* mRNA is expressed at high levels in normal epithelial cells and at lower levels in the lamina propria (I). Arrows indicate epithelial labelling and arrowheads indicate the labelling of immune cell of the lamina propria. CL, crypt of Lieberkühn; EP, epithelium; LP, lamina propria. Bar=10 µm.

FIGURE 2. Localisation of *hsst1-5* mRNA in tumoural colonic tissue (patient no. 2) by *in situ* hybridisation. (A, C, E, G, I) Tissue sections were hybridised with *hsst1-5* antisense riboprobes; (B, D, F, H, J) tissue sections were hybridised with *hsst1-5* sense riboprobes (control). Exposure time was as described in the legend of Fig. 1. *Hsst1* mRNA is highly expressed as foci by stromal cells (A). *Hsst2* mRNA is barely expressed in tumoural and in stromal cells (C). *Hsst3* is expressed at a moderate level by transformed epithelial cells and stromal cells (E). *Hsst4* is preferentially expressed as foci by stromal cells (G). *Hsst5* mRNA is very intensely expressed in tumoural cells and at a lower level in stromal cells (I). Arrows indicate tumoural labelling and arrowheads indicate the labelling of immune cell of the stroma. T, tumoural cells. Bar=10 µm.

FIGURE 3. Expression of *hsst5* mRNA in tumoural cells and normal mucosa sample from patient no. 3. (A) normal tissue; (B) tumoural tissue (Dukes' stage B). Marked labelling of the tumour can be noted (arrows). LP, lamina propria; CL, crypt of Lieberkühn; T, tumour; M, muscle. Bar = 10 μ m.

hybridisation signal was 2- to 4.5-fold higher in tumoural tissues than in normal tissue (Fig. 4A). In 88% of the area analysed in normal tissue less than 30 grains/0.1 mm² were found whereas there was, in 66% of the area analysed in tumoural tissue, more than 45 grains/0.1 mm². Statistical comparison by the Kolmogorov–Smirnov test indicated a significant ($P < 0.001$) shift towards higher values for *hsst5* mRNA in tumours as compared with normal tissue (taken at a distance > 3 cm from the tumour). Interestingly, a significant difference ($P < 0.001$) was also found between morphologically normal tissues taken at distance from the tumour and morphologically normal tissue adjacent to the tumours (Fig. 4B).

The tumoural colonic epithelial cell line HT29-D4 showed the same expression pattern of

hsst as in primary tumours with a high level of *hsst5* mRNA, detected after 3 days of exposure (88–132 grains/0.1 mm²) (Fig. 5A) and a low level of expression of *hsst2* mRNA, which could only be detected after 21 days of exposure (Fig. 5B).

Receptor assays

The binding of [¹²⁵I]Tyr^o-D-Trp⁸-SS14 to HT29-D4 cell line was found to be temperature- and time-dependent. The total binding was found to be 20% with 50% specific binding. No displacement was obtained with gastrin (not shown). Scatchard analysis of the displacement of [¹²⁵I]Tyr^o-D-Trp⁸-SS14 by native SS14 indicates that HT29-D4 possess a single class of SS binding sites ($K_d = 524$ nM, $B_{max} = 1$ fmol/10⁶ cells). In competition binding studies, SS28 and BIM23268 (an analogue that shows preferential specificity towards *hsst5*) effectively inhibited binding of [¹²⁵I]Tyr^o-D-Trp⁸-SS14 (IC_{50} : 15 and 157 nM respectively), while BIM23197 (an analogue that shows preferential specificity towards *hsst2*) was ineffective ($IC_{50} > 1$ μ M) (Fig. 6).

DISCUSSION

In view of the many biological actions of SS, particularly in the gut, where SS inhibits endocrine and exocrine secretion, electrolyte and enzyme secretion, electrolyte transport, intestinal nutrient absorption and cell proliferation, an important question that arises is whether these effects are subtype selective. Because of the lack of good *sst* subtype-selective antagonist, studies on the expression pattern of *sst* in brain and peripheral tissue have been performed to approach this question. They have revealed an overlapping but characteristic pattern of expression that is subtype, tissue and also species specific (Patel & Srikant 1997). We present here the first study of the cellular localisation of the five *hsst* mRNAs in a large set of normal and tumoural human colonic tissue. We found a widespread expression of the five *hsst* mRNA subtypes in human colonic tissue, particularly expressed in both epithelial and in lamina propria cells. Interestingly, among the different subtypes, *hsst5* was by far the most highly expressed and its expression was increased in tumoural cells.

Few studies have approached *hsst* distribution in normal human colonic tissue (Lewin 1992, Radulovic *et al.* 1992, Miller *et al.* 1993, Warhurst *et al.* 1995, Buscail *et al.* 1996, Laws *et al.* 1997). Binding studies have shown the presence of *hsst* in

FIGURE 4. Semi-quantitative analysis of *in situ* hybridisation signal of *hsst5* mRNA. Quantification was performed using a computerised image analysis system in three different tissue types; tumoural tissue, morphologically normal tissue adjacent to tumoural tissue and morphologically normal tissue taken at a distance from the tumours. Each frequency histogram shows pooled data obtained from five patients (ten fields measured per tissue type per patient). (A) In tumours, silver grain density of *hsst5* mRNA hybridisation signal shifts towards higher values as compared with normal tissues. (B) A shift towards higher values is also found in the morphologically normal tissue adjacent to tumour as compared with normal tissue. Statistical comparison by the Kolmogorov–Smirnov test indicates a significant ($P < 0.001$) difference between normal and tumoural or morphologically normal tissue adjacent to tumoural tissue.

normal human mucosa (Radulovic *et al.* 1992, Miller *et al.* 1993). Buscail *et al.* (1996) have shown by RT-PCR the expression of *hsst2*, *hsst4* and *hsst5* in normal human mucosa. As shown by Laws *et al.* (1997), we demonstrated in a study performed by RT-PCR that all five *hsst* were detected in normal tissues, *hsst5*, *hsst1* and *hsst2* mRNA being the most frequently detectable (Vuaroqueaux *et al.* 1999). These non-quantitative results are in accord with our *in situ* hybridisation results showing a high expression of *hsst5* mRNA in epithelial cells, and of *hsst1* mRNA in stroma while *hsst2* was found to be widely expressed in mucosa but at a low level. Using *in situ* hybridisation, Laws *et al.* (1997) found that *hsst2* mRNA was widely distributed in normal mucosa and stroma in 90% of samples while *hsst5* was present only in half of normal mucosal samples. The difference between our results and those of Laws is most probably related to the difference of sensitivity of the probes used. Several studies have been performed in rats. Using RT-PCR, Warhust *et al.* (1996) have shown that crypt epithelium expressed *sst1* and *sst2* mRNA and, at lower levels,

sst5 mRNA. However, in rat colon adenocarcinoma (DHD/K12) *sst2*, *sst3* and *sst5* were identified (Gouyon *et al.* 1995). A very detailed *in situ* hybridisation study in the rat gastrointestinal tract has been performed by Krempels *et al.* (1997) showing a widespread expression of rat *sst* mRNAs and suggesting a large SS regulatory system in the gastrointestinal tract. Similar to what we found, they showed that all tissues contained mRNA for several *sst* subtypes. A clear expression of *sst3* mRNA was demonstrated, rather comparable with what we found. There is also a close resemblance between *sst1* and *sst4* expression in rat and in human colonic tissue but *sst5* expression was far higher in human tissue in our study than in rats. This difference is not surprising as species-specific distribution of *sst* has previously been shown, the difference of expression being particularly marked for *sst5* (Hoefland & Lambert 1996, Thoss *et al.* 1996, Patel 1997).

Interestingly, we found expression of *hsst1–4* in immune cells of both lamina propria and stroma, *hsst1* being the most expressed. This underlines the

FIGURE 5. Expression of *hsst5* and *hsst2* mRNA in the epithelial colonic tumoural cell line HT29-D4. (A and B) Cell culture hybridised with *hsst5* and *hsst2* antisense riboprobes respectively. (A) High level of *hsst5* mRNA was detected with antisense probe after 3 days of exposure. (B) Very low level of *hsst2* mRNA, detectable only after 21 days of exposure. Bar=10 μ m.

relevance of the immunomodulatory functions of SS (Pawlikowsky *et al.* 1987, Van Hagen *et al.* 1994), which is mainly inhibitory, even though SS has also been shown to amplify the immune response in peculiar experimental conditions (Pawlikowski *et al.* 1989). SS has been shown to act in a paracrine and/or autocrine fashion to inhibit lymphocyte proliferation (Aguila *et al.* 1996), and to inhibit T cell activation by interfering with the antigen-independent pathways CD2 and CD28 (Casini *et al.* 1997). Furthermore, in the gut, it was shown that SS can inhibit immunoglobulin production by mononuclear cells of lamina propria and that these cells display specific high-affinity ($K_d=2.1 \pm 0.34$ nM) *hsst*, whose subtype was not characterised (Fais *et al.* 1991). Our data suggest that *hsst1* may be a major actor.

Looking for therapeutic bases for SS analogue treatment of colorectal cancer, we studied *hsst* expression in 22 tumours. We show that the five *hsst* mRNAs were expressed in all the tumours. The tumoural status did not modify the expression of *hsst1*–4 although in some cases tumours seemed more labelled because of the higher cell density. By contrast, an enhanced labelling for *hsst5* mRNA

was found in tumoural cells, silver grain density being 2- to 4.5-fold higher in tumoural tissues than in normal ones. Of interest, *hsst5* mRNA was retained in advanced tumours. Using a non-quantitative RT-PCR, Buscail *et al.* (1996) have previously noted a very frequent expression of *hsst5* in tumours (in 71% of tumours whatever the stage of the tumours) while *hsst2* was less frequently detected especially in advanced tumours (three out of six in Dukes' B stage, one out of five in Dukes' C stage, none out of three in Dukes' D stage). Our work underlines the importance of *hsst5* in tumours. The group of Primerose (Laws *et al.* 1997) found also an increased frequency of *hsst5* expression in early-stage tumours as compared with normal mucosa (75 vs 45%) but this increase of frequency was lacking in late-stage tumours.

Although mRNA steady-state levels do not necessarily correlate with the amount of protein synthesised, the existence of large amounts of *hsst5* mRNA was in favour of the presence of high levels of *hsst5*. The recent availability of SS analogues with preferential specificity for *hsst5* allowed us to show the presence of *hsst5* binding sites and a very low level of *hsst2* receptor on HT29-D4. Indeed BIM23268, an SS analogue with a higher affinity for *hsst5* than for *hsst2* and *hsst3* (40- and >10 000-fold respectively) (Shimon *et al.* 1997) was more effective than SS14 itself ($IC_{50}=157$ nM vs 628 nM for SS14) in displacing [125 I]Tyr^o-D-Trp⁸-SS14 bound to HT29-D4 cells. Furthermore, BIM23197, an SS analogue with a higher affinity for *hsst2* than for *hsst5* and *hsst3* (50- and 140-fold respectively) did not affect the binding of iodinated SS14 to HT29-D4 cells. Our results are in good agreement with the recently published immunohistochemical studies showing the presence of the *hsst5* receptor protein in colonic tissue (Estève *et al.* 1998).

Our results show a high expression of *hsst5* mRNA in human tumoural colonic tissue, while *hsst5* protein is the predominant *hsst* protein subtype in a tumoural colonic cell line, and raise the question of the potential benefit of *hsst5* analogues in colonic cancer therapeutic approaches. Indeed, SS analogues such as octreotide and RC160 have been shown to decrease tumoural markers and the growth of human colonic tumours transplanted in rodents and to reduce metastatic development (Qin *et al.* 1991, 1992, Dy *et al.* 1992, Dy & Morris 1993, Ruzsiewicz *et al.* 1993, Davies *et al.* 1995). These antiproliferative effects of SS are mediated both indirectly through inhibition of secretion of hormones and growth factors involved in tumour growth and directly through *hsst* located on tumour tissue (Lamberts *et al.* 1991, Patel & Srikant 1997).

FIGURE 6. Displacement of [125 I]Tyr 0 -D-Trp 8 -SS14 binding to epithelial colonic tumoural cell line HT29-D4. Cells were incubated for 2 h at 4 °C with 0.25 nM iodinated peptide in the absence (total) or in the presence of increasing concentrations (10^{-11} – 10^{-5} M) of SS14, SS28, BIM23268 or BIM23197. Values are the mean $\pm$ s.e. of three determinations. Results are expressed as the percentage of total specific binding.

Schematically, sst can activate protein tyrosine phosphatases inducing dephosphorylation of growth factor receptor kinase and then inhibit MAP kinase activity (Pan *et al.* 1992, Buscail *et al.* 1994, Patel & Srikant 1997, Pollak & Schally 1998). Several sst subtypes have been implicated in SS antiproliferative effects (Buscail *et al.* 1994, Sharma *et al.* 1996, 1999, Cordelier *et al.* 1997, Patel & Srikant 1997, Florio *et al.* 1999). Even though SS-induced apoptosis has been shown to occur via the *hsst3* subtype in cycling cells (Sharma *et al.* 1996), a large body of experimental evidence suggests involvement of *hsst5* in SS-induced antiproliferative effects (Cordelier *et al.* 1997, Sharma *et al.* 1999). A cytostatic effect of SS via *hsst5* that occurred through induction of the retinoblastoma protein and

G(1) cycle arrest was recently described (Sharma *et al.* 1999). Moreover, *hsst5*, the subtype over-expressed in colonic tumours, can inhibit the proliferation induced by cholecystokinin (CCK) (Cordelier *et al.* 1997). Indeed, when stably expressed in CHO cells, *hsst5* mediates the antiproliferative effect of SS analogues, via the successive inhibition of soluble guanylate cyclase, protein kinase G and p42 MAP kinase activities (Cordelier *et al.* 1997). It is interesting to note that we have previously shown in the tumours analysed in this study the presence of CCK-B long and short isoform and CCK-C receptors (Biagini *et al.* 1997).

In conclusion, our results suggest that studying the effect of *hsst5* analogues in colorectal cancers may be an interesting approach.

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REFERENCES

- Aguila MC, Rodriguez AM, Aguila-Mansilla HN & Lee WT 1996 Somatostatin antisense oligonucleotide-mediated stimulation of lymphocyte proliferation in culture. *Endocrinology* **137** 1585–1590.
- Biagini P, Monges G, Vuaroqueaux V, Parriaux D, Cantaloube F & De Micco P 1997 The human gastrin/cholecystokinin receptors: type B and type C expression in colonic tumors and cell lines. *Life Sciences* **61** 1009–1018.
- Buscail L, Delesque N, Estève J-P, Saint-Laurent N, Prats H, Clerc P, Robberecht P, Bell GI, Liebow C, Schally AV, Vaysse N & Susini C 1994 Stimulation of tyrosine phosphatase and inhibition of cell proliferation by somatostatin analogues: mediation by human somatostatin receptor subtypes SSTR1 and SSTR2. *Proceedings of the National Academy of Sciences of the USA* **91** 2315–2319.
- Buscail L, Saint-Laurent N, Chastre E, Vaillant JC, Gespach C, Capella G, Kalthoff H, Lluís F, Vaysse N & Susini C 1996 Loss of sst2 receptor gene expression in human pancreatic and colorectal cancer. *Cancer Research* **56** 1823–1827.
- Cascinu S, Del Ferro E, Grianti C, Ligi M, Ghiselli R, Foglietti G, Saba V, Lungarotti F & Catalano G 1997 Inhibition of tumor cell kinetics and serum insulin growth factor I by octreotide in colorectal patients. *Gastroenterology* **113** 767–772.
- Casini C, Lattuada D, Perego C, Fransco P & Marelli O 1997 Inhibitory effect of somatostatin on human T lymphocytes proliferation. *International Journal of Immunopharmacology* **19** 721–727.
- Cordelier P, Estève J-P, Bousquet C, Delesque N, O'Carroll AM, Schally AV, Vaysse N, Susini C & Buscail L 1997 Characterization of the antiproliferative signal mediated by the somatostatin receptor subtype sst5. *Proceedings of the National Academy of Sciences of the USA* **94** 9343–9348.
- Davies N, Kynaston H, Yates J, Nott DM, Nash J, Taylor BA & Jenkins SA 1995 Octreotide inhibits the growth and the development of three types of experimental liver metastases. *British Journal of Surgery* **82** 840–843.
- Delean A, Munson PJ & Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *American Journal of Physiology* **235** E97–E102.
- Dukes CE & Bussey HJR 1958 The spread of rectal cancer and its effect on prognosis. *British Journal of Cancer* **12** 309–320.
- Dutour A, Kumar U, Panetta R, Ouafik L'H, Fina F, Ramakrishnan S & Patel Y 1998 Expression of somatostatin receptor subtypes in human brain tumors. *International Journal of Cancer* **76** 620–627.
- Dy DY & Morris DL 1993 Somatostatin inhibits both *in vivo* and *in vitro* carcinoembryonic antigen secretion by human colon cancer. *European Journal of Surgical Oncology* **19** 168–172.
- Dy DY, Whitehead RH & Morris DL 1992 SMS 201-995 inhibits *in vitro* and *in vivo* growth of human colon cancer. *Cancer Research* **52** 917–923.
- Estève JP, Rochaix P, Voigt JJ, Schally AV, Vaysse N, Susini C & Buscail L 1998 Role of nitric oxide in the antiproliferative signal mediated by the somatostatin receptor sst5. *Gastroenterology* **114** G4651.
- Fais S, Annibale B, Boirivant M, Santoro A, Pallone F & Delle Fave G 1991 Effects of somatostatin on human intestinal lamina propria lymphocytes. Modulation of lymphocytes activation. *Journal of Neuroimmunology* **31** 211–219.
- Florio T & Schettini 1996 Multiple intracellular effectors modulate physiological function of the cloned somatostatin receptors. *Journal of Molecular Endocrinology* **17** 89–100.
- Florio T, Yao H, Carey KD, Dillon TJ & Stork PJS 1999 Somatostatin activation of mitogen-activated protein kinase via somatostatin receptor 1. *Molecular Endocrinology* **13** 24–37.
- Goldberg RM, Moertel CG, Wiehand HS, Krook JE, Schutt AJ, Veeder MH, Milliard JA & Dalton R 1995 A phase III evaluation of a somatostatin analogue (octreotide) in the treatment of patients with asymptomatic advanced colon carcinoma. *Cancer* **76** 962–966.
- Gouyon BA, Reyl-Desmars F, Le Romancer M, Pigeon C, Lewin MJM & Lehy T 1995 *In vivo* effect of somatostatin analogue, lantreotide, and/or GRP antagonist, BIM 26226, on the growth of colon cancer peritoneal carcinomatosis in the rat. *International Journal of Oncology* **7** 1167–1173.
- Grino M & Zamora AJ 1998 An *in situ* hybridization histochemistry technique allowing simultaneous visualization by the use of confocal microscopy of three cellular mRNA species in individual neurons. *Journal of Histochemistry and Cytochemistry* **46** 753–759.
- Hoefland LJ & Lambert SWJ 1996 Somatostatin receptors and disease: role of receptor subtypes. *Baillière's Clinical Endocrinology and Metabolism* **10** 163–176.
- Iftikhar SY, Watson SA & Morris DL 1991 The effect of long acting somatostatin analogue SMS 201–995 therapy on tumour kinetic measurements and serum tumour marker concentrations in primary rectal cancer. *British Journal of Cancer* **63** 971–974.
- Iftikhar SY, Thomas WM, Rooney PS & Morris DL 1992 Somatostatin receptors in human colorectal cancer. *European Journal of Surgical Oncology* **18** 27–30.
- Krempels K, Hunyady B, O'Carroll AM & Mezey C 1997 Distribution of somatostatin receptors messenger RNAs in the gastrointestinal tract. *Gastroenterology* **112** 1948–1960.
- Lamberts SWJ, Krenning EP & Reubi JC 1991 The role of somatostatin receptors and its analogs in the diagnosis and treatment of tumors. *Endocrine Reviews* **12** 450–482.
- Laws S, Gough AC, Evans AA, Bains MA & Primerose JN 1997 Somatostatin receptors subtype mRNA expression in human colorectal cancer and normal colonic mucosa. *British Journal of Cancer* **75** 360–366.
- Lewin MJM 1992 The somatostatin receptor in the GI tract. *Annual Review of Physiology* **54** 455–468.
- Magnan E, Cataldi M, Guillaume V, Mazzocchi L, Dutour A, Compte Delvoix B, Giraud P & Oliver C 1993 Neostigmine stimulates growth hormone-releasing hormone release into

- hypophysial portal blood of conscious sheep. *Endocrinology* **132** 1247–1251.
- Miller GV, Preston SR, Woodhouse LF, Farmery SM & Primerose JN 1993 Somatostatin binding in human gastrointestinal tissues: effect of cations and somatostatin analogues. *Gut* **34** 1351–1356.
- Munson PJ & Rodbard D 1980 LIGAND: a versatile computerized approach for characterization of ligand-binding system. *Analytical Biochemistry* **107** 220–239.
- Palmer Smith J & Solomon TE 1988 Effects of gastrin, proglumide, and somatostatin on growth of human colon cancer. *Gastroenterology* **95** 1541–1548.
- Pan MG, Florio T & Stork PJS 1992 G protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science* **256** 1215–1217.
- Patel YC 1997 Molecular pharmacology of somatostatin receptor subtypes. *Journal of Endocrinological Investigation* **20** 348–367.
- Patel YC & Srikant CB 1997 Somatostatin receptors. *Trends in Endocrinology and Metabolism* **8** 398–405.
- Pawlikowski M, Zelazowski P & Stepień H 1989 Enhancement of human lymphocytes natural killer activity by somatostatin. *Neuropeptides* **13** 75–77.
- Pawlikowski M, Stepień H, Kunert-Radek J, Zelazowski P & Shally AV 1987 Immunomodulatory action of somatostatin. *Annals of the New York Academy of Sciences* **496** 233–239.
- Pollak MN & Schally AV 1998 Mechanism of antineoplastic action of somatostatin analogs. *Proceedings of the Society for Experimental Biology and Medicine* **217** 143–152.
- Qin Y, Shally AV & Willems G 1991 Somatostatin analogue RC-160 inhibits the growth of transplanted colon cancer in rats. *International Journal of Cancer* **47** 765–770.
- Qin Y, Shally AV & Willems G 1992 Treatment of liver metastases of human colon cancers in nude mice with somatostatin analogue RC-160. *International Journal of Cancer* **52** 791–796.
- Radulovic SS, Milovanovic SR, Cai RZ & Shally AV 1992 The binding of bombesin and somatostatin and their analogs to human colon cancers. *Proceedings of the Society for Experimental Biology and Medicine* **200** 394–401.
- Reubi JC, Horisberger U, Waser B, Gebbers JO & Laissue J 1992 Preferential location of somatostatin receptors in the germinal centers of human gut lymphoid tissue. *Gastroenterology* **103** 1207–1214.
- Reubi JC, Horisberger U & Laissue J 1994 High density of somatostatin receptors in veins surrounding human cancer tissue: role in tumor-host interaction. *International Journal of Cancer* **56** 681–688.
- Reubi JC, Mazzucchelli L, Hennig I & Laissue JA 1996 Local up-regulation of neuropeptide receptors in host blood vessels around human colorectal cancers. *Gastroenterology* **110** 1719–1726.
- Ruszniewski P, Lehy T, Reyl-Desmars F, Le Roux S & Lewin MJM 1993 Octreotide (SMS 201–995) inhibits the growth of colon peritoneal carcinomatosis in BDIX rats. *Regulatory Peptides* **43** 141–147.
- Sharma K, Patel YC & Srikant CB 1996 Subtype-selective induction of wild-type p53 and apoptosis, but not cell cycle arrest, by human somatostatin receptor 3. *Molecular Endocrinology* **10** 1688–1695.
- Sharma K, Patel YC & Srikant CB 1999 C-terminal region of human somatostatin receptor 5 is required for induction of Rb and G(1) cell cycle arrest. *Molecular Endocrinology* **13** 82–90.
- Shimon I, Taylor JE, Dong JZ, Bitonte RA, Kim S, Morgan B, Coy DH, Culler MD & Melmed S 1997 Somatostatin receptor subtype specificity in human fetal pituitary cultures: differential role of SSTR2 and SSTR5 for growth hormone, thyroid-stimulating hormone, and prolactin regulation. *Journal of Clinical Investigation* **99** 789–798.
- Stewart GJ, Connor JL, Lawson JA, Preketes A, Kings J & Morris DL 1995 Octreotide reduces the kinetic index, proliferating cell nuclear antigen-maximum proliferative index, in patient with colorectal cancer. *Cancer* **76** 572–578.
- Thoss VS, Perez J, Probat A & Hoyer D 1996 Expression of five somatostatin receptor mRNA in the human brain and pituitary. *Archives of Pharmacology* **354** 411–419.
- Van Hagen PM, Krenning EP, Kwekkeboom DJ, Reubi JC, Anker-Lugtenburg PJ, Lowenberg B & Lamberts SWJ 1994 Somatostatin and the immune and haematopoietic system; a review. *European Journal of Clinical Investigation* **24** 91–99.
- Vuaroqueaux V, Dutour A, Briard N, Monges G, Grino M, Oliver C & Ouafik L'H 1999 No loss of sst receptors gene expression in advanced stages of colorectal cancer. *European Journal of Endocrinology* **140** 362–366.
- Warhurst G, Higgs NB, Grigor MR, Ross I & Barbezat GO 1995 Expression of multiple somatostatin receptor genes in human colonic epithelial cells. *Biochemical Society Transactions* **23** 18S.
- Warhurst G, Higgs NB, Fakoury H, Warhurst AC, Garde J & Coy DH 1996 Somatostatin receptor subtype 2 mediates somatostatin inhibition of ion secretion in rat distal colon. *Gastroenterology* **111** 325–333.

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