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Expression of the Breast Cancer Associated Gene pS2 and the Pancreatic Spasmolytic Polypeptide Gene (hSP) in Diffuse Type of Stomach Carcinoma

Birgit Theisinger, Cornelius Welter, Gerhard Seitz, Marie-Christine Rio, Rich Lathe, Pierre Chambon and Nikolaus Blin

Expression of the pancreatic spasmolytic peptide (hSP) gene and pS2 (a gene isolated from oestrogen-induced breast carcinoma cells) were analysed in 36 samples of human stomach carcinoma. 17 tumours were investigated at the RNA level (by northern blots) as well as at the gene product level (by immunochemistry). Since pS2 had been shown to be expressed in normal stomach mucosa its activity in carcinoma samples was expected. Surprisingly, strong pS2 immunoreactivity was noted in the diffuse carcinoma type, whereas the intestinal type displayed weak reactivity. The tumour samples showing strong immunostaining expressed the regular 0.6 kb pS2 RNA band and weak staining was paralleled by aberrant transcripts. Additionally, only in tumour samples with regular pS2 transcription was the typical 0.7 kb hSP RNA band seen; samples with aberrant pS2 bands did not express hSP at all. This is the first demonstration of hSP gene activity in a human tumour.
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INTRODUCTION

THE HUMAN pS2 gene, transcriptionally controlled by oestrogen in a subclass of oestrogen receptor positive breast carcinomas [1] is also expressed, independently of oestrogen, in normal stomach mucosa [2]. Additionally, it has been noted to be active in pancreatic carcinoma but not in the corresponding healthy tissue (B.T. *et al*). The gene's partial homology to the pancreatic spasmolytic polypeptide gene [2, 3] suggests similar function of both proteins in affecting cell metabolism. Since cDNAs for both sequences, pS2 and hSP, are available [4, 5] we initiated a comparison of gene organisation and expression of both sequences in healthy tissue of the stomach in addition to primary stomach carcinomas and some metastases. Activity of hSP was examined whenever RNA from tumours was available, in particular in those tumour samples which displayed altered pS2 expression as judged at the mRNA level (by northern blots) and protein level (by immunostaining), and finally in some rare cases when primary tumours and metastases were available.

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MATERIAL AND METHODS

Tissue samples

The tumour and stomach mucosa specimens were frozen in N_2 within 30 minutes of operation. All samples were examined histologically. Histopathological classification was according to Lauren [6]. In addition to primary carcinomas of the stomach (n = 33), 3 metastases were obtained.

DNA and RNA analyses

Simultaneous isolation of DNA and RNA from all tissues was performed as described [7]. Gene organisation and copy number in tumour cells was monitored by the restriction pattern using restriction endonucleases and by the intensity of DNA bands in

Southern blots. Gene expression was tested by northern blots. Gene sequence inserts were excised from recombinant molecules [4, 5] and labelled by primer extension (random priming kit, Gibco/BRL) (10^6 cpm/ng DNA). They were used to probe the blotting filters. *Hin*dIII fragmented lambda DNA was used for sizing DNA bands and bacterial rRNA for size determination of RNA bands. For RNA quantitation, an 28S rRNA oligonucleotide served as a control [8]. For Southern blots, nylon filters (Zeta, BioRad) were applied; northern blots were performed on nitrocellulose sheets (Schleicher & Schüll). All hybridisation and washing steps were standard procedures.

Immunostaining

pS2 immunostaining of tissue sections was essentially performed as described earlier [1]. The monoclonal antibody against the pS2 protein (p2802) [2] was applied at a 1:2000 dilution for peroxidase anti-peroxidase (PAP) and 1:6000 for alkaline phosphatase anti-alkaline phosphatase (APAAP). Controls by replacing the primary antibody were run in parallel.

RESULTS

Expression of pS2

Transcription of the pS2 gene, shown to be expressed and secreted by surface epithelial cells of the mucosa [2], usually leads to a mRNA banding at 0.6 kb. We observed this transcript in 11 out of 17 stomach carcinoma cases. In an additional 5 cases, aberrant transcription bands (0.8 and 0.5 kb) were noted; in 1 tumour sample the regular band appeared next to an additional, larger band (0.9 kb) (Fig. 1A). As expected, all tumour tissues displaying the 0.6 kb band showed distinct pS2 immunostaining (Fig. 2A). In all 5 tumour samples with irregular pS2 transcripts, immunoreactivity was still positive but visibly reduced (Fig. 2B). In all samples of normal stomach tissue, a regular transcription and marked reaction of the pS2 antibody were observed. In 2 cases, the initial stomach carcinomas plus their metastases were available for the RNA study. It is noteworthy that in 1 case the primary tumour expressed a regular pS2 band and the metastasis showed no transcription at all (Fig. 3A). In one additional sample, in the tumour as well as in the metastasis, pS2 expression was observed, however, leading to aberrant transcripts (data not shown). When pS2 expression patterns were inspected in more detail by subclassifying all

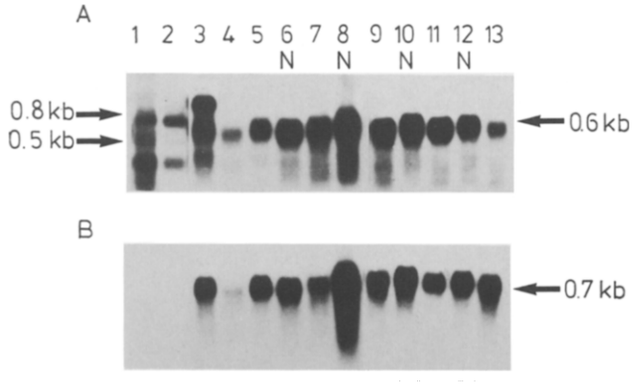

Fig. 1. Transcriptional activity of pS2 (1A) and hSP (1B) in a set of stomach carcinoma and normal stomach mucosa (N) samples. Total RNA was blotted to filters and the filters were hybridised with the pS2 probe and reused for hSP hybridisation. The size (in kb) of regular transcripts is indicated at the right side; arrows at the left side point to aberrant transcripts. The samples indicated by numbers are explained in Table 2.

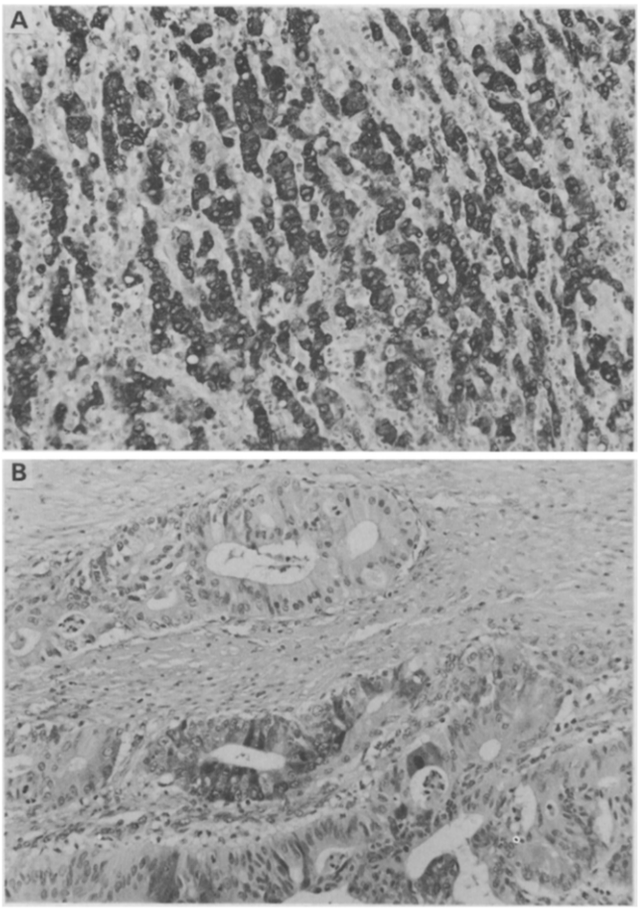

Fig. 2. pS2 immunostaining of stomach carcinoma. (A) Diffuse type of carcinoma showing strong immunoreactivity; (B) intestinal type of carcinoma showing immunoreactivity only in isolated areas: note the unstained group of tumour cells in the upper part.

36 stomach carcinomas investigated, a remarkable correlation became visible. Whereas most of the carcinomas of the intestinal type showed weak immunostaining, stomach cancer of the diffuse type displayed strong immunoreactivity (Table 1). In cases where mRNA was available weak immunoreaction correlated with aberrant transcripts.

Expression of hSP

The porcine pancreatic spasmolytic polypeptide (PSP) was reported to display sequence homology to pS2 [2, 3]; both proteins, however, are encoded by separate genes. The human equivalent to PSP, the hSP gene, was cloned most recently and yields a transcript of about 700 bp [5]. Our RNA blots from the pS2 experiment were reused to examine the hSP activity. All samples from normal stomach tissue and stomach carcinoma with regular pS2 transcription showed the 0.7 kb hSP band. Additionally, the tumour sample with the regular plus the larger transcript expressed the hSP gene, too (Fig. 1B). On the contrary, the stomach carcinomas which displayed aberrant transcripts and reduced immunoreactivity for pS2 conspicuously did not show any hSP transcription at all. Metastases with active but irregular pS2 or missing transcription were hSP negative (Fig. 3B) despite the fact that a control using 28S rRNA in all cases showed undegraded RNA (Fig. 3C).

Gene characterisation

To analyse the structure of both genes involved in our study, in particular in the cases of aberrant pS2 and failing hSP

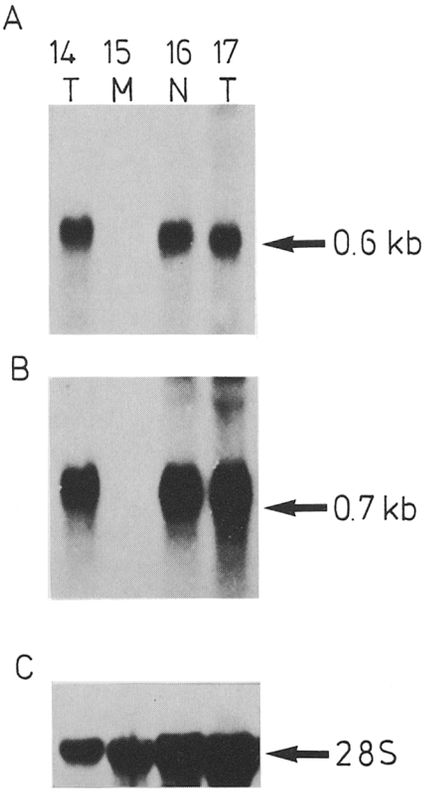

Fig. 3. Transcriptional activity in a set of normal stomach mucosa (N), primary stomach carcinoma (T) and corresponding metastasis (M). An additional tumour sample is shown to the right. The northern blots were successively probed with pS2, hSP and 28S rRNA. (A) pS2 hybridisation, (B) hSP hybridisation (C) 28S rRNA hybridisation as a RNA quality control.

transcription genomic DNA was fragmented using *Eco*RI, *Bam*HI and *Pst*I restriction endonucleases. However, all samples, healthy as well as neoplastic tissues, displayed comparable DNA bands. This result indicated that gross changes of the sequences probed did not occur, irrespective of transcriptional activity (data not shown).

DISCUSSION

When investigating the pS2 gene, its regulation and its biological role, computer analysis revealed a strong sequence homology between the pS2 protein and a porcine pancreatic spasmolytic polypeptide (PSP). In the latter a cysteine-rich domain of pS2 is tandemly duplicated [2]. Moreover, amphibian spasmolysins seem to belong to this homology group; for all peptides a common structural domain, a trefoil loop, was suggested [3] and, most recently, computer searching indicated a common core of 5 conserved cysteine residues, termed the P domain [5].

Table 1. Correlation of pS2 immunoreactivity to subtypes of stomach carcinoma

	Immunoreactivity		
Tumour type	<25	25–75	>75%
Diffuse	4	3	10
Intestinal	15	3	1

Table 2. hSP and pS2 expression in gastric carcinoma

Patient	Sample	Lane	MRNA (kb) pS2	MRNA (kb) hSP	pS2 immuno-staining	Lauren classification [6]
1	T	7	0.5 + 0.8	—	+	Intestinal
	N	8	0.6	0.7	+++	
2	T	17	0.6	0.7	+++	Diffuse
3	T	2	0.5 + 0.8	—	+	Intestinal
4	T	1	0.5 + 0.8	—	+	Intestinal
5	T		0.5 + 0.8	—	+	Intestinal
	M		0.5 + 0.8	—	+	Intestinal
6	T	3	0.6 + 0.9	0.7	+	Intestinal
7	T	4	0.6	0.7	++	Diffuse
8	T	5	0.6	0.7	+	Intestinal
	N	6	0.6	0.7	+++	
9	T		0.6	0.7	++	Intestinal
	N		0.6	0.7	+++	
10	T	9	0.6	0.7	+	Intestinal
		N 10	0.6	0.7	+++	
11	T		0.6	0.7	+++	Diffuse
	N		0.6	0.7	+++	
12	T		0.6	0.7	+++	Diffuse
	N		0.6	0.7	+++	
13	T		0.6	0.7	+++	Diffuse
	N		0.6	0.7	+++	
14	T	11	0.5 + 0.8	—	+	Intestinal
	N	12	0.6	0.7	+++	
15	T		0.6	0.7	+++	Diffuse
	N		0.6	0.7	+++	
16	T	14	0.6	0.7	+++	Diffuse
	N	16	0.6	0.7	+++	
	M	15	—	—	+	
17	T	13	0.6	0.7	++	Diffuse
	N		0.6	0.7	+++	

T = tumour, N = normal mucosa, M = metastasis.
+ = <25%, ++ = 25–75%, +++ = >75% positive tumour cells.

The biological function of pS2 and hSP still remains unknown. Due to the present availability of specific probes we initiated comparative analysis of their expression in healthy and neoplastic tissue. Since pS2 transcription and secretion was noted in normal stomach mucosa and stomach carcinoma and, more recently, hSP was described to be coexpressed in stomach mucosa as well, the need arose to investigate both genes in normal tissue, tumours and their metastases.

Next to the expected coexpression of pS2 and hSP in healthy stomach mucosa [2, 5] an unexpected, strong correlation for both genes became evident: only tumours wth a regular pS2 transcript showed hSP activity. Tumour samples and their metastases with aberrant pS2 transcription did not indicate any hSP expression. Most remarkably, when pS2 expression was set in correlation with subtypes of stomach carcinomas, the diffuse type displayed the strongest immunoreactivity, whereas the intestinal type, in general, showed weak immunostaining.

Since, in all cases investigated, the gross gene structure obviously remained unaltered it is tempting to speculate that the coordinated activity of both genes may be regulated via comparable or even identical steps which may include the genes' regulatory regions and specific factors. Coordinated secretion of structurally similar pS2 and hSP in normal mucosa cells suggests their comparable role, possibly in contributing to glycoprotein degradation as discussed by Tomasetto *et al.* [5]. Correlated

secretion in malignant cells of the gastrointestinal tract but not in breast carcinoma may further allow the speculation that a common regulatory pathway exists for pS2 and hSP in these steroid hormone receptor negative cells.

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Circumvention of Doxorubicin Resistance in Multi-drug Resistant Human Leukaemia and Lung Cancer Cells by the Pure Antioestrogen ICI 164384

X.F. Hu, G. Nadalin, M. De Luise, T.J. Martin, A. Wakeling, R. Huggins and J.R. Zalcberg

ICI 164384, a new steroidal antioestrogen, entirely devoid of oestrogenic activity, modulates doxorubicin resistance *in vitro*. At non-cytotoxic concentrations, ICI 164384 potentiated the cytotoxicity of doxorubicin in a dose-dependent manner in both the classical multi-drug resistant (MDR) human leukaemia cell lines CEM/VLB 100 and CEM/VLB 1000 and the human small cell lung cancer cell line H69 LX4. ICI 164384 had no effect on the two respective parental cell lines, CEM/CCRF and H69 P. None of these cell lines expressed the oestrogen receptor. In comparative studies at concentrations ranging from 1.25 to 10 μmol/l, ICI 164384 was significantly more effective (1.2–6-fold) than tamoxifen in reducing the IC_{50} of doxorubicin in the CEM/VLB 100 line. In resistant cells, ICI 164384 increased ^{3}H-daunomycin accumulation in a dose-dependent manner and was significantly more effective than tamoxifen at concentrations ranging from 2.5 to 10 μmol/l. ICI 164384 reduced the efflux of daunomycin from resistant cells more effectively than tamoxifen. These studies suggest that ICI 164384 is an effective modulator of MDR.
Eur J Cancer, Vol. 27, No. 6, pp. 773–777, 1991

INTRODUCTION

IT IS widely accepted that multi-drug resistance (MDR), described in several tumour types [1] and numerous cell lines [2–4], is a major obstacle in cancer chemotherapy [5]. Based on *in vitro* studies, the classical MDR phenotype may be defined by the cross-resistance of cells to a number of structurally unrelated cytotoxic drugs [3] and the increased expression of P-glycoprotein [6, 7]. Although the exact function of this membrane protein is unknown, it is thought to be responsible for the reduced cellular accumulation of drugs in resistant cells [8, 9] as a result of increased energy-dependant drug efflux [10, 11].

A number of biochemical modulators capable of reducing the level of resistance in tumour cell lines have been reported. These include a diverse group of structurally unrelated agents such as cyclosporin [12, 13], calcium channel blockers [14] and antioestrogens such [15] as tamoxifen.

Tamoxifen, a non-steroidal antioestrogen, was first noted to modulate the resistance of P388/ADR, a doxorubicin-resistant murine leukaemia cell line [15]. More recently, tamoxifen has also been shown to increase the drug sensitivity of a human doxorubicin-resistant breast cancer line [16]. Although its mechanism of action remains unclear, early studies suggested that

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