Association of pS2 (TFF1) release with breast tumour proliferative rate: *in vitro* and *in vivo* studies

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Abstract. Although cytosolic expression of the protein pS2 (TFF1) is considered to be a marker of oestrogen receptor (OR) function, there exists some clinical data to suggest an inverse relationship of cytosolic pS2 to tumour proliferation. Although secreted from breast cancer cells, the relationship of pS2 secretion to tumour natural history has been little studied. The mechanisms and kinetics of pS2 release and its relation to tumour cell proliferation were studied in a human breast cancer cell line MCF-7 and verified in a preliminary clinical study. Stimulation by stripped serum or oestradiol resulted in parallel increases of proliferation and pS2 release in both time course and dose–response experiments. Direct pharmacological alterations of proliferation were followed by identical changes in pS2 release. The relationship between serum pS2 levels and tumour proliferative activity when analysed as a function of steroid status showed a slope of 0.56 in OR+ vs. 0.19 in OR– tumours. It is concluded that pS2 release from breast cancer cells is associated with their proliferation and measurement of serum pS2 levels might be a good predictor of tumour proliferative state and could permit noninvasive monitoring of this tumour parameter.

INTRODUCTION

In steroid receptor positive tumours both oestrogens and growth factors stimulate proliferation, invasion and the production/secretion of a number of proteins, while in oestrogen receptor negative tumours growth factors are the primary driving force for these processes (Lippman *et al.* 1986). Disturbed cell proliferation leading to uncontrolled growth is a key phenotypic characteristic of cancer making its first appearance at the earliest identifiable steps in carcinogenesis (Evans 1991). One aspect of the alterations in the natural processes of cancer cells is the production and/or secretion of substances specific to the tumour state: the tumour markers.

The cystine rich protein pS2 is a second generation tumour marker that has been studied extensively in both clinical (Foekens *et al.* 1993, Gion *et al.* 1993, Predine, Spyrtos & Prud'Homme 1992, Henry *et al.* 1991, Schwartz *et al.* 1991, Cappelletti *et al.* 1992, Thor *et al.* 1992, Marsigliante *et al.* 1995) and cell culture (Nunez *et al.* 1987, Cavailles *et al.* 1989, Kida

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et al. 1989, Nunez *et al.* 1989, Rio & Chambon 1990, Miyashita *et al.* 1994, van Agthoven *et al.* 1994) studies. It belongs to a family of small secretory proteins characterized by three intrachain disulphide bonds, forming a trefoil motif. Three genes clustered in human 21q22.3 encoding for three different trefoil proteins have been isolated (Kayademir *et al.* 1998). Human pS2 (trefoil factor family 1, TFF1) is a trefoil, 60 amino acid peptide, which forms dimers and may stimulate gut repair (Marchbank *et al.* 1998). It was first described as an oestrogen-induced protein in the human breast cancer cell line MCF-7 (Nunez *et al.* 1987) and its production and secretion have since been considered to be tightly regulated by oestrogen receptor function. pS2 cytosolic presence has been observed to be positively related to hormone responsiveness (Schwartz *et al.* 1991, Predine *et al.* 1992, Correale *et al.* 1993, Foekens *et al.* 1994, Sauer & Naess 1994) and to be associated with more favourable prognosis (Predine *et al.* 1992, Foekens *et al.* 1990, Thompson *et al.* 1993, Manning *et al.* 1994, Besse *et al.* 1995, Rajakariar & Walker 1995, Soubeyran *et al.* 1995).

However, pS2 expression has also been reported to be strongly induced by growth factors (Miyashita *et al.* 1994, Cavailles, Garcia & Rochefort 1989) and even by analogues of gonadotropin-releasing hormone (Neri *et al.* 1991). Further, there are some clinical data suggesting a weak inverse relationship of intracellular pS2 to tumour growth rate (Cappelletti *et al.* 1992, Correale *et al.* 1993). Although secreted in large amounts by tumour cells (Kida *et al.* 1989, Nunez *et al.* 1989, Miyashita *et al.* 1994), pS2 has been considered clinically only as an intracellular marker. Taken together, these reports underline the necessity for a re-evaluation of the hormone sensitivity and mechanisms of pS2 release and an evaluation of its possible significance as a serum tumour marker. The possible use of serum pS2 would have the advantage of being a relatively noninvasive measurement, and a repeatable assay and to allow monitoring of patient/tumour state.

To elucidate further the biological mechanism(s) behind the release of pS2 and to clarify the relationship of its release with proliferative activity in breast cancer, an *in vitro* study was conducted on the oestrogen receptor positive (OR+) human breast cancer cell line MCF-7. To corroborate these data *in vivo*, the relationship between serum pS2 levels and primary tumour proliferative rate was determined in a series of patients with a wide range of breast tumour stages. This paper demonstrates that pS2 release is tightly associated with proliferation both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells

MCF-7 human breast cancer cells (Soule *et al.* 1973) were routinely maintained as a monolayer in a humidified atmosphere of 95% air, 5% CO₂ at 37°C in DMEM/HAMS F-12 with 50 U/ml penicillin, 50 µg/ml streptomycin, 300 mg/l L-glutamate and 10% fetal calf serum (FCS). Culture media were changed every 2 days and cells were serially passaged every 5–7 days by releasing the cells with DMEM/HAMS F-12 plus 0.05% trypsin, 0.02% EDTA and seeded at 10⁶ cells per 75 cm² flask.

Growth and pS2 release experiments

Stock cultures of exponentially growing MCF-7 monolayers were brought into suspension as above and aliquots of 4000 cells in 200 µl were plated in wells of 96-well growth plates (GIBCO Milano, Italy) in complete medium for 24 h. Cells were then washed once with phosphate-buffered saline (PBS) and incubated for 24 h in serum-free medium (serum

starvation) Preliminary experiments established that this 24 h incubation stopped growth and the cells were metabolically blocked Cells were then treated with the simple growth medium used for starvation (S), 10^{-11} M oestradiol (+O2), 10% FCS stripped of steroids (+SS), or 10% FCS stripped of steroids plus 10^{-11} M O2 (+O2, +SS) FCS was stripped of steroids by dextran-coated charcoal treatment as previously described, which reduced whole serum oestrogen levels to approximately 10^{-14} M (Tedone *et al* 1996) Cell growth and pS2 present in the medium were measured at various times from 30 min to 2 days For doseresponse experiments, after serum starvation, the cells were treated with O2 (10^{-9} M) or 10% SS Control cells were run in parallel using the same culture conditions without O2 or SS

Patients

Fifty previously untreated women with histologically confirmed primary breast carcinoma, radically treated by surgery in the Senology Unit of the Oncology Institute of Bari, Italy were considered in this study Serum samples were obtained by venous puncture 1–7 days before primary surgery The serum was frozen at -20°C until processing The mean age and range were 49.5 ± 8.8 years and 41–67 years, respectively Patients were classified as premenopause (44%), paramenopause (9%) and postmenopause (48%), when women were menstruating normally, the last menses was within the previous 2 years or longer, respectively Node involvement was defined on the basis of histological examination 50% were node negative, 42% node positive and 8% not known Fifty-five per cent were OR+ and 45% OR– tumours

Cordycepin and EIPA treatment

Stock solutions (1 mg/ml) of cordycepin (3'-deoxyadenosine), a specific inhibitor of the polyadenylation necessary for the maturation of mRNA from hnRNA, which prevents the appearance of mRNA in the cytoplasm (Nakazato, Edmonds & Kopp 1974), were prepared in 10% ethanol Stock solutions (0.1 M) of ethylisopropylamiloride (EIPA), a specific inhibitor of membrane Na^+/H^+ exchange, which prevents cellular alkalization necessary for the initiation of growth after serum deprivation (Wakabayashi *et al* 1997), were prepared in dimethylsulphoxide Working solutions were serially diluted in growth medium After serum starvation cells were treated with either simple medium, 10% SS plus 0–10 $\mu\text{g}/\text{ml}$ cordycepin or 2 μM EIPA Samples of media were collected and proliferation measured as described

Proliferation assays

Proliferation was simultaneously measured in each sample using three independent techniques that assay different cellular processes as previously described (Tedone *et al* 1996)

1 cells were released by trypsinization as described above and their number counted in a Burkner Chamber,

2 a [^3H]thymidine (^3H -TdR) incorporation assay was performed at various incubation times in culture depending upon the specific experimental design (all assays were performed in quadruplicate), and

3 at the indicated incubation times the culture medium was replaced with 100 μl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) at 1 mg/ml in PBS (all the assays were performed in triplicate)

Tumour proliferative activity

Tumour tissue proliferative activity was measured by an autoradiographic ^3H -TdR incubation assay The fresh tumour sample was minced into 5–8 fragments of about 1 mm^3 and incubated

for 1 h in a Dubnoff shaker at 37°C in complete McCoy's medium plus 20% FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 222 kBq/ml (6 µCi/ml) of ³H-TdR. The fragments were then processed according to the usual autoradiographic procedure. The ³H-TdR labelling index (TLI) was the ratio between the number of ³H-TdR labelled cells and the total number of tumour cells observed with an optical microscope. The laboratory where the autoradiographic TLI analysis was performed participates in the Program of Quality Control for ³H-TdR-LI coordinated by the Italian Society of Basic and Applied Cell Kinetics.

pS2 levels

The levels of pS2 released into the culture medium and in blood serum levels were measured by a commercially available two site immunoradiometric (IRMA) assay kit obtained from Cis bio international (Gif sur Yvette, France).

OR and PgR

A portion of each samples was immediately frozen in liquid nitrogen and stored at -80°C for no more than 1 month. Tumour cell cytosol for hormone receptor analysis was obtained by dissolving powered tumour biopsy tissue in a pH 7.4 buffer containing: 10 mM monothioglycerol, 10 mM sodium molybdate and 1.5 mM EDTA. This homogenate was centrifuged at 105 000 *g* for 60 min and the supernatant removed. OR and PgR were assayed using the dextran-coated charcoal (DCC) methods as outlined by the European Organization for Cancer Research and Treatment (EORTC Breast Cancer Cooperative Group 1980). The cut-off value for OR and PgR analyses was 10 fmol/mg cytosol protein. The laboratory where the analyses were performed participates in the Italian Program of Hormone Receptor Assay Quality Control.

Kinetic analysis

The kinetic coefficients for stripped serum-dependent pS2 release and proliferation were estimated by the nonlinear curve-fitting regression utilizing the iterative Marquardt procedure on the KALEIDAGRAPH® (Abelbeck Software, Boston, MA, USA) program. The data were fitted to the Michaelis-Menten equation: $R = (R_{\max} [SS]) / (K_s + [SS])$ in which *R* is the rate of either pS2 release or proliferation, [SS] is the experimental concentration of stripped serum, *R*_{max} is the maximum rate of these processes and *K*_s is the stripped serum concentration that yields one-half *R*_{max}.

RESULTS

Time course of stripped serum and oestradiol stimulation of proliferation and pS2 release

Figure 1 shows the time course of stimulation of MCF-7 cell proliferation by steroid hormone (oestradiol, O2) or stripped serum (SS) addition as measured simultaneously by three independent methods. Proliferation in this MCF-7 cell line was stimulated by either oestradiol alone (10⁻¹¹ M) or by stripped serum alone (10%) and simultaneous addition of both resulted in a further stimulation of proliferative activity. At long time periods the three independent measurements (cell number, ³H-TdR and MTT) gave equivalent results suggesting that proliferation was in equilibrium. At short incubation times the different biological aspects did not have the same time kinetics: cell number increased significantly only after 6 h of treatment while increases in ³H-TdR incorporation and MTT hydrolysis were already measurable at 1 h and 2 h, respectively

Figure 1. Time course of MCF-7 proliferation. Monolayers cultures of MCF-7 cells were starved for 24 h and then subjected to the same 'control' conditions (●), 10^{-11} M oestradiol (▲), 10% stripped serum (■) or both together (◆). At the indicated times cell proliferation was determined as a change in the number of cells (a), the incorporation of ³H-thymidine (b) or the metabolism of MTT (c). Values are mean ± SE, $n = 4$.

The accumulation of pS2 in the culture medium during a representative proliferation experiment is shown in Figure 2. A rapid release of pS2 was observed after all the three treatments and closely reflected the stimulation of cell proliferation observed in Figure 1. In order to ascertain time-dependent changes in marker secretion, pS2 accumulated in the medium during the first hour, or during the first 24 h and for 1 h after 24 h of stimulation was determined. One-hour releases of pS2 were similar at all the times from stimulation (Table 1).

Figure 2. Time course of the accumulation of pS2 in growth medium. Monolayer cultures of MCF-7 cells were subjected to the same conditions as in Figure 1 and at the indicated times growth medium was collected and pS2 measured as in *Materials and methods*. Values are mean $\pm$ SE, $n = 4$.

Table 1. pS2 accumulation in the growth medium at different times of stimulation in MCF-7 cells

	1 h	1–24 h	(per h)	24–25 h
–Serum	48 ± 2.4	1278 ± 51	(53)	73 ± 2.7
+O ₂ (10^{-11} M)	76 ± 3	1710 ± 85	(71)	94 ± 5.3
+SS (10%)	520 ± 22	9258 ± 388	(386)	508 ± 29
+SS, +O ₂	653 ± 41	$12\,612 \pm 428$	(525)	773 ± 27

Values are in pg/ml pS2 in medium harvested from 6 wells of a 96-well plate. Values in parentheses are the pS2 released in the first 24 h divided by 24 as an estimate of the hourly release.

Dose–response of stripped serum and oestradiol on proliferation and pS2 release

The above time course data suggest that pS2 release in these breast cancer cells may be related to stimulation of proliferation in both oestrogen-dependent and oestrogen-independent receptor-mediated processes. To gain further insight into the possible relationship between growth factor and oestrogen-stimulated proliferation and pS2 release, the kinetics of stripped serum- and oestradiol-dependent stimulation of both proliferation and pS2 release were compared.

Figure 3 shows that the dose effect of both stripped serum (a) and oestradiol (b) on proliferation were identical to that of pS2 release (in these MCF-7 cells). Michaelis–Menten half-maximum stripped serum stimulation values (K_s) of pS2 release and cell proliferation were statistically identical (1.05 ± 0.46 vs. $1.15 \pm 0.32\%$ SS for pS2 release and ^3H -TdR incorporation, respectively, $P < 0.01$). Oestradiol affected both pS2 release and proliferation in a complex but identical manner with an increase of values to a maximum at an oestradiol concentration of between 10^{-11} and 5×10^{-11} M and a decrease at higher oestradiol concentrations (Figure 3b).

Effect of cordycepin and EIPA on stripped serum-stimulated proliferation and pS2 release

In order to further corroborate the association of pS2 release to proliferation, the effect of the specific pharmacological inhibition of proliferation on pS2 was analysed. The proliferative

Figure 3. Dose-effect of stripped serum and oestradiol on proliferation and pS2 release. Monolayer cultures of MCF-7 cells were starved for 24 h and then subjected to various concentrations of stripped serum (a) or oestradiol (b). The release of pS2 (●) was measured after 6 h incubation while the rate of MTT metabolism (■) was measured after 24 h incubation. Values are mean $\pm$ SE, $n = 3$.

rate of these cells was pharmacologically modified by various concentrations of cordycepin (3-deoxyadenosine), a specific inhibitor of mRNA maturation (polyadenylation) or EIPA, a specific inhibitor of the Na^+/H^+ exchanger which has been shown to inhibit proliferation by not permitting the necessary cytoplasmic alkalization (Wakabayashi, Shigekawa & Pouyssegur 1997).

In Figure 4 the effect of 10% SS plus 0–10 $\mu\text{g}/\text{ml}$ cordycepin on proliferation as measured by MTT and pS2 release is shown. Incubation in the presence of cordycepin greatly modified

Figure 4. Dose-effect of cordycepin on proliferation and pS2 release. Monolayer cultures of MCF-7 cells were starved for 24 h and then incubated without or with 10% stripped serum and 010 ng/ml cordycepin. The release of pS2 (●) and rate of MTT metabolism (■) were measured as in Figure 3. Values are mean $\pm$ SE, $n = 3$.

both cell growth and pS2 release; SS, i.e. growth factor-dependent proliferation, and pS2 release were further stimulated by the lowest cordycepin concentration (1 μ g/ml) by more than 150% and about 200%, respectively. Further increases in cordycepin concentration resulted in 55% decrease in proliferation and 70% inhibition of pS2 release with respect to values observed in the cells stimulated by only 10% stripped serum. Similar results were observed when cell proliferation was evaluated as cell number or by MTT approach (data not shown).

The effect of EIPA on the stimulation of proliferation and pS2 release is shown in Figure 5. The presence of 2 M EIPA strongly inhibited both the re-initiation of proliferation and the increase in pS2 after the period of starvation.

Clinical relationship between pS2 release and proliferation

The relationship between serum pS2 and tumour proliferative rate (TLI) was analysed in a group of 50 patients with primary breast tumours with different oestrogen receptor status (Figure 6). A significant relationship between serum pS2 level and the cell proliferative rate was observed. When tumours were stratified according to oestrogen receptor status, a higher association between the two biological aspects was observed in OR+ than in OR- tumours. In particular, the slopes for the relationship were 0.56 ($r = 0.60$) vs. 0.19 ($r = 0.65$) for OR+ and OR- tumours, respectively ($P < 0.01$). Serum pS2 levels were neither related to node status, menopausal status nor to tumour OR level in the OR+ tumours (data not shown).

Figure 5. Effect of EIPA on proliferation and pS2 release. Monolayer cultures of MCF-7 cells were starved for 24 h and then incubated without or with 10% stripped serum and 2 μ M EIPA. The release of pS2 (striped bar) and rate of [3 H]thymidine (solid bar) were measured as in Figure 3. Values are mean $\pm$ SE, $n = 3$.

Figure 6. Relationship between tumour proliferative rate (TLI) and serum pS2 in 50 patients with primary breast cancer. Tumours were divided into oestrogen receptor positive (OR+, ●, solid line, $n = 27$, slope = 0.56, $r = 0.60$) or oestrogen receptor negative (OR-, ▲, dashed line, $n = 23$, slope = 0.19, $r = 0.65$).

DISCUSSION

In the current study, the MCF-7 human breast cancer cell line was used to determine the hormone sensitivity of pS2 release and the relationship between pS2 release and alterations in proliferation. The growth of the particular MCF-7 line used is regulated by oestrogen and peptide growth factors added as serum stripped of steroids and further stimulated following the joint exposure of the two stimuli. A physiological concentration of oestrogen resulted in a doubling time little more than 1 day, similar to that reported by others for MCF-7 cells (Kida *et al.* 1989, Pagliacci *et al.* 1991). A high concordance was observed among the results by the three assays used to evaluate cell growth, suggesting that the variations induced by different treatments on cell number are a result of proliferation changes rather than other process, such as necrosis.

The sensitivity of these cells to either oestradiol or stripped serum potentially allows the separation of cellular processes underlining the stimulation induced by the different types of hormones (Tedone *et al.* 1996, Tedone *et al.* 1997). However this experimental manipulation has not been fully utilized to quantitatively analyse the relative contribution of steroid hormones and growth factors to pS2 release. The results from the present study clearly show that both oestradiol and stripped serum are individually able to stimulate the release of large amounts of pS2 and that the growth is further increased by the joint treatment. Taken all together, these findings show that pS2 release in these cells is dependent on the function of the receptors for both steroid hormones and growth factors. pS2 in tumour cells is still primarily regarded as an oestrogen-regulated protein and there is little experimental evidence on the relative contribution of steroid hormones and growth factors to its regulation (Cavaillès *et al.* 1989, Neri *et al.* 1991, Miyashita *et al.* 1994). The data from the present study support the hypothesis that pS2 secretion is not strictly dependent on OR function. Moreover, the time course of stimulation of proliferation and pS2 release as well as the dose-response to both stripped serum and oestradiol were practically identical. These observations suggest a direct relationship between receptor-stimulated proliferation and stimulated pS2 release and imply that there may be a causal relationship between proliferation and pS2 release. In particular, the pS2 release and proliferation patterns in response to increasing oestradiol concentrations were similar to those reported by Kida *et al.* 1989 for MCF-7 cells. These authors, however, did not analyse the potential link between the two aspects. Cordycepin, which acts on the transcription process independently of any receptor interference (Nakazato *et al.* 1974) and EIPA, a specific inhibitor of membrane Na^+/H^+ exchange (Wakabayashi *et al.* 1997), necessary for cytoplasmic alkalization, produced similar alterations of pS2 release and proliferation following stripped serum stimulation. These concomitant alterations of pS2 release by direct, non-receptor related manipulation of proliferation, provide further evidence for a direct relationship between proliferative rate and pS2 release.

The time course of pS2 accumulation shows that pS2 secretion is tightly coupled to its production and that it is relatively constant over time. This tight association has been previously reported (Cavaillès *et al.* 1989, Miyashita *et al.* 1994) and it was suggested to be post-translationally regulated (Miyashita *et al.* 1994).

These *in vitro* data suggest a direct, causal action of proliferation on pS2 release and therefore that pS2 release could probably represent an alternative means to determine proliferative activity of this cell line. The intracellular regulatory mechanisms for these two processes and how they are linked remain to be elucidated.

The finding of a significant relationship between serum pS2 and *in situ* tumour proliferative

rate, to the authors' knowledge never previously reported, confirms and enhances the hypothesis of an inverse relationship between cytosolic pS2 and S phase cell fraction (Soule *et al* 1973, Cappelletti *et al* 1992). The significant difference observed between the slopes of the relationship in OR+ and OR- breast tumours corroborates the *in vitro* synergistic effect between oestradiol and stripped serum in OR+ MCF-7 cells observed in the present study and that observed between oestradiol and the growth factor bFGF by Miyashita *et al* 1994. The lack of a relationship between pS2 and OR levels and the influence of the oestrogen receptors on the relation between tumour proliferation and serum pS2 suggest that the oestrogen receptor modulates proliferation by a complex mechanism not directly related to pS2 release.

These *in vivo* preliminary data need to be corroborated by studies on a larger series of breast cancers and in other tissues. If confirmed in the clinical situation, pS2 determination could be of clinical usefulness, and represent a noninvasive, repeatable test to collect information on the proliferative state of the tumour not only before but also during and after clinical treatment. A recent study suggested that pS2 levels could be a predictor of response to tamoxifen treatment (Soubeyran *et al* 1996).

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