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pS2 transfection of murine adenocarcinoma cell line 410.4 enhances dispersed growth pattern in a 3-D collagen gel

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SUMMARY

We describe the first model system employing human *pS2* gene transfer and expression in a non-pS2-expressing cell line, mouse mammary adenocarcinoma 410.4, in order to analyse the potential effect of human trefoil peptide pS2 in glandular epithelium. Two selected clones, AA4 and AD4, were established and shown to have incorporated the pS2 cDNA sequence into the genome, express pS2 containing transcript and produce the pS2 peptide. When grown in 3-D collagen gels both transfectants show striking morphological changes compared to the vector control clone (VA5). VA5 forms large cohesive spherical aggregates with rare coarse spicular outgrowths, accompanied by prominent hyalinised extracellular matrix deposition. pS2 transfectants form poorly cohesive, stellate colonies with very little or no matrix deposition, radiating long cords composed of single elongated cells, an effect previously observed in other cell lines with hepatocyte growth factor. pS2 transfection had no demonstrable effect on proliferation and this is not a morphogenetic phenomenon, as tubulogenesis is not seen. Motility assays suggest that the pS2 'dispersant' effect in collagen gels is due to an increase in cell motility. There were no measurable alterations in either E-cadherin expression or E-cadherin-dependent cell-cell aggregation. pS2 may play a role in maintenance and restitution of mucosal integrity by accelerating migration/dispersion.

Key words: pS2, Trefoil peptide, Migration, Cell motility

INTRODUCTION

In 1982 the human *pS2* gene was cloned from an expression library of the oestrogen-dependent human breast adenocarcinoma cell line MCF-7 (Masiakowski et al., 1982). The oestrogen-upregulated product of this gene is a 60 amino acid mature peptide, formed from an 84 amino acid precursor (Jackowlew et al., 1984; Rio et al., 1988; Mori et al., 1988). Analysis of the 5′ flanking region of the *pS2* gene revealed a complex enhancer region responsive to oestrogen, epidermal growth factor (EGF), tumour promoter 12-tetradecanoyl-phorbol 13-acetate, c-Ha-ras and c-jun proteins (Nunez et al., 1989). Several groups have isolated what is now known to be the same peptide, which has been variously termed BCEI, pNR-2/-105, Md2 and EGF-immunoreactive factor (Prud'homme et al., 1990; May and Westley, 1986, 1988; Mori et al., 1988; Skilton et al., 1989). The homology of pS2 with porcine pancreatic spasmolytic polypeptide (PSP) and a protein from the skin of *Xenopus laevis* led to the proposal of a new peptide motif, termed the trefoil motif or P-domain (Thim, 1989; Tomasetto et al., 1990), composed of 38-39 amino acids in which six cysteine residues are disulphide-linked to form a distinct three-loop structure. From NMR spectroscopic and X-ray diffraction studies on PSP (Carr, 1992; Gajhede et al., 1993) it is clear that trefoil peptides have a unique super-secondary structure distinguishing them from other highly disulphide cross-linked domains such as EGF (Gregory and Preston, 1977) and insulin-like growth factor 1 (Blundell and Humbel, 1980; Warne and Laskowski, 1990). To date three trefoil peptides have been identified in humans. These are the single-trefoil domain-containing pS2, intestinal trefoil factor (hITF), and human spasmolytic polypeptide (hSP), a peptide with a double-trefoil motif (Podolsky et al., 1993; Hauser et al., 1993; Tomasetto et al., 1990).

pS2, hSP and hITF are highly expressed in regional-specific patterns throughout the gastrointestinal tract (Rio et al., 1988; Tomasetto et al., 1990; Podolsky et al., 1993). Thus, although the *pS2* gene was found originally in a human breast cancer cell line it is expressed constitutively in antrum of the normal stomach and the peptide is secreted into the gastric juice (Rio et al., 1988). hSP is widely expressed in gastric foveolar epithelium and gastric glands (Hanby et al., 1993), while hITF is confined largely to small intestinal and colonic goblet cells (Podolsky et al., 1993). Mouse spasmolytic polypetide and pS2 as well as rat spasmolytic polypetide and intestinal trefoil factor have the same distribution as is found in humans (Lefebvre et al., 1993; Suemori et al., 1991; Tomasetto et al., 1990). Indeed there is a higher degree of homology among the same class of trefoils between species than there is between different trefoils from the same species. Thus there is 67%

homology between human and mouse pS2 and only a 37% homology between human pS2 and hITF (Lefebvre et al., 1993; Podolsky et al., 1993). The degree of evolutionary conservation in both trefoil structure and distribution clearly indicates that they have an important function, but their real physiological role is unclear.

Early studies with purified PSP indicated that it may have weak physiological effects, including inhibition of intestinal muscular contraction and gastric acid secretion (Jørgensen et al., 1982), and a weak proliferative effect on HCT 116 and MCF-7 cell lines in vitro (Hoosein et al., 1989). A possible clue to trefoil function emerged with the discovery of increased expression in a number of physiological and pathological conditions. Of particular interest is the association of pS2 and hSP expression with mucosal injury and ulceration (Wright et al., 1990a,b, 1993). Wright et al. (1990b) demonstrated increased pS2 and hSP expression in the ulcer-associated cell lineage (UACL) formed in Crohn's disease. It has been proposed that trefoil peptides may be involved in the maintenance of mucosal integrity and may have a role in ulcer healing, tentatively based on topological evidence. Some evidence for this presumed role has recently come from the observation that hSP and ITF can apparently accelerate restitution in an in vitro 'wounding' model (Dignass et al., 1994). In addition, it has been shown in an experimental in vivo model of ulceration in rat stomach that it is rSP and rITF that are upregulated in response to injury (Alison et al., 1995). Indeed, the trefoil expression is apparent before EGF and TGFα peptides, which are more typically associated with ulcer healing.

pS2 is expressed in adenocarcinomas of the colon, breast, pancreas, stomach, gall bladder and ovary (Henry et al., 1991; Seitz et al., 1991), and in cell lines derived from these tissues. Thus pS2 expression has been demonstrated in human cell lines of the breast: MCF-7, T47D and ZR75; stomach: Kato3, MKN45 and MKN28; and pancreas: CAPAN2 and BXPC3 (Mori et al., 1988; Takahashi et al., 1990; our unpublished observations). In view of this, to examine the effect of pS2 in isolation, we selected the murine mammary cell line 410.4 because it does not express pS2 (there is no cross-hybridisation with human pS2 cDNA probe at low stringency). In this paper we show how the transfection of human pS2 cDNA into the mouse 410.4 cell line causes changes in growth patterns in collagen matrix that could be consistent with its putative physiological role.

MATERIALS AND METHODS

Materials

410.4, a murine mammary adenocarcinoma cell line (Dexter et al., 1978), screened for mycoplasma infection, was obtained from our cell culture stock. Full-length pS2 cDNA as a BamHI/EcoRI insert in the pGem1 vector was provided by R. Playford (ICRF, London, UK). All reagents were purchased from Sigma (Dorset, UK) unless otherwise stated. The mBCEI$_1$ antibody (Prud'homme et al., 1990) was a gift from Prof. E. Milgrom (Faculty of Medicine Paris-Sud, Cedex, France) and the GE1 antibody (Elia et al., 1995) was kindly provided by G. Elia (ICRF, London, UK).

Cell culture

Unless otherwise indicated, all cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) supplied by Gibco BRL Ltd, Paisley, UK.

Transfection

Prior to transfection the 410.4 cell line was grown in increasing concentrations of puromycin (1-10 μg/ml) to ascertain the level of resistance and death. At a concentration of 2.5 μg/ml no viable cells were observed after 7 days in culture. The transfection vector pBppS2 was constructed by insertion of the 490 bp BamHI/EcoRI fragment containing pS2 cDNA into the retroviral expression vector pBabe Puro (Morgenstern and Land, 1990) (see Fig. 1, below), kindly provided by H. Land (ICRF, London, UK). Transfection was carried out by a modification of the CaPO$_4$ method (Chen and Okayama, 1987) using the Stratagene mammalian transfection kit (Stratagene, Cambridge, UK). Duplicate 90 mm tissue culture dishes were seeded with 2×10^4 410.4 cells per dish and at 20% confluency 10 μg of either pBpps2 (for pS2 transfection) or pBabe Puro (for vector control transfection), as a CaPO$_4$-DNA precipitate, was added. After 18 hours the medium was removed and the culture was washed with Dulbecco's PBSA, pH 7.2, and fresh medium was applied. After a further 24 hours of culture the cells were split and seeded into 10 Petri dishes at 1000 cells per dish. Cells were allowed to settle for 24 hours and then subjected to puromycin selection (2.5 μg/ml). Puromycin-resistant clones were isolated by ring cloning.

Southern blotting

Genomic DNA was prepared from cells grown to confluency in 15 cm Petri dishes. Cells were washed with ice-cold Dulbecco's PBSA, pH 7.2, before addition of 4 ml of lysis buffer (10 mM Tris buffer, pH 8.0, containing 100 mM NaCl, 10 mM EDTA, 0.5% SDS and proteinase K 50 μg/ml). Lysis was carried out at 37°C for 5 hours. The cell lysates were then extracted twice with phenol followed by choloroform/isoamyl alcohol (24:1, v/v), and the genomic DNA was ethanol precipitated. For Southern analysis 20 μg of genomic DNA was digested with KpnI and then run out on a 0.6% (w/v) agarose gel in TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8, buffer). The DNA was then transferred and fixed to Genescreen Plus hybridisation Transfer membrane (Du Pont Ltd, Herts, UK) by the salt transfer protocol (Sambrook et al., 1989).

Northern blotting

Total cellular RNA was isolated by the acid guanidium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) as modified by Cinna/Biotecx (Biogenesis Ltd, Poole, UK). For northern analysis 20 μg of the RNA samples were denatured at 65°C for 5 minutes in a solution containing 50% formamide and 2.2 M formaldehyde. The samples were run out on a 0.8% agarose gel using formaldehyde-containing buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, 4.75% formaldehyde). RNA quality and loading were assessed by ethidium bromide staining. Samples were transferred to Genescreen Plus hybridisation Transfer membrane in the presence of 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) and crosslinked in a UV Stratalinker 2400 (Stratagene, Cambridge, UK).

Hybridisation

Membranes from northern and Southern blots were probed with the 490 bp BamHI/EcoRI insert containing the full-length pS2 cDNA. The probe was labelled with [α-^{32}P]dCTP (Amersham Life Sciences, Bucks, UK) by random priming (Sambrook et al., 1989). Hybridisation was carried out at 42°C overnight in 50% formamide, 10% dextran sulphate, 1% SDS, 1 M NaCl and 20 μg/ml denatured salmon sperm DNA. The membranes were washed in 2× SSC for 5 minutes at room temperature, 2× SSC, 1% SDS for 30 minutes at room temperature, 2× SSC, 1% SDS for 30 minutes at 65°C, 0.2× SSC, 1% SDS for 30 minutes at 65°C and finally in 0.1× SSC, 1% SDS for 30 minutes at 65°C. The membranes were visualised after exposure to Hyperfilm-MP film (Amersham Life Sciences, Bucks, UK).

The level of E-cadherin expression was assessed by probing northern blots of total RNA of cells grown in collagen gel with the 2.5 kb EcoRI fragment of mouse E-cadherin cDNA from the

pBatEM2 vector (Nose et al., 1988) kindly provided by Dr M. Takeichi (Institute of Basic Biology, Kyoto University, Japan).

Immunoprecipitation

Cells for immunoprecipitation (IP) were grown to confluence in 15 cm dishes. Cells were washed in ice-cold Dulbecco's PBSA, pH 7.2, and then lysed for 30 minutes on ice with 1 ml of 50 mM Tris-HCl, pH 7.5, buffer containing 100 mM NaCl, 0.5% Nonidet P40, 0.2 mM Na_3VO_4, 50 mM NaF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 100 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone and 100 µg/ml 4-(2-aminoethyl)-benzenesulphonyl fluoride. Lysates were transferred to Eppendorf tubes and centrifuged at 13,000 g for 5 minutes. A 50 µl sample of pre-clearing complex, Protein G-Sepharose (Pharmacia Biotech Ltd, Herts, UK), incubated with pooled normal mouse immunoglobulins for 1 hour at 4°C, was added to the supernatants and incubated for 1 hour at 4°C on a rotator. A 1 µg sample of GE1 antibody was added to the recovered supernatant. Following a 1 hour incubation at 4°C, 50 µl of Protein G-Sepharose was added and a further 1 hour incubation was carried out. The beads were then washed 5 times with TBST (25 mM Tris-HCl, 150 mM NaCl, pH 7.2, containing 0.5% Tween 20) and finally resuspended in 50 ml of lysis buffer (0.5 M Tris-HCl, pH 6.8, buffer containing 10% SDS, 500 mM EDTA, β-mercaptoethanol and 5% glycerol). The lysates were boiled for 5 minutes, centrifuged at 13,000 g for 5 minutes and supernatants run out on a 15% SDS-PAGE gel and electroblotted onto nylon membranes (Bio-Rad Laboratories Ltd, Herts, UK). The membranes were washed in TBST, blocked with 5% dried skimmed milk in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.2) for 1 hour at room temperature. The membranes were incubated with $mBCEI_1$ (2.5 µg/ml) in TBST overnight, then washed in TBST (3× 10 minutes). The membranes were incubated for 1 hour at room temperature with biotinylated rabbit anti-mouse IgG antibodies (Dako Ltd, Bucks, UK) and then developed using the ABC method (Dako Ltd, Bucks, UK) followed by an ECL chemiluminescence assay (Amersham Life Sciences, Bucks, UK) and visualised by exposure to Hyperfilm-MP film (Amersham Life Sciences, Bucks, UK).

Collagen gels

Quadruplicate collagen type 1 gels using Vitrogen 100 (Celtrix Pharmaceuticals, Santa Clara, CA) and bovine dermal collagen were made up according to the manufacturer's instructions. Briefly, 8 vol. of collagen stock solution was mixed with 1 vol. of 10× DMEM, neutralised with 0.1 M NaOH and made up to 10 vol. with sterile distilled water in a sterile flask on ice. A 0.5 ml sample of appropriate cells was added to 5 vol. of the gel solution to give a final concentration $1×10^4$ cells per ml, and 1 ml aliquots were dispensed into 35 mm tissue culture dishes and allowed to gel for about 15 minutes at 37°C before addition of culture medium. For surface collagen gel growth studies gels were set up as above except that the cells ($1×10^4$ cells per gel) were added after the collagen gels had set. All collagen gels were fed with DMEM/10% FBS containing 2.5 mg/ml puromycin every 72 hours. Growth was assessed every 24 hours by examination under an Olympus IMT-2 phase-contrast microscope and followed for a maximum of 21 days. Collagen gels were fixed at weekly intervals in 10% formol saline overnight, processed and subsequently paraffin-embedded and serially sectioned (5 µm sections).

Histology and immunocytochemistry

Sections were routinely stained with haematoxylin and eosin (H and E). For fibronectin, type IV collagen and laminin detection sections were digested with protease for 20 minutes at 37°C and then stained immunohistochemically using a diaminobenzidine peroxidase-antiperoxidase technique using a 1 in 100 dilution of either anti-human laminin rabbit polyclonal antibody (EURO-PATH, Bude, UK) or anti-human fibronectin rabbit polyclonal antibody (Dako Ltd, Bucks, UK). The anti-human collagen type IV rabbit polyclonal antibody (EURO-PATH, Bude, UK) was used at a 1 in 1000 dilution

and developed using the ABC method. All of the antibodies cross-react with mouse proteins.

Quantification of colony type

A total of 300 colonies for each clone were counted from H and E-stained en face sections of collagen gels after 14 days growth (two separate sets of four collagen gels). The colonies were assigned to one of three categories: circumscribed, branched or dispersed (for a description see Results).

Cell aggregation assay

For the aggregation assay cells were grown in T75 cm^3 flasks to 70-80% confluency. The cells were washed with PBSA prewarmed to 37°C and then incubated with 5 ml of a non-enzymic cell-dissociation medium (Sigma, Dorset, UK) for 10 minutes at 37°C. Cells were washed with Ca^{2+}- and Mg^{2+}-free DMEM to obtain a single cell suspension. A total of $3×10^6$ cells were suspended in 3 ml of DMEM containing 0.8% FBS and incubated in a gyration shaker rotating at 100 rpm at 37°C. Duplicate 10 µl samples were withdrawn after 0, 15, 30, 45 and 60 minutes of incubation and the number of single cells was counted. The assay was repeated a total of 5 times and the data presented as $(N_t/N_o)×100$, where N_t and N_o refer to the number of single cells at time t and 0, respectively.

Motility assay using time-lapse videomicroscopy

Time-lapse videomicroscopy experiments were performed using Olympus IMT 1 or 2 microscopes that were enclosed within environmental chambers. High resolution monochrome CCD cameras (Sony M370CE) were attached to each microscope. Images were recorded using broadcast quality videorecorders (Sony Betacam PVW 2800) that were driven externally by animation controllers (BAC 900 from EOS Electronics AV Ltd, Barry, S. Wales).

For the motility assay cells were plated on 35 mm dishes at 10^4 cells per dish, 24 hours before the start of the assay. A field containing between 30 and 40 cells was chosen for each cell line. Images were recorded at one frame every minute for 24 hours. The recordings were downloaded onto an S-VHS videorecorder (Mitsubishi B-82), which was linked to an Apple MacIntosh 6100 computer, and the cells that remained within the observed field for the duration of the film were tracked using Cell Motility software (EOS Electronics). A printout of the track of each cell was obtained together with calculation off the mean distance and speed for each cell line.

RESULTS

Characterisation of transfected clones

For transfection the pS2 cDNA, a 490 bp BamHI/EcoRI restriction fragment, was inserted into the pBabe Puro retroviral expression vector as is shown in Fig. 1. In this vector the promoter in the 5′ Mo MuLV long terminal repeat (LTR) is used to transcribe the $pS2$ inserted gene and the internal SV40 early promoter expresses puromycin drug resistance marker, both transcripts sharing a common termination and poly-adenylation signal in the 3′ LTR. Transfectants were selected by their ability to grow in media supplemented with puromycin at 2.5 µg/ml, a concentration that was shown to kill 410.4 cells. Two pS2 cDNA transfected clones (AA4, AD4) and one clone (VA5) from transfection with pBabe Puro were isolated by ring cloning. Genomic DNA isolated from all three clones (AA4, AD4 and VA5) and the parental cell line was digested with KpnI, Southern blotted and hybridised with a pS2 cDNA probe. The resulting blot (Fig. 2A) shows that both AA4 and AD4 display a single band of 3 kb; by contrast, neither the parental
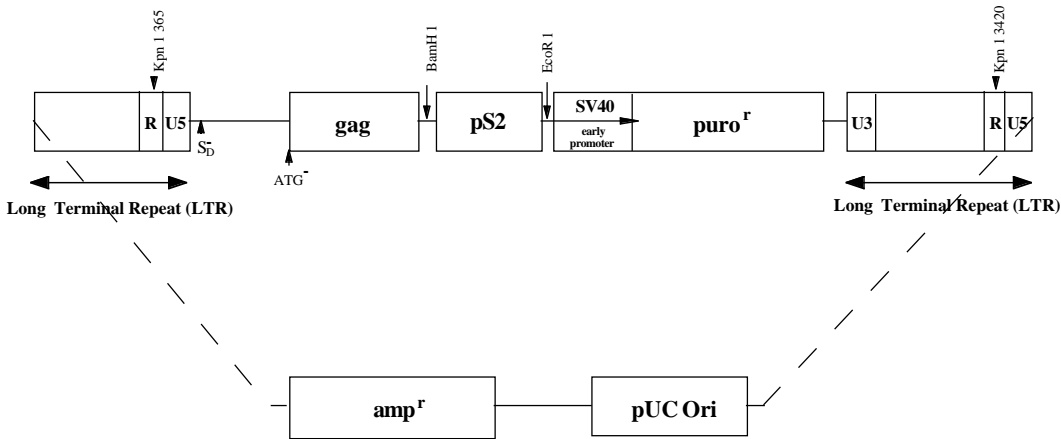
Fig. 1. Map of pBppS2 transfection vector.

cell line nor the vector control VA5 clone shows any bands. The 3 kb band is in keeping with the expected size of the *Kpn*I fragment of the pBppS2, since there is one *Kpn*I site in each of the LTRs (see Fig. 1), and indicates no gross rearrangement or truncation of integrated DNA copy. This clearly demonstrates that both pS2 transfected clones have incorporated the pS2 cDNA into the 410.4 genome.

The presence of pS2 transcripts in the clones was determined by northern analysis of total RNA. The northern blot analysis for pS2 containing transcripts (Fig. 2B) shows clear 3.5 kb bands in both the AA4 and AD4 clones that correspond to the expected full-length transcript from 5′ viral LTR (see Fig. 1). The presence of an extra band at 2 kb in the AD4 clone tract may be due to a splicing event from the cryptic termination or a poly(A) site at the 3′ end of the pS2 cDNA insert (Dai et al., 1993; Kirschmeier et al., 1988). This band is also observed in the AA4 clone on prolonged exposure of the northern blot (data not shown). Levels 6-fold higher, as determined by laser densitometry (Molecular Dynamics, Bucks, UK), of the transcript are present in the AD4 clone. No bands were evident with VA5 or 410.4 RNA samples.

To ascertain whether the transcripts were appropriately translated immunoprecipitation was carried out on the cell lysates using the GE1 anti-pS2 antibody. Fig. 2C shows a western blot of the immunoprecipitates developed using the anti-pS2 mBCEI$_1$ antibody. Bands at approximately 7 kDa were shown by AD4 and AA4 but not 410.4 and VA5. The band size matches that obtained for pS2 immunoprecipitation from MCF-7 cells (Elia et al., 1995). It is also evident that the AD4 clone expresses greater amounts of pS2 peptide than the AA4 clone. From these results we can say that both AD4 and AA4 contain pS2 cDNA at the genomic level, produce pS2-containing transcripts and produce the correct translation product.

Growth on plastic and in type 1 collagen gels

When grown on plastic the pS2 transfected clones AD4 and AA4 showed the same morphology as the vector control transfectant (VA5) and the parental cell line. No difference in cytological characteristics or growth rate measured by DNA fluorimetry (Rao and Otto, 1992) was evident (data not shown).

Phase contrast microscopy (7-14 days)

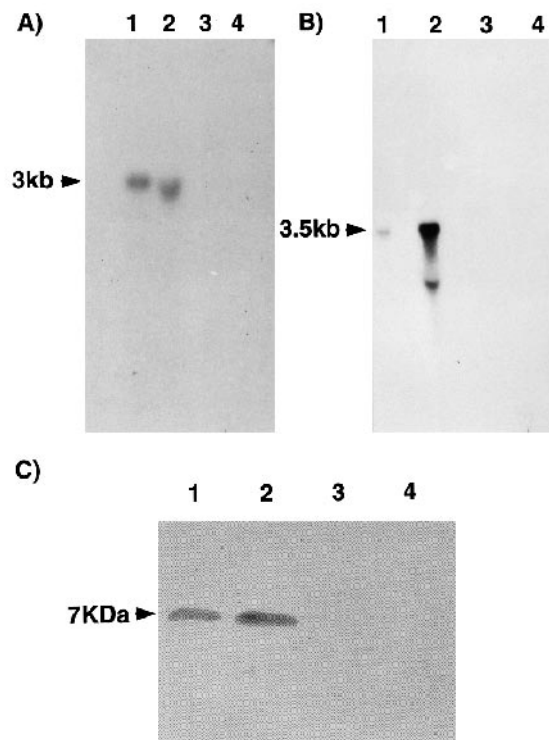To establish whether expression of pS2 would lead to differ-

Fig. 2. Characterisation of transfectants. (A) Southern blot of genomic DNA from transfectants and parental cell line. A 20 μg sample of genomic DNA was digested with *Kpn*I and run out on a 0.6% agarose gel in TBE. The DNA was transferred to a GeneScreen nylon membrane and probed with ^{32}P-labelled 490 bp pS2 cDNA. (B) Northern blot of total RNA from transfectants and parental cell lines. A 20 μg sample of total RNA was run out on 0.8% agarose formaldehyde gel and transferred to a GeneScreen nylon membrane. The membrane was probed with ^{32}P-labelled 490 bp pS2 cDNA. (C) Immunoprecipitation of pS2 peptide from transfected clones. GE1 immunoprecipitates from cell lysates were run out on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was developed with the anti-pS2 antibody mBCEI[1]. Throughout, lane 1 refers to AA4 clone, lane 2 to AD4 clone, lane 3 to the vector control (VA5) and lane 4 to the parental cell line.

ences in growth pattern the clones were propagated in type 1 collagen matrix. Phase-contrast microscopy showed marked differences in the growth pattern of the transfectants as early
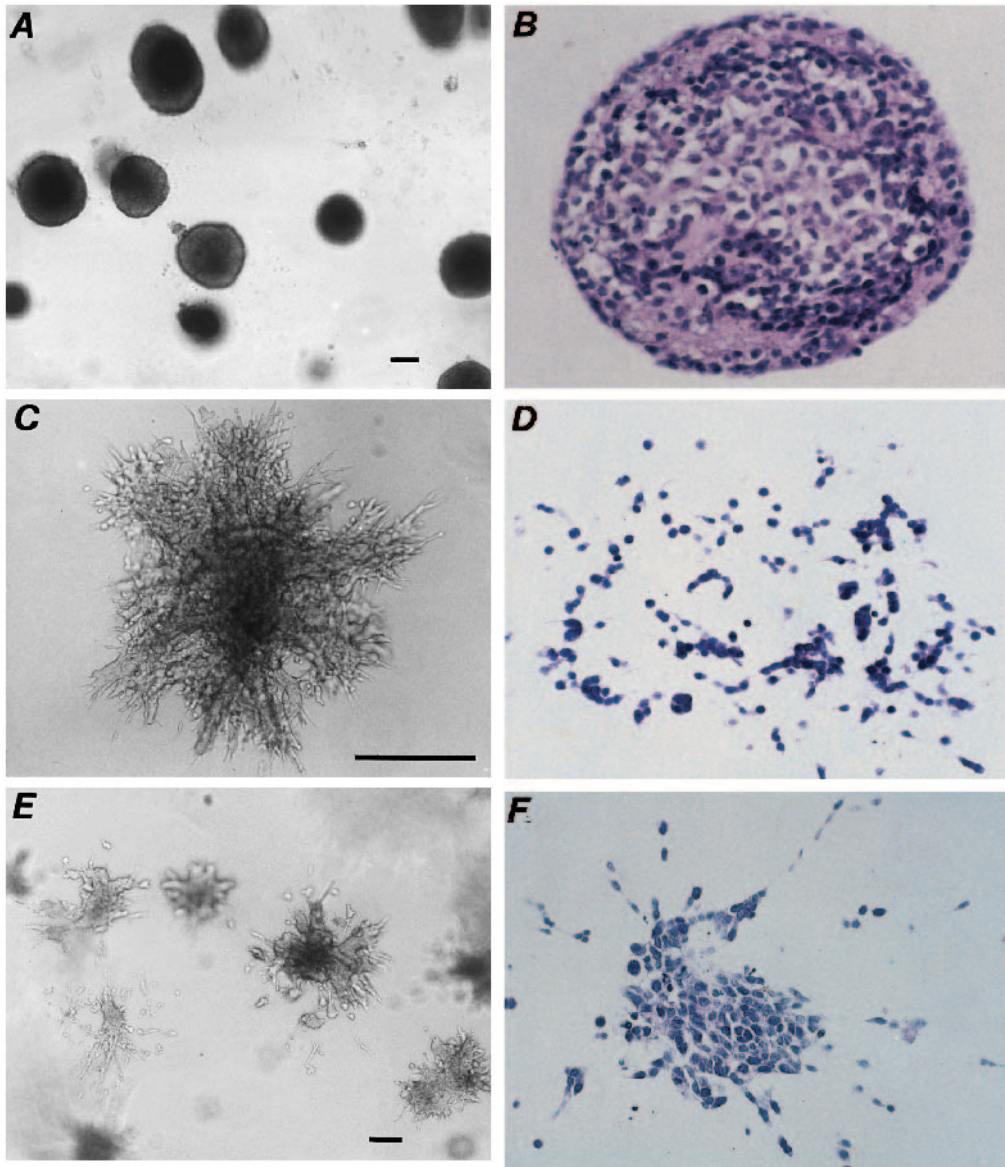
Fig. 3. Growth of transfectants in collagen type 1 gels. (A) A phase-contrast photograph of VA5 colonies formed after 14 days growth in collagen gel. (B) H and E-stained en face section of a typical VA5 colony. (C) A phase-contrast photograph of a typical AD4 colony after 14 days growth in collagen gel. (D) H and E-stained en face section of a typical AD4 colony. (E) A phase-contrast photograph of AA4 colonies formed after 14 days growth in collagen gel. (F) H and E-stained en face section of typical AA4 colony. Bars, 150 µm.

as after 7 days of growth. Both the parental and vector control (VA5) cell lines formed small tight compact spherical colonies (Fig. 3A), while the pS2 transfectants formed dispersed stellate structures with peripheral thin extensions (Fig. 3C,E). These differences became even more pronounced after 14 days in culture.

Histology of cells grown in collagen (7-14 days)

H and E-stained sections of the collagen gels (Fig. 3B) show that VA5 forms tight compact colonies with circumscribed margins, composed of large cells with abundant cytoplasm. In addition there is prominent extracellular matrix deposition in the colony shown up as hyaline eosinophilic aggregates. Occasional less circumscribed colonies with broad short projections into the gel were observed. The parental cell line had a similar appearance. Sections of the transfectants show that the dispersed structures with radiating cords are essentially composed of extensive cords of elongated single cells migrating out into the gel (Fig. 3D,F). No tubular formations

were observed and thus this process is not regarded as a morphogenetic phenomenon. No matrix deposition was evident. Confirmation that pS2 was expressed in cells grown in collagen was obtained from northern analysis of pS2 RNA extracted from cells grown in collagen gel. A similar level of pS2-containing transcript was produced as was observed with cells grown on plastic (data not shown).

Quantitative analysis of structures

Three types of colonies were quantified on the gel sections. Circumscribed colonies were typified by VA5. Branching colonies had outgrowths into the gel, which are short, often clubbed and composed of rounded or polygonal cells, which are often over 2 cells in width. Dispersed colonies had radiating cords of cells, usually elongated, with no more than 1-2 cells at the extremities. Fig. 4 shows a quantitative analysis of the colony type formed by the transfectants (day 14). The vector control VA5 showed predominantly circumscribed structures (94.5%) with some branching (4.2%), and very little in the way
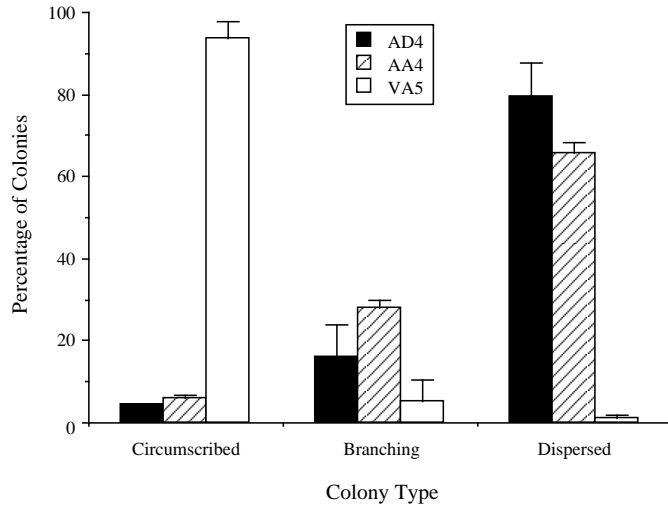
Fig. 4. Quantification of colony types formed by transfectants in collagen gels. The type of colonies formed by the pS2 transfectants AD4 and AA4 and the vector control VA5 were counted in H and E-stained en face sections from two separate sets of four collagen gels after 14 days growth (300 colonies were counted). The results represent the mean ± s.e.m.

of dispersed colonies (1.3%) was evident. Some of these may even have arisen by intracolony cell death and regeneration, as they appeared to contain cellular debris. Only 3% of AD4 and 5% of AA4 colonies were circumscribed, with a majority being dispersed, 80% in the case of AD4 and 68% for AA4. There would appear to be a correlation between the extent of dispersion and the level of pS2 expression. The higher pS2 expressing clone AD4 showed a greater tendency to form dispersed colonies than the lower expressor AA4.

Growth on type 1 collagen gels

When the clones were grown on the surface of collagen gels a similar difference was observed in the growth pattern of the pS2 transfectants compared to the vector control. Under phase-contrast the parental and VA5 cell lines (vector control) formed large circumscribed spherical colonies showing very little lateral migration, whereas the transfectants spread over the gel surface. Each gel was longitudinally serially sectioned and stained. Sections of the parental gel showed large spherical colonies protruding above the surface of the gel, with very prominent deposits of ECM forming hyaline eosinophilic aggregates (Fig. 5A). The pS2 transfected clones spread out over the surface of the gel with no nodular growths or ECM deposition (Fig. 5B,C). Significantly, we found no evidence of invasion into the collagen gel by VA5, AD4 or AA4 clones.

Immunocytochemistry (data not shown) on sections did not reveal any significant difference in the amount of laminin, fibronectin and collagen type IV staining between the pS2 transfected clones and the vector control, although these components tended to be more spread out in close approximation to the cells in the transfectants. However, the majority of the eosinophilic ECM did not show specific immunoreactity for specific matrix components, which is predictable from studies on hyalinised ECM in human tissues.

It has been reported that dispersant growth patterns of cells are mediated by changes in their cadherin-based cell adhesion ability (Behrens et al., 1993) and that E-cadherin expression is

Table 1. Motility assay

Cell line	Cell motility (μm/h)
VA5	12.64±1.13
AD4	29.87±1.75

The results represent the mean speed (μm/h) determined for 40 cells of each cell line ± s.e.m.

frequently down-regulated in highly invasive, poorly differentiated carcinomas (Frixen et al., 1991; Behrens et al., 1991; Pignatelli et al., 1992). The level of E-cadherin expression of the transfectants was assessed by northern analysis of total RNA extracted from cells grown in collagen gel using a mouse E-cadherin cDNA probe (Nose et al., 1988). A single band of 4.5 kb was observed in all RNA samples and there was no difference in the level of expression in the pS2 transfectants compared to the VA5 and parental cell lines as determined by densitometric analysis (data not shown). To determine whether the observed changes were due to changes in E-cadherin-mediated cell adhesion, the calcium-dependent cell-cell aggregation of the pS2 transfectants was compared with that of the vector control. From Fig. 6 it is evident that pS2 expression does not result in any changes in the calcium-dependent cell-cell aggregation. It is therefore unlikely that the observed dispersant growth pattern of the pS2 transfectants changes are due to alteration in E-cadherin function.

It is possible that the changes in growth pattern of 410.4 cells as a result of pS2 expression could be explained by pS2 causing an increase in cell motility. To test this a motility assay was carried out on AD4 pS2 transfectant and the vector control VA5. The results show that the pS2 transfectant has a 2.4-fold increase in motility over that of the vector control cell line (Table 1).

DISCUSSION

We describe the first model system employing human *pS2* gene transfer and expression in a non-pS2-expressing cell line. There is a high degree of conservation between human and mouse pS2 (Lefebvre et al., 1993), and we could not show any cross-hybridisation of human pS2 probes in the parental cell line at low stringency. Our findings were that both the AD4 and AA4 clones had integrated the pS2 cDNA into the 410.4 genome, and produced pS2-containing transcripts and the mature pS2 peptide. The AA4 transfectant showed lower levels of pS2 transcript and peptide compared to the AD4 clone.

From the growth in collagen type 1 gels it is clear that pS2 expression in the two transfectants resulted in a more dispersed pattern of growth. The vector control clone (VA5), like the parental cell line, typically formed cohesive islands of cells showing no invasion into the collagen matrix, with prominent ECM deposition. The pS2 transfectants tended to form poorly cohesive stellate colonies from which radiate thin cords of elongated cells into the collagen matrix, with no visible ECM deposition. The extent to which the transfectants formed these dispersed structures appeared to correlate with the level of pS2 expression, with the lower pS2 expressor, AA4, showing a smaller degree of dispersion.

Assay of cellular motility using videomicroscopy clearly

showed a marked increase in the speed and distance travelled by the AD4 transfectant. Since dispersion might be a consequence of reduced intercellular cohesion, which is primarily mediated by E cadherin (Takeichi, 1991), we performed cell-cell aggregation assays, which showed no differences between vector controls and transfectants. Moreover, there were no differences in E cadherin expression as determined by northern analysis.

Thus pS2 appears to exert a motogenic effect on 410.4 cells, but not a mitogenic or a morphogenetic effect. A similar motogenic or 'scatter' effect is induced by hepatocyte growth factor (HGF), which has been shown to dissociate layers of epithelial cells, increasing their motility and invasiveness (Stoker et al., 1987; Weidner et al., 1990). However, HGF is also a mitogen for kidney tubular epithelium, keratinocytes, endothelial cells and melanocytes (Rubin et al., 1991; Bussolino et al., 1992), and can function as a morphogen stimulating the three-dimensional organisation of Madin-Darby canine kidney cells (MDCK) (Montesano et al., 1991). It has also been shown to promote the progression of carcinoma cells towards a malignant invasive phenotypes (Weidner et al., 1990).

Early studies with PSP suggested that this trefoil had a mitogenic activity towards MCF-7 and HCT 116 cell lines (Hoosein et al., 1989). However, the levels of PSP required to elicit a response were 10 times higher than the expected range for most growth factors. We have been unable to reproduce this effect and have found no mitogenic activity for hSP in a number of cell lines (unpublished observations). Recently, Dignass et al. (1994) also failed to observe any mitogenic activity for hSP, rITF and hITF on a panel of cell lines. In this study, expression of pS2 had no effect on the proliferation of the 410.4 cell line. It therefore seems unlikely that trefoils are mitogens. Since we did not demonstrate any invasive action of pS2-expressing cells from the surface into the collagen matrix, it may be that pS2, unlike HGF, induces a spreading rather than an invasive phenotype. Indeed, we have demonstrated that pS2 expression results in increased cell motility.

The changes in growth pattern of the 410.4 cell line brought about by pS2 expression were associated with an alteration in ECM deposition. Prominent deposits of ECM were shown by the vector control. Both the pS2 transfectants showed very little in the way of visible ECM when propagated in or on the surface of collagen gels. No quantitative differences in type IV collagen, fibronectin or laminin were apparent apart from redistribution commensurate with the alteration in growth pattern. It may be that other components of the ECM associated with cell migration and spreading, such as tenascin, vitronectin or laminin fragments, possibly in the non-immunoreactive parts of the ECM, could be responsible for these effects.

It has been demonstrated that the motogenic and morphogenic activity in epithelial cells induced by HGF is associated with degradation of ECM. Thus the HGF-induced morphogenesis of MDCK cells when grown in collagen type 1 is repressed by protease inhibitors (Montesano et al., 1991). It is conceivable that pS2 expression leads to an increased expression of proteases, resulting in a degradation in ECM, which leads to a loosening of cell interaction, an area that we are currently investigating.

Is there any relationship between these in vitro effects of pS2 and its physiological role? Trefoil peptides are a normal component of the gastrointestinal mucosa. The expression of trefoil peptides in response to mucosal injury has led to the suggestion that they participate in the ulcer-healing response. Thus pS2 and hSP expression is found in the ulcer-associated cell lineage (UACL) in chronic ulcerative conditions such as Crohn's disease (Wright et al., 1990a,b, 1993). This differentiating cell lineage buds from the bases of intestinal crypts adjacent to the ulcer, ramifying and anastomosing in the submucosa and finally fusing with the villous epithelial surface via terminal ducts (Wright et al., 1990a). UACL development with concomitant pS2/hSP expression has also been observed at other sites of chronic endodermal injuries such as ducts in chronic pancreatitis or biliary obstruction, and in nasal polyps (Wright et al., 1990b; Seitz et al., 1991).

Healing of ulcerative lesions occurs throughout the gastrointestinal tract and is characterised by the ability to reconstitute the mucosa followed by the re-formation of specialised structures. When a mucosal defect occurs, it is initially covered by a layer of necrotic cells, fibrin and mucus termed the 'mucoid cap'. This mucoid cap may be of particular importance in the stomach in re-establishing a pH gradient so that the cells of the surviving mucosa are maintained at a neutral pH rather than that of the acidic gastric juice (Wallace and Whittle, 1986). Epithelial cells at the margin of the defect migrate to re-establish a continuous epithelial layer. This process is termed 'epithelial restitution'. Restitution occurs within the first hour following injury and over the next 48 hours cellular proliferation and differentiation in adjacent glands occur, re-establishing the normal architecture. In ulcer healing it is the re-epithelialisation of the damaged area that is the important first response and this is achieved by epithelial cells from the wound edges migrating into the area, and does not involve a proliferative response (at least initially). Indeed it has been shown that healing of mucosal erosions (injury not involving the full thickness of the mucosa) in rats is very rapid. Silen and Ito (1985) have demonstrated complete re-epithelialisation of totally desquamated surfaces within 1 hour by migration of cells from neighbouring pits before cell proliferation and inflammation.

Our observation that pS2 has the potential to induce a migratory response, taken together with the topographical expression of pS2 in response to ulcerative damage, supports a role for trefoil peptides in ulcer healing. Our results are compatible with the recent observation that showed that two trefoil peptides, intestinal trefoil factor (ITF) and human spasmolytic polypetide (hSP), accelerated restitution of wounded monolayers in an in vitro restitution assay (Dignass et al., 1994). This study also demonstrated that the effect is not necessarily species-specific, since hITF was as effective as its rat equivalent in the restitution assays carried out on rat intestinal epithelial cell lines. Further support for the proposed role comes from the observation of an up-regulation of rSP and rITF at ulcer margins in experimentally induced gastric ulcers in rat (Alison et al., 1995). This expression of the trefoils in response to injury occurs prior to the expression of the EGF and TGFα molecules traditionally associated with ulcer healing. It is conceivable that pS2, as well as other trefoils, initiates the healing process by induction of a migratory response in the epithelial population around the margins of the ulcer. This would explain the high levels of pS2 and hSP expression in the gastric mucosa, since a high level of restitutive peptides would be necessary to enable a rapid re-estab-
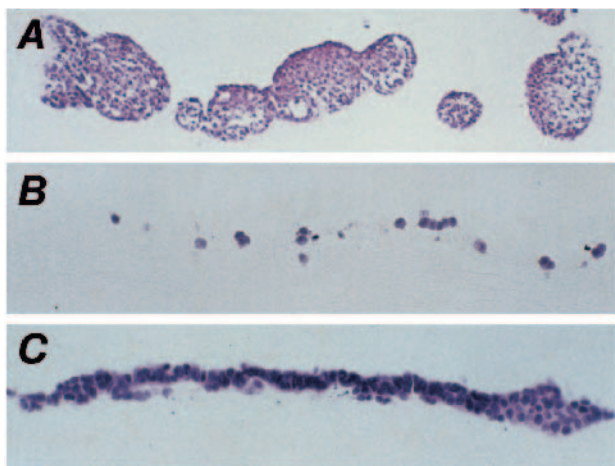
Fig. 5. Growth on collagen type 1 gels. (A) H and E-stained section of parental cells grown on collagen gel. (B) H and E-stained section of AD4 clone grown on collagen gel. (C) H and E-stained section of AA4 clone grown on collagen gel. (In all figures the collagen gels were embedded on edge after 14 days in culture and the sections are oriented with the collagen gel at the bottom of the field.)
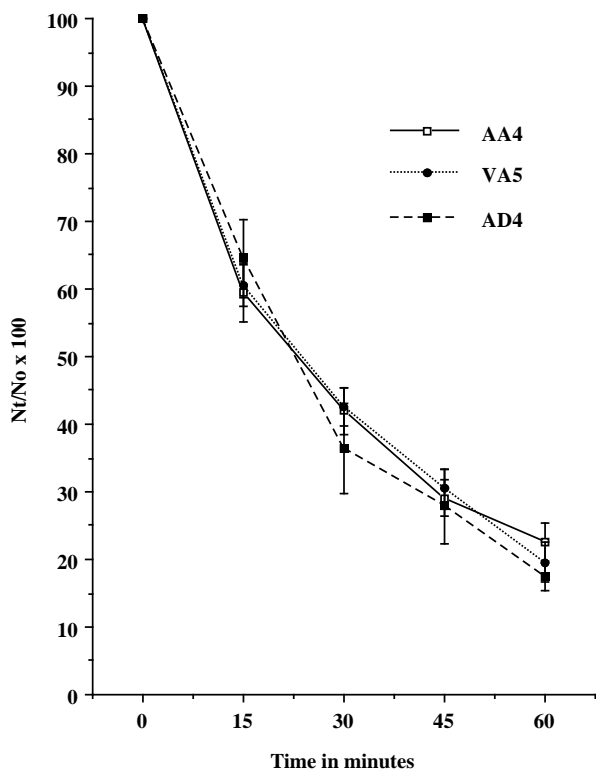

Fig. 6. Cell aggregation assay. N_t and N_0 refer to the number of single cells at time t and time 0, respectively. The results represent the mean values of five separate experiments ± s.e.m.

lishment of mucosal integrity in a particularly hostile environment. Presumably, the formation of UACL at sites of more extensive damage provides a large pool of trefoils to facilitate restitution.

Our data are therefore consistent with a role for trefoil peptides in the maintenance and restoration of mucosal integrity following mucosal injury and ulceration.

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