PERGAMON

European Journal of Cancer 36 (2000) 1171–1179

European Journal of Cancer

www.ejconline.com

Analysis of growth factor-dependent signalling in human epithelioid sarcoma cell lines: clues to the role of autocrine, juxtacrine and paracrine interactions in epithelioid sarcoma

C.-D. Gerharz *, U. Ramp, P. Reinecke, C. Schardt, U. Friebe, M. Déjosez, T. Nitsch, H.E. Gabbert

Institute of Pathology, University Hospital, Moorenstr. 5, 40225 Düsseldorf, Germany

Received 21 December 1999; received in revised form 7 January 2000; accepted 13 January 2000

Abstract

Human epithelioid sarcoma (ES) is an extremely aggressive soft tissue tumour of unknown histogenesis. Although growth factor-dependent signalling cascades significantly affect the biological behaviour of malignant tumours, little is known so far about their role in human ES. The present investigation, therefore, analyses the coexpression and function of different growth factors and their receptors in the human ES cell line GRU-1 and its clonal subpopulations (GRU-1A, GRU-1B and GRU-1C). As shown by Northern blot, flow cytometry, immunocytochemistry and MTT assay, all ES cell lines expressed transforming growth factor (TGF)-α and the epidermal growth factor receptor (EGF-R). Although no response to exogenous TGF-α was observed, antagonistic anti-EGF-R antibodies (at 20 μg/ml) induced significant ($P < 0.05$) growth inhibition in all cell lines. All cell lines showed coexpression of platelet-derived growth factor (PDGF)-A and the corresponding receptors. Neutralisation of ES-derived PDGF by anti-hPDGF antibodies resulted in significant ($P < 0.05$) growth inhibition of all clonal subpopulations. Although all cell lines expressed TGF-β_1 as well as TGF-β type I and type II receptors (TGF-BI-R and TGF-BII-R), growth inhibition ($P < 0.05$) by exogenous TGF-β_1 was achieved in the clonal subpopulations only and not in the parental cell line. No ES cell line expressed acidic fibroblast growth factor (FGF) but stimulation of FGF type 3 and type 4 receptors (FGF-3R and FGF-4R) by exogenous acidic FGF (aFGF) resulted in a marked ($P < 0.05$) acceleration of proliferation in all cell lines. In conclusion, our investigation suggests an intricate network of autocrine, juxtacrine and paracrine signalling between ES tumour cells and adjacent non-neoplastic stromal cells.

Keywords: Epithelioid sarcoma; TGF-α; PDGF; FGF; TGF-β

1. Introduction

Human epithelioid sarcoma (ES) is a peculiar malignant soft tissue tumour of unknown histogenesis exhibiting a mixed phenotype with large polygonal cells imparting an epithelial aspect as well as mesenchymal spindle-shaped cells [1–5]. From the clinical point of view, ES is an extremely aggressive tumour showing a high rate of recurrence and metastasis [1]. Since conventional chemotherapy and irradiation have proved to be ineffective, the prognosis of ES beyond the reach of curative surgery is poor [1,6–8]. Therefore, the characterisation of the complex network of growth factors active in ES might provide important new insights into the mechanisms determining its biological behaviour and eventually facilitate the design of novel concepts in tumour therapy [9].

Although initial immunohistochemical studies indicated a strong expression of the epidermal growth factor receptor (EGF-R) in human ES [10,11], further investigations into the coexpression of different growth factors and their receptors as well as the functional intactness of the corresponding signal transduction pathways have not to date been performed in this tumour type. The aim of our investigation, therefore, was to analyse the coexpression of different growth factors and their receptors in appropriate *in vitro* models, which became

* Corresponding author. Tel.: +49-211-81-00; fax: +49-211-811-8353.

0959-8049/00/$ - see front matter
PII: S0959-8049(00)00053-8

available with the development of the ES cell line GRU-1 and its clonal subpopulations in our laboratory [12–16]. These clonal subpopulations have previously been shown to differ in morphology, invasiveness and response to retinoic acid, tumour necrosis factor (TNF)-α and paclitaxel [13–16]. In the present study, these clonal cell lines were used to provide additional clues to the biological implications of tumour heterogeneity for the expression and function of growth factors in ES. Because ES exhibits a peculiar mixed phenotype with both epithelial and mesenchymal features of differentiation, we focused on growth factors known to affect both epithelial and mesenchymal cells [17].

2. Materials and methods

2.1. Cells and culture

The human ES cell line GRU-1 and its clonal subpopulations (GRU-1A, GRU-1B, and GRU-1C) were established in our laboratory [12,13]. The cell lines were maintained at 37°C in an atmosphere with 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin.

2.2. Immunohistochemical and immunocytochemical analysis

Formalin-fixed, paraffin-embedded tissue of the three different ES was analysed by immunohistochemistry. For immunocytochemical analysis, tumour cells of the parental cell line and its clonal subpopulations were seeded onto microscopic slides and fixed *in situ* by exposure to methanol (5 min) and acetone (10 s) at −20°C. The slides were stained for EGF and TGF-α (Ab-3, Oncogene Science, Schwalbach, Germany), EGF-R (E-30, Merck, Darmstadt, Germany), fibroblast growth factor type 4 receptor (FGF-4-R) (C-16, Santa Cruz, Santa Cruz, USA), platelet-derived growth factor (PDGF) (PC-21, Oncogene Science), platelet-derived growth factor receptor (PDGF-R) (GR-14, Oncogene Science), transforming growth factor (TGF)-β1 (V, Santa Cruz), TGF-β type I receptor (TGF-β I-R) (V-22, Santa Cruz) and TGF-β type II receptor (TGF-β II-R) (H-567, Santa Cruz) by the avidin–biotin complex peroxidase method. Proper negative controls were performed for all antibodies.

The percentage of positive tumour cells was graded as follows: 0, none; 1, <10%, 2, 10–50%; 3, 51–80%; and 4, >80%. Immunostaining intensity was rated as follows: 0, none; 1, weak; 2, moderate; and 3, strong. To estimate the protein expression, a score was calculated in which the per cent positive rating was multiplied by the intensity rating: (+) score 1–3, +, score 4–6, ++, score 8–9; and +++, score 12.

2.3. DNA and RNA extraction

Aliquots of the cell lines were kept at −70°C until DNA and RNA preparation. Genomic DNA and total cellular RNA were isolated by the guanidine–thiocyanate method with minor modifications. After caesium chloride ultra-centrifugation, genomic DNA was extracted with phenol/chloroform and finally precipitated with ethanol. Total cellular RNA was further extracted according the method of Chirgwin and associates [18].

2.4. Northern blot analysis

The concentration of the purified total cellular RNA was measured by spectrophotometry at 260 nm and the quality verified in an ethidium bromide stained agarose gel. Northern blot analysis was carried out for 20 μg RNA of each sample under denaturating conditions electrophoretically separated on a 1% formaldehyde gel. Prior to the transfer of the RNA to Nylon membranes, the gel was stained with ethidium bromide and the equality of RNA amounts loaded in each lane was verified under ultraviolet (UV) light and photographed. In a second control, complete transfer of the RNA from the gel to the Nylon membranes was again verified under UV light. Afterwards, the RNA was hybridised with specific DNA probes. The DNA was labelled by incorporation of [^{32}P]dCTP using an oligo-labelling kit (Pharmacia, Freiburg, Germany). Hybridisation, stringent washing procedures and fluorography were carried out as previously described [19]. Fluorography was carried out by exposure of Kodak X-Omat film for 10 days to the dried filters at −70°C in conjunction with intensifying screens. The probes were obtained from the purified inserts of the following plasmids: TGF-α (ATCC no.: 59950; pBR 327), insert: *Eco*RI. EGF (ATCC no.: 59958; pUC 9), insert: *Eco*RI. EGF-R (ATCC no.: 57346; pBR 322), insert: *Cla*I. PDGF-A (C.H. Heldin; pUC 13), insert: *Eco*RI. PDGF-B (C.H. Heldin; pUC 13), insert: *Eco*RI. PDGF-α-R (C.H. Heldin; pUC 19), insert: *Eco*RI/*Acc*I. PDGF-β-R (C.H. Heldin; pUC 19), insert: *Pst*I. TGF-β_1 (S. Qian; pBluescript), insert: *Hin*dIII/*Xba*I. TGF-β I-R (C.H. Heldin; pSV7d), insert: *Eco*RI. TGF-β II-R (R.A. Weinberg; pcDNAI), insert: *Eco*RI. TGF-β III-R (J. Massague; pGEM 4Z), insert *Eco*RI. Acidic FGF (aFGF) (ATCC no.: 53336; pUC 18), insert: *Eco*RI. FGF-3 receptor (FGF-3-R) (ATCC no.: 65796; pGEM-3Zf+), insert: *Eco*RI. FGF-4-R (ATCC no.: 65790; pGEM-3Zf+), insert: *Eco*RI. All experiments were done twice with different Nylon membranes and the results obtained were reproducible. Proper positive controls were performed in all blots.

2.5. Enzyme-linked immunosorbent assay (ELISA) analysis

For the determination of TGF-α or TGF-β_1, supernatants were obtained from confluent cultures of GRU-1 and its subpopulations incubated for 96 h in 5 ml (TGF-β_1) or 10 ml (TGF-α) of serum-free standard growth medium (DMEM). Sandwich enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's recommended procedures (TGF-α: Dianova, Hamburg, Germany, no. QIA 05, FRG; TGF-β_1: Quantikine TM, no. DB 100, R&D Systems, Europe). All samples were run in duplicate: Colour intensity was measured at 490 nm (TGF-α) or 450 nm (TGF-β_1) using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The minimal detectable concentrations were found to be 10 pg/ml for TGF-α or 5 pg/ml for TGF-β_1. Activation of latent TGF-β_1 to immunoreactive TGF-β_1 was achieved by acidification according to the manufacturer's recommended procedure and all samples were measured before and after activation. No detectable levels of biologically active TGF-β_1 or TGF-α were observed in standard growth medium (DMEM supplemented with 10% FCS).

2.6. Flow cytometry

Tumour cells were harvested before reaching the plateau phase of growth, washed twice in phosphate buffered saline (PBS)/0.1% bovine serum albumin (BSA) and 10% DMEM and adjusted to a cell number of 2×10^6/ml. Indirect immunostaining of EGF-R was performed using the specific anti-EGF-R antibody (clone 528,GRO1L, Dianova; concentration: 0.1 μg/2×10^5 cells) for 1 h at 4°C. Cells were washed twice and incubated with goat anti-mouse 5-([4,6-dichlorotriuzin-2-yl]-amino)-fluorescein (DTAF)-conjugated IgG (No. 115-015-062, Dianova; concentration: 0.25 μg/2×10^5 cells). The isotype control was performed with a non-relevant mouse IgG (No. 115-000-003, Dianova; concentration: 0.1 μg/2×10^5 cells). Flow cytometry was performed with a Cytoron absolute (Ortho Diagnostic Systems, Neckargemünd, Germany). Cells were analysed for cell volume, right-angle light scatter and green fluorescence. Data were acquired in the list mode. Green fluorescence was collected with a logarithmic amplifier and analysed with the software program Immunocount (Ortho Diagnostic Systems, Neckargemünd, Germany). Cells were gated on forward and sideward scatter to exclude debris. Anti-EGF-R stained cells were compared with unstained cells and cells stained with the irrelevant mouse IgG as isotype control. As a internal control for EGF-R expression the cell line A-431 (positive control) and the cell line U-937 (negative control) were used, both purchased from the European Culture Collection. All experiments were done twice and the results were reproducible.

2.7. Assessment of growth properties after exposure to exogenous growth factors or antagonistic antibodies

TGF-α (Hermann Biermann, Bad Nauheim, Germany) was prepared as a 100 μg/ml stock solution in PBS and 0.1% BSA and then added to the cultures to yield a final concentration of 1 ng/ml and 10 ng/ml, respectively. PDGF-AA, PDGF-BB and aFGF (Hermann Biermann) were prepared as a 10 μg/ml stock solution in 0.1% BSA and 4 mM HCl and then added to the cultures to yield final concentrations of 10 ng/ml and 100 ng/ml, respectively. TGF-β_1 (Hermann Biermann) was prepared as a 2 μg/ml stock solution in 1 mg/ml BSA and 4 mM HCl and then added to the cultures to yield a final concentration of 1 ng/ml and 10 ng/ml, respectively. The antagonistic anti-EGF-R antibody Ab-1 (=MoAb-528 [20]; Dianova), which inhibits ligand binding was added to the cultures to yield a final concentration of 2 μg/ml and 20 μl/ml, respectively. The anti-hPDGF antibody (R+D Systems, Wiesbaden, Germany), which neutralises the biological activity of all human PGDF isoforms (AA, AB, BB), was added to the cultures to yield a final concentration of 0.2 μg/ml and 2.0 μg/ml, respectively.

2.8. In vitro *proliferation assay*

Tumour cells in the exponential growth phase were counted via a haemocytometer and then transferred to 96 microwell titre plates (Gibco) at 10 000 cells per well in 0.1 ml of standard growth medium. Preliminary studies had demonstrated that this cell number did not result in confluent cultures at the end of the observation period of 168 h and produced the optimal absorbance value for the 3-[4,5-dimethylthiazol-2-yl]2,5 diphenyltetrazolium bromide (MTT) assay. After 24 h, the tumour cells were exposed to exogenous growth factors in growth medium containing either 1% FCS (TGF-α, PDGF-AA, PDGF-BB, aFGF) or 10% FCS (TGF-β_1). The first column of each microwell plate served as a blank and the second was used as a control containing tumour cells in standard growth medium with 1% FCS or 10% FCS, respectively, but without the growth factor supplement. The plates were incubated for another 72 and 168 h (TGF-β_1 only) at 37°C and 5% CO_2 without further renewal of growth medium. The number of viable tumour cells was then estimated using the colorimetric MTT assay as described by Mosman [21]. The MTT assay is based on the reduction of MTT by a mitochondrial succinyldehydrogenase in viable cells to a formazan derivative that can be measured spectrophotometrically. Briefly, 0.25 mg of MTT dissolved in PBS (Serva, Heidelberg, Germany) was added to each well and incubated for another 4 h at 37°C. Formazan crystals were dissolved by exposure to DMSO for 10 min and colour intensity was determined on a micro-

culture plate reader (Titertek Multiscan Plus MK II) at 570 nm. The per cent viability of each well was calculated from the following:

$$\text{per cent viability} = \frac{\text{absorbance of test} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100$$

The data presented are the mean±standard deviation (S.D.) from eight replicate wells per microtitre plate and three replicate microtitre plates per cell line. Microscopic inspection of the wells confirmed that decreased absorbance values correlated with decreased cell number. Data of the MTT assays were analysed by means of Student *t*-tests. A *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. Expression of growth factors and their receptors in the original tumour

Immunohistochemical analysis of the original ES demonstrated a very weak cytoplasmatic staining for EGF-R and PDGF-R, a moderate expression of TGF-α, PDGF, TGF-β_1 as well as TGF-β type I-R and II-R and a strong expression for the FGF-4-R (Fig. 1, Table 1). Of note, a closely corresponding expression pattern was observed in two other epithelioid sarcomas retrieved from our files (Table 1). No expression of EGF was found in any tumour.

3.2. Expression and function of growth factors and their receptors in vitro *EGF/TGF-α and EGF-R*

Northern blot analysis revealed no expression of EGF in any cell line (data not shown). TGF-α mRNA was observed in GRU-1 and its clonal subpopulations with a low to moderate expression level (Fig. 2). Immunocytochemistry revealed weak to moderate levels of TGF-α protein in approximately 50% of the tumour cells (Table 1). Secretion of TGF-α into the supernatant could be demonstrated for GRU-1B and GRU-1C by ELISA, the TGF-α concentrations ranging from 40 to 70 pg/ml per 10^7 tumour cells (Table 2). No TGF-α protein secretion could be detected in the supernatants of GRU-1 and GRU-1A, although both cell lines had exhibited TGF-α mRNA and protein expression.

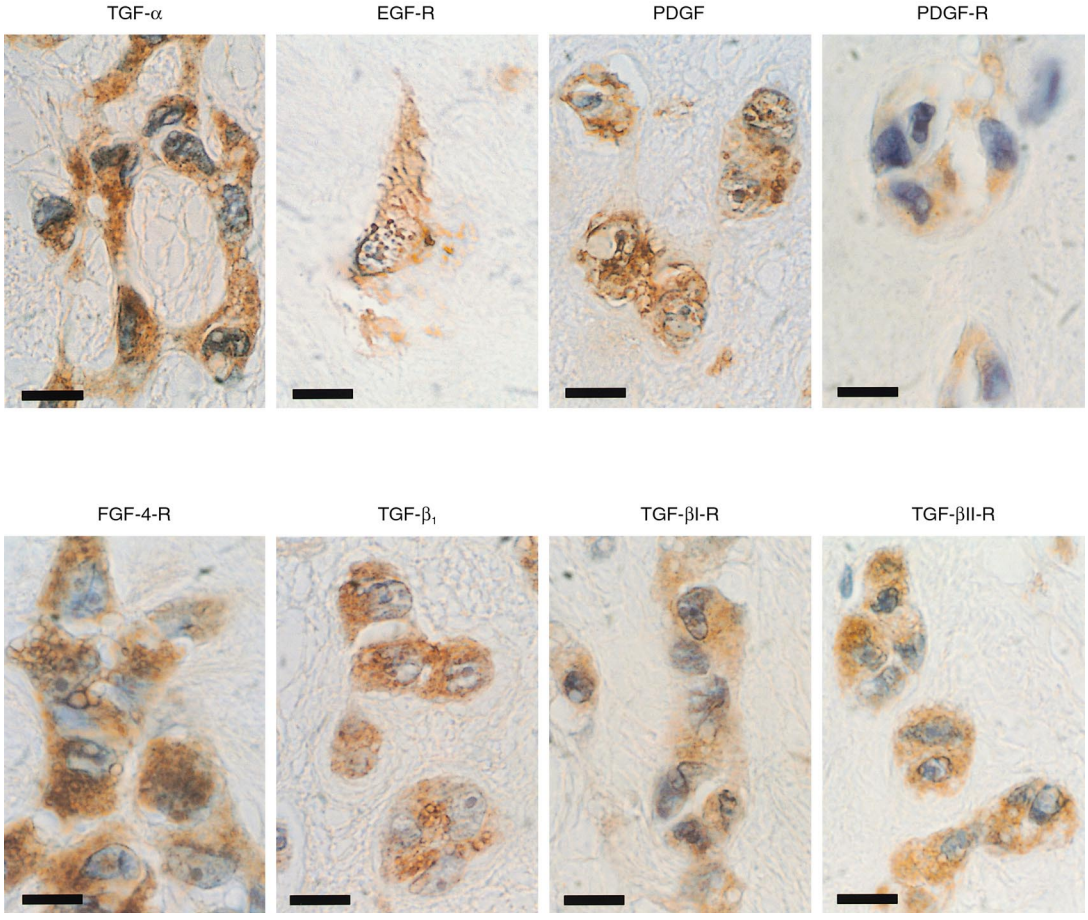

Fig. 1. Immunohistochemical analysis of growth factors and their receptors in the original epithelioid sarcoma (ES). Positive immunostaining indicates expression of growth factors and their receptors *in vivo*. Bar = 20 μm.

Table 1
Expression of growth factors and their receptors as revealed by immunocytochemical analysis[a]

	TGF-α	EGF-R	PDGF	PDGF-R	FGF-4-R	TGF-β_1	TGF-β I-R	TGF-β II-R
Original tumour *in vitro*	++	(+)	++	(+)	+++	++	++	++
GRU-1	+	(+)	++	+	++	+	(+)	+
GRU-1A	++	(+)	++	+	+	++	++	++
GRU-1B	++	(+)	++	++	++	++	++	+
GRU-1C	++	(+)	++	++	++	++	++	++
ES-1	(+)	(+)	++	(+)	+	+	+	+
ES-2	(+)	(+)	++	(+)	+	+	++	+

[a] Preserved expression pattern of growth factors and their receptors when comparing the original tumour and its cell lines. Closely corresponding expression pattern in tissue samples from two other epithelioid sarcomas originating in a 7-year-old girl with a tumour 3 cm in diameter (=ES-1) and a 17-year-old boy with a tumour 20 cm in diameter (=ES-2).

TGF(-α,β,β_1,βI,βII), transforming growth factor (alpha, beta, beta type I and II; R, receptor; EGF-R, epidermal growth factor receptor; PDGF, platelet-derived growth factor; ES, epithelioid sarcoma. Intensity rating: (+), score 1–3; +, score 4–6; ++, score 8–9; +++, score 12.

EGF-R mRNA was observed in all cell lines, the expression being most pronounced in GRU-1A and GRU-1B (Fig. 2). Flow cytometric analysis demonstrated EGF-R expression in all cell lines (Table 3), the mean fluorescence intensities varying from 6.0 (GRU-1C) to 9.5 (GRU-1A). In all cell lines, immunocytochemistry revealed low levels of EGF-R expression in less than or equal to 50% of tumour cells (Table 1).

Exogenously added TGF-α (10 ng/ml) resulted in a very weak growth stimulation ($P < 0.05$) of doubtful biological significance in GRU-1B, whereas no effects were observed in the other cell lines (Fig. 3a). In contrast, the inhibition of TGF-α binding to its receptor by the antagonistic anti-EGF-R antibody Ab-1 (at 20 μg/ml) resulted in a significant ($P < 0.05$) dose-dependent growth inhibition in all cell lines (Fig. 3b).

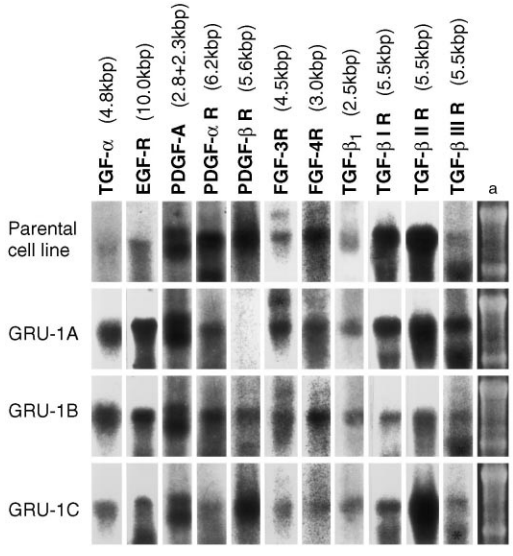

Fig. 2. Northern blot analysis of growth factors and their receptors in GRU-1 and its subpopulations. Specific signals indicate expression of growth factors and their receptors *in vitro* (each lane contains 20 μg of total RNA. [a]ethidium bromide stained agarose gel with 18S and 28S RNA.

3.3. PDGF-A/B and PDGF-α/-β-Rs

Northern blot analysis revealed a strong PDGF-A expression in all cell lines (Fig. 2), whereas PDGF-B mRNA was not observed in any cell line (data not shown).

PDGF-α-R mRNA (Fig. 2) was present in every cell line, the expression being most pronounced in the parental cell line GRU-1 and least pronounced in GRU-1C. A varying PDGF-β-R mRNA expression (Fig. 2) was evident in GRU-1, GRU-1B and GRU-1C, whereas no PDGF-β-R mRNA was observed in GRU-1A. In all cell lines, immunocytochemistry revealed moderate PDGF expression in at least 50% of the cells and a weak to moderate staining for PDGF-R in at least 25% of the cells (Table 1). (It has to be noted that the antibodies used for the detection of PDGF or PDGF-R do not discriminate between the different types of ligand and receptor.)

Exogenously added PDGF-AA or PDGF-BB (10 and 100 ng/ml) exhibited no growth-modulating effects in

Table 2
Enzyme-linked immunosorbent assay (ELISA) analysis of transforming growth factor (TGF)-α secretion into the supernatant[a]

Cell line	TGF-α (pg per ml and 10^7 cells)
GRU-1	n.d.
GRU-1A	n.d.
GRU-1B	40
GRU-1C	70

[a] Data were obtained from confluent cultures incubated for 96 h in 10 ml serum-free Dulbecco's modified Eagle's medium (DMEM) growth medium and represent the mean of two replicate analyses.

n.d., not detectable.

any cell line (data not shown). In contrast, the neutralisation of ES-derived PDGF ligands by an anti-hPDGF antibody (at 2 μg/ml) resulted in a significant ($P < 0.05$) dose-dependent growth inhibition of all clonal subpopulations (Fig. 3c).

3.4. aFGF and FGF-3- and 4-Rs

No expression of aFGF mRNA was detected in any cell line (data not shown). In contrast, all cell lines exhibited a faint to moderate FGF-3-R and -4-R

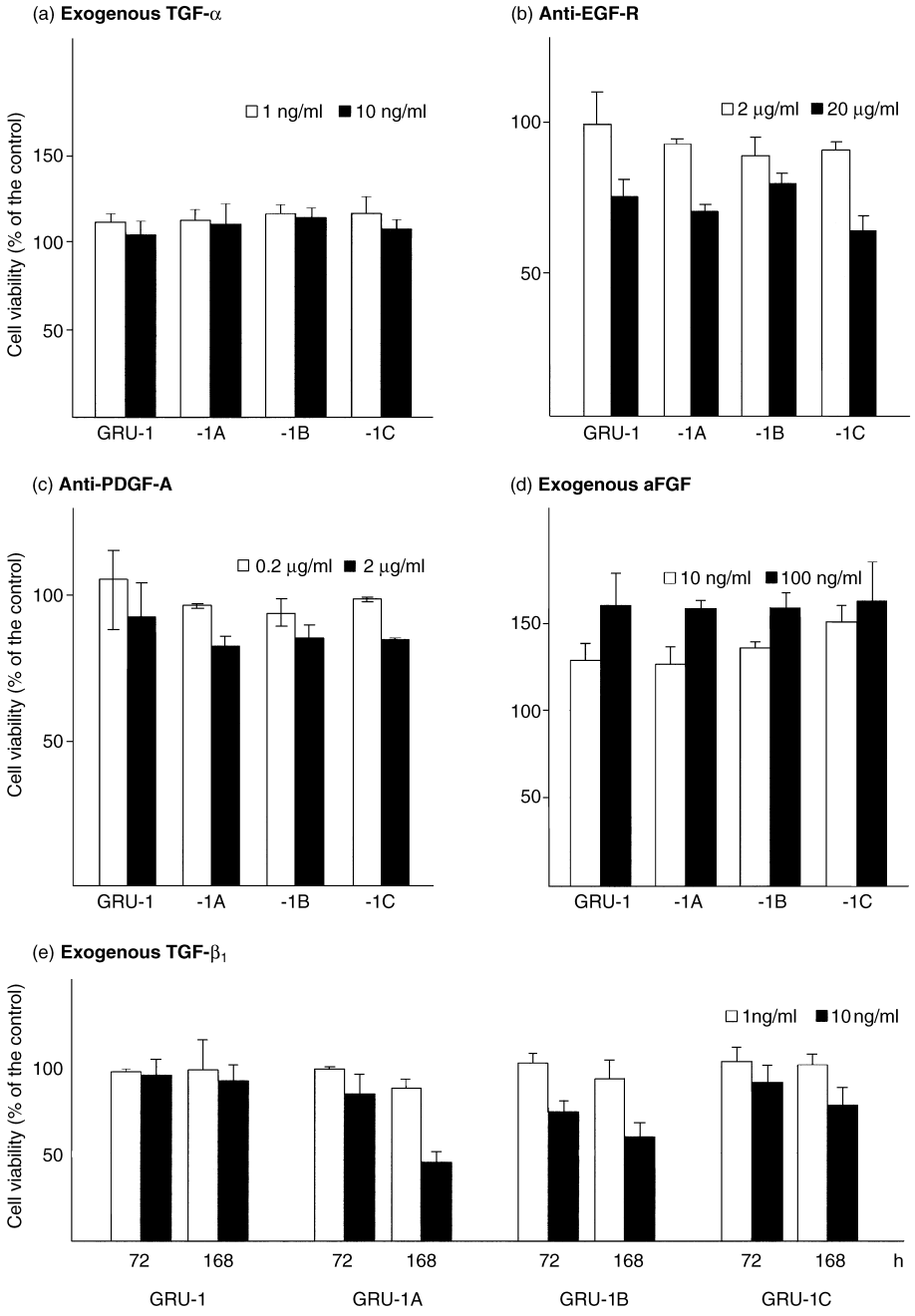

Fig. 3. Effects of exogenously added transforming growth factor (TGF)-α, acidic fibroblast growth factor (aFGF) or TGF-β_1 on proliferation of GRU-1 and its subpopulations. (a) Very weak growth stimulatory effects ($P < 0.05$) of doubtful biological significance in GRU-1B cells after exposure to TGF-α for 72 h. (b) Dose-dependent growth inhibition ($P < 0.05$) in all ES cell lines by the blocking of epidermal growth factor (EGF) receptor activation by antagonistic Ab-1 antibodies for 72 h. (c) Dose-dependent growth inhibition ($P < 0.05$) in all clonal subpopulations after neutralisation of platelet-derived growth factor (PDGF) ligands by hPDGF antibodies for 72 h. (d) Dose-dependent growth stimulation ($P < 0.05$) in all ES cell lines after exposure to aFGF for 72 h. (e) Dose-dependent growth stimulation ($P < 0.05$) in all clonal subpopulations, but not in the parental cell line GRU-1 after exposure to TGF-β_1 for 168 h. Data represent the mean cell number±standard deviation (per cent of the control) of three replicate experiments.

Table 3
FACS analysis of epidermal growth factor receptor (EGF-R) protein expression[a]

Cell line	Mean fluorescence intensities	
	EGF-R	Isotype control
GRU-1	6.4	1.4
GRU-1A	9.5	3.0
GRU-1B	8.6	2.4
GRU-1C	6.0	2.4
A-431	46.4	2.8
U-937	2.0	1.8

[a] Data represent the mean fluorescence intensity of 2×10^6 cells using an EGF-R-specific antibody (isotype control with a non-relevant mouse antibody; A-431, positive control, U-937, negative control.

Table 4
Enyzme-linked immunosorbent assay (ELISA) analysis of transforming growth factor (TGF)-β_1 secretion into the supernatant[a]

Cell line	TGF-β_1 (pg per ml and 10^6 cells)
GRU-1	70
GRU-1A	80
GRU-1B	n.d.
GRU-1C	500

[a] Data were obtained from confluent cultures incubated for 96 h in 5 ml serum-free Dulbecco's modified Eagle's medium (DMEM) growth medium and represent the mean of two replicate analyses.
n.d., not detectable.

mRNA expression (Fig. 2). Immunocytochemistry confirmed weak to moderate levels of FGF-4-R protein expression in GRU-1 and its subpopulations in at least 50% of tumour cells (Table 1).

Exogenously added aFGF (10 and 100 ng/ml) resulted in a significant ($P<0.05$) dose-dependent stimulation of proliferation in all cell lines (Fig. 3d). The maximum of growth stimulation was observed in GRU-1C showing a stimulation of cell growth up to 164 + 22% of the control after 72 h.

3.5. TGF-β_1 and TGF-β type I-, II- and III-Rs

Northern blot analysis revealed a faint TGF-β_1 expression in all cell lines (Fig. 2), which was confirmed by immunocytochemistry showing weak to moderate levels of TGF-β1 in at least 50% of tumour cells (Table 1). Secretion of TGF-β1 into the supernatant could be demonstrated for GRU-1, GRU-1A and GRU-1C by ELISA, the TGF-β_1 concentrations per 10^6 tumour cells ranging from 70 pg/ml (GRU-1) to 500 pg/ml (GRU-1C) (Table 4). TGF-β_1, however, was secreted as a biologically inactive complex only, which had to be activated by acidification prior to measurement by ELISA. (It is important to note that no detectable levels of biologically active TGF-β_1 were observed in standard growth medium supplemented with 10% FCS.)

mRNA of TGF-β I-, II- and III-Rs was detected in all cell lines but at differing expression levels (Fig. 2). Immunocytochemistry revealed weak to moderate levels of TGF-β I-R and II-R protein in all cell lines in at least 50% of tumour cells (Table 1).

Exogenously added biologically active TGF-β_1 (10 ng/ml) resulted in a significant ($P<0.05$) inhibition of proliferation in all subpopulations, but not in the parental cell line (Fig. 3e). The maximum of growth inhibition was observed in GRU-1A showing a reduction of cell growth to 44±6% of the control after 168 h.

4. Discussion

Our study clearly demonstrated that ES expresses a complex pattern of growth factors and their receptors both *in vivo* and *in vitro*. Analysis of the actual function of the corresponding signal transduction further confirmed the existence of an intricate network of autocrine, juxtacrine and paracrine signalling between ES tumour cells and adjacent non-neoplastic stroma cells.

Thus, TGF-α expression could be detected in all ES cell lines, although TGF-α secretion into the supernatant was found in two clonal subpopulations only. This observation indicated interclonal heterogeneity in the activation and secretion of TGF-α, which is generated as a membrane-bound precursor molecule prior to further processing by enzymatic cleavage [22]. But even without secretion, juxtacrine actions of membrane-bound TGF-α could be suspected, because EGF-R expression was found in all our cell lines, confirming previous reports on EGF-R expression in ES [10,11]. In fact, the marked growth-inhibitory effects of antagonistic anti-EGF-R antibodies indicated the existence of effective autocrine and juxtacrine growth stimulation by ES-derived TGF-α in all our cell lines. In conclusion, TGF-α expression by ES cells *in vivo* could be relevant for autocrine and juxtacrine growth stimulation as well as for paracrine stimulation of angiogenesis [23] and tumour invasion [24].

Similar conclusions could be drawn from our analysis of the PDGFs. Based on our Northern blot and immunocytochemical data, ES could be supposed to respond to PDGFs, either produced by the tumour cells or provided by the microenvironment. Although exposure to exogenous PDGF-AA or PDGF-BB failed to reveal any effects on tumour cell proliferation, effective autocrine growth stimulation became evident from our experiments showing growth inhibition of all our ES subpopulations after neutralisation of endogenous PDGF ligands by anti-hPDGF. *In vivo*, ES-derived PDGF-AA might also be relevant for paracrine signalling between tumour cells and their stromal microenvironment, either

stimulating angiogenesis or the massive deposition of collagen characteristic for ES [23,25,26].

Although aFGF expression could not be demonstrated in our ES cell lines, all cell lines showed expression of FGF-3-R and -4-R. Therefore, paracrine signalling could become effective between ES cells and stromal cells of the microenviroment, which are known to release FGFs [27]. This assumption was supported by our experiments showing that exposure to exogenous aFGF resulted in a significant dose-dependent stimulation of proliferation in all cell lines. This response, however, was at variance with the retardation of proliferation previously observed in another ES cell line after exposure to FGF [28]. These opposite effects of FGF in different ES cell lines further underline the complexity of growth regulation in ES.

The important role of paracrine interactions between ES and non-neoplastic stroma cells was also underlined by our analysis of the TGF-β_1 system. Thus, our ES expressed TGF-β_1 as well as the type I and type II receptors both *in vivo* and *in vitro*, which would permit autocrine TGF-β_1-triggered signal transduction. Exposure to exogenous biologically active TGF-β_1, however, could not inhibit the proliferation of the parental cell line GRU-1, thereby indicating escape from negative growth control by TGF-β_1, one of the most potent inhibitory growth factors identified so far [29,30]. Because ES cells were shown to secrete TGF-β_1 exclusively in a biologically inactive complex, GRU-1 cells are not confronted with a biologically active TGF-β_1 *in vitro*. In consequence, there is no selection pressure, which could favour the aquisition of TGF-β_1 resistance *in vitro*. The TGF-β-resistant phenotype of GRU-1 cells, therefore, is very likely not a cell culture artefact, but rather had developed in the original tumour.

Nevertheless, our cloning experiments revealed that the TGF-β-resistant parental cell line GRU-1 still harboured clonogenic subpopulations, which responded to biologically active TGF-β_1 with significant growth inhibition. Of course, it is tempting to speculate that these TGF-β_1-responsive subpopulations represent remnants of an earlier stage of tumour progression in ES. Thus, it is highly unlikely that mutational events leading to TGF-β_1-resistance in the parental cell line could have been reversed by consecutive mutations in the clonal subpopulations GRU1-A-, -B, and -C. Nevertheless, it was perplexing that our cloning experiments failed to establish TGF-β_1-resistant clonal subpopulations as well. The clonal subpopulations of our study, however, differ in many additional aspects, not directly related to TGF-β_1 responsiveness, including cytoskeletal architecture, growth properties and invasive behaviour [13–16]. Therefore, as yet undefined selective advantages imposed by our cloning procedure might have favoured the selection of exclusively TGF-β_1-responsive subpopulations.

In this context, it is important to emphasise that the secretion of biologically inactive TGF-β_1 *in vitro* cannot argue against a role of tumour-derived TGF-β_1 for paracrine action on stroma cells *in vivo*. Although little is currently known about TGF-β_1 activation *in vivo* [30], *in vitro* activation has been achieved by acidification or exposure to proteases like plasmin or cathepsin D [29]. It is reasonable, therefore, to assume that *in vivo* activation of ES-derived TGF-β_1 will happen in the acidic microenvironment of ischaemic tumours or in areas rich in proteases secreted by either the tumour cells or inflammatory cells [30–32]. As a consequence, ES-derived TGF-β_1 could indirectly accelerate tumour progression by its immunosuppressive or angiogenic potential [33,34].

In conclusion, our investigation revealed a complex pattern of expression and functional activity of growth factors and their receptors in the human ES cell line GRU-1 and its clonal subpopulations. Although these observations suggest intricate autocrine, juxtacrine and paracrine interactions between ES cells and non-neoplastic stromal cells, further investigations will have to define the expression of growth factors and their receptors in a larger series of this rare tumour entity.

Acknowledgements

We thank C.H. Heldin, J. Massague and R.A. Weinberg for providing us with the plasmids used in this study. Our appreciation is expressed to Mrs A. Florange-Heinrichs, Mrs H. Balven, Mr Ringler and Mr Rinschede for their excellent technical assistance. We are indebted to Dr Willers for his statistical evaluations.

References

1. Chase DR, Enzinger FM. Epithelioid sarcoma. Diagnosis, prognostic indicators and treatment. *Am J Surg Pathol* 1985, **9**, 241–263.
2. Gerharz CD, Moll R, Meister P, Knuth A, Gabbert HE. Cytoskeletal heterogeneity of an epithelioid sarcoma with expression of vimentin, cytokeratins and neurofilaments. *Am J Surg Pathol* 1990, **14**, 274–283.
3. El-Naggar AK, Garcia GM. Epithelioid sarcoma. Flow cytometric study of DNA content and regional DNA heterogeneity. *Cancer* 1992, **69**, 1721–1728.
4. Ishida T, Oka T, Matsushita H, Macinami R. Epithelioid sarcoma: an electron-microscopic, immunohistochemical and DNA flow cytometric analysis. *Virch Arch A Pathol Anat* 1992, **421**, 401–408.
5. Sonobe H, Furihata M, Iwata J, *et al*. Morphological characterization of a new human epithelioid sarcoma cell line, ES020488, *in vitro* and *in vivo*. *Virch Arch B Cell Pathol* 1993, **63**, 219–225.
6. Evans HL, Baer SC. Epithelioid sarcoma: a clinicopathologie and prognostic study of 26 cases. *Semin Diag Pathol* 1993, **10**, 286–291.
7. Halling AC, Wollan PC, Pritchard DJ, *et al*. Epithelioid sarcoma: a clinico-pathologic review of 55 cases. *Mayo Clin Proc* 1996, **71**, 636–642.
8. Ross HM, Lewis JJ, Woodruff JM, Brennan MF. Epithelioid sarcoma: clinical behaviour and prognostic factors of survival. *Ann Surg Oncol* 1997, **4**, 491–495.

9. Brinkmann U, Pastan I. Immunotoxins against cancer. *Biochem Biophys Acta* 1994, **1198**, 27–45.
10. Gusterson B, Cowley G, Mcilhinney J, *et al*. Evidence for increased epidermal growth factor receptors in human sarcomas. *Int J Cancer* 1985, **36**, 689–693.
11. Reeves B, Fisher C, Smith S, *et al*. Ultrastructural, immunocytochemical and cytogenetic characterization of a human epithelioid sarcoma cell line (RM-HS1). *J Natl Cancer Inst* 1987, **78**, 7–18.
12. Gerharz CD, Moll R, Ramp U, *et al*. Multidirectional differentiation in a newly established human epithelioid sarcoma cell line (GRU-1) with co-expression of vimentin, cytokeratins and neurofilament proteins. *Int J Cancer* 1990, **45**, 143–152.
13. Engers R, Gerharz CD, Moll R, *et al*. Interclonal heterogeneity in a human epithelioid–sarcoma cell line (GRU-1). *Int J Cancer* 1994, **59**, 548–553.
14. Engers R, Van Roy F, Heymer T. Growth inhibition in clonal subpopulations of a human epithelioid sarcoma cell line by retinoic acid and tumor necrosis factor alpha. *Br J Cancer* 1996, **73**, 491–498.
15. Engers R, Gerharz CD, Donner A, *et al*. In vitro invasiveness of human epitheloid–sarcoma cell lines: association with cell motility and inverse correlation with the expression of tissue inhibitor of metalloproteinases. *Int J Cancer* 1999, **80**, 406–412.
16. Reinecke P, Knopf C, Schmitz M, Schneider EM, Gabbert HE, Gerharz CD. Growth inhibitory effects of paclitaxel (Taxol®) on human epithelioid sarcomas *in vitro:* heterogeneity of response and the multidrug resistance phenotype. *Cancer* 2000, **88**, 1614–1622.
17. Pusztal L, Lewis CE, Lorenzen J, Mcgee OD. Growth factors: regulation of normal and neoplastic growth. *J Pathol* 1993, **169**, 191–201.
18. Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979, **18**, 5294–5299.
19. Ramp U, Jaquet K, Reinecke P, *et al*. Aquisition of TGF-β_1 resistance: an important progression factor in human renal cell carcinoma. *Lab Invest* 1997, **76**, 739–749.
20. Masui H, Kawamoto T, Sato D, *et al*. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res* 1984, **44**, 1002–1007.
21. Mosman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 1983, **65**, 55–64.
22. Carpenter G, Wahl MI. The epidermal growth factor family. In Sporn MB, Roberts AB, eds. *Peptide Growth Factors and their Receptors. I. Handbook of Experimental Pharmacology*. Berlin, Springer, 1990, 69–172.
23. Folkman I, Klagsbrun M. Angiogenic factors. *Science* 1987, **235**, 442–447.
24. Yudok K, Matsui H, Kanamori M, *et al*. Effects of epidermal growth factor on invasiveness through the extracellular matrix in high- and low-metastatic clones of RCT sarcoma *in vitro*. *Jpn J Cancer Res* 1994, **85**, 63–71.
25. Heldin CH, Westermark B. Platelet-derived growth factor: mechanism of action and possible *in vivo* function. *Cell Regulation* 1990, **1**, 555–566.
26. Forsberg K, Valyi-Nagy I, Heldin CH, *et al*. Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc Natl Acad Sci USA* 1993, **9**, 393–397.
27. Biard A, Böhlen P. Fibroblast growth factors. In Sporn MB, Roberts AB, eds. *Peptide Growth Factors and their Receptors. I. Handbook of Experimental Pharmacology*. Berlin, Springer, 1990, 369–418.
28. Roche H, Julia AM, Jozan S, *et al*. Characterization and chemosensitivity of a human epithelioid sarcoma cell line (SARCCR 2). *Int J Cancer* 1993, **54**, 663–668.
29. Roberts AB, Sporn MB. The transforming growth factor-beta. In Sporn MB, Roberts AB, eds. *Peptide Growth Factors and their Receptors. I. Handbook of Experimental Pharmacology*. Berlin, Springer, 1990, 419–472.
30. Kekow J, Wiedemann GJ. Transforming growth factor beta: a cytokine with multiple actions in oncology and potential clinical applications. *Int J Oncol* 1995, **7**, 1427–1432.
31. Barnard JA, Lyons RM, Moses HL. The cell biology of transforming growth factor beta. *Biochem Biophys Acta* 1990, **1032**, 79–87.
32. Wrana JL, Attisano L, Wieser R, *et al*. Mechanism of activation of the TGF-β receptor. *Nature* 1994, **370**, 341–347.
33. Jachimczak P, Bogdahn U, Schneider J, *et al*. The effect of transforming growth factor β-specific phosphorothioate-antisense oligo deoxynucleotides in reversing immunosuppression in malignant glioma. *J Neurosurg* 1993, **78**, 944–951.
34. Pepper MS, Vasalli DJ, Orci L, Montesano R. Biphasic effect of transforming growth factor β_1 on *in vitro* angiogenesis. *Exp Cell Res* 1993, **204**, 356–363.