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European Journal of Cancer 36 (2000) 932–941

European Journal of Cancer

www.ejconline.com

Differential response to transforming growth factor (TGF)-α and fibroblast growth factor (FGF) in human renal cell carcinomas of the clear cell and papillary types

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Received 9 August 1999; received in revised form 9 November 1999; accepted 12 November 1999

Abstract

The clear cell and the papillary types of human renal cell carcinoma (RCC) are distinct tumour entities with marked differences in their biological properties. Because growth factors are considered to affect profoundly the biological behaviour of malignant tumours, we compared the expression and function of transforming growth factor (TGF)-α and fibroblast growth factor (FGF) in both types of RCCs. Both *in vivo* and *in vitro* expression of TGF-alpha, epidermal growth factor-receptor (EGF-R), FGF-2 and FGF type 3- and 4-receptors was found in RCCs of both types. However, marked differences between clear cell and papillary RCCs became evident for TGF-α secretion, which could be demonstrated in 20 out of 24 (83%) clear cell RCCs but in only two out of four (50%) papillary tumours. Moreover, the mean TGF-α secretion rate in clear cell RCCs significantly ($P < 0.05$) exceeded that of papillary RCCs. Because the expression of growth factor receptors could not prove the corresponding signalling cascades were functional, tumour cell proliferation was tested after exposure to exogenous TGF-α or FGF-1. These experiments demonstrated that papillary RCCs did not respond significantly to exogenous TGF-α or FGF-1, whereas eight (33%) (TGF-α) and 11 (46%) (FGF-1) out of 24 clear cell RCCs responded with significant ($P < 0.05$) growth stimulation. In conclusion, our investigation presents data indicating that TGF-α and FGF are functionally involved in the progression of clear cell RCCs, directly stimulating proliferation by autocrine and/or paracrine actions. In contrast, TGF-α and FGF did not directly stimulate the proliferation of our papillary RCCs, thereby suggesting functional defects or a blockade in the corresponding signalling cascades. This differential functionality might contribute to the more aggressive behaviour of clear cell RCCs.

Keywords: Human RCC; TGF-α; FGF; Signal transduction pathways

1. Introduction

Until recently, human renal cell carcinoma (RCC) had been considered to be a single tumour entity, showing variable histomorphological patterns [1]. However, based on distinct cytomorphological criteria, a refined subclassification of human RCC was introduced, separating the clear cell and the papillary types of RCC, which together make up more than 80% of all RCCs [2,3]. This morphological separation between these two types of RCC was further substantiated by differences in the intermediate filament composition, enzyme synthesis and most importantly, by recent progress in molecular pathology demonstrating distinct cytogenetic aberrations for each tumour type [4–13].

The particular molecular mechanisms underlying the neoplastic proliferation of these two RCC types are not yet conclusively known. We recently demonstrated that a significant proportion of RCCs of both types have acquired resistance to the growth-inhibitory effects of transforming growth factor (TGF)-β_1 suggesting TGF-β_1-resistance as an important progression factor in human RCCs [14]. However, the balance of growth regulation in tumours can also be profoundly disturbed by the aberrant expression of and/or response to growth-stimulatory factors. Thus, autocrine growth stimulation

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0959-8049/00/$ - see front matter
PII: S0959-8049(00)00030-7

by TGF-α has been demonstrated in a small panel of clear cell RCCs [15–18]. Fibroblast growth factor (FGF)-2 is provided by stromal cells in tumours and is produced by RCC cells as well [19–22]. Moreover, an increased serum level of FGF-2 has been suggested to be a prognostic factor for renal carcinoma patients [23]. However, since the receptor status and functional intactness of the corresponding signal transduction pathways have not systematically been analysed, with the exception of preliminary data on a single RCC cell line [24], the actual functional contribution of FGF-2 to the neoplastic proliferation of RCCs is so far largely circumstantial.

The aim of the present investigation, therefore, was to compare the functional contribution of TGF-α and FGF-2 with the deregulated growth of the clear cell and papillary types of RCC.

2. Materials and methods

2.1. Cells and culture

All cell lines used in this study were derived from typical representatives of the clear and papillary types of renal cell carcinoma (RCC), established in our laboratory as previously described [11,14,25,26]. Staging of the primary tumours (Table 1) was done according to the TNM classification (UICC) and grading was performed according to Störkel and colleagues [27]. Since initially the Mainz-classification of RCCs was used [2,27], the cell lines of the papillary type were named 'chromphi'. The cell lines were maintained at 37°C in an atmosphere with 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and

Table 1
The status of the transforming growth factor-α, (TGF-α) epidermal growth factor-receptor (EGF-R) and the fibroblast growth factor (FGF) system in human renal cell carcinoma (RCC) cell lines of the clear cell and papillary/chromophilic types

Cell lines	Staging and grading of original tumours	TGF-alpha		EGF-R			FGF-2		FGF-3-R	FGF-4-R	
		IC	ELISA	IC	FACS[a] EGF-R	Isotype control	IC	ELISA	IC	RT-PCR and NB	IC
clearCa-											
1	pT3a, G2	+	n.d.	(+)	33	2	+	30	(+)	+	(+)
2	pT3a, G3	+ + +	283	+	24	3	+	1	(+)	+	(+)
3	pT3a, G2	+ +	189	+	15	2	+	13	(+)	+	+
4	pT2, G1	+ +	n.d.	+	22	2	+	80	(+)	+	+
5	pT3b, G3	+ +	49	(+)	10	2	+ +	90	(+)	+	+
7	pT2, G3	+	1656	+	19	3	+	20	(+)	+	(+)
8	pT3b, G2	+ +	803	+	26	4	+ +	120	+	+	+
9	pT2, G1	+	n.d.	+	25	3	+	70	(+)	+	+ +
10	pT3b, G2	(+)	302	+	20	5	+ +	80	(+)	+	+
11	pT2, G2	+	98	+ +	19	2	+ +	20	+	+	+ +
12	pT3a, G2	+	95	+	17	3	+	10	+	+	+
13	pT2, G2	+ +	1248	(+)	27	4	+	20	(+)	+	(+)
14	pT3b, G3	+	n.d.	+	9	3	+ +	20	+	+	+ +
15	pT3a, G2	+	437	+ +	28	3	+	10	+ +	+	+ + +
16	pT3a, G2	(+)	636	+	16	3	+ +	30	(+)	+	+
17	pT3a, G3	+	17	+	35	3	+ +	30	+	+	+
18	pT2, G2	+ +	121	+ +	19	3	+ +	2	+	+	+
19	pT2, G2	+ +	441	+	45	4	+	10	+	+	+
20	pT3a, G3	+	142	+ +	16	2	+	40	+	+	+
21	pT3a, G2	+ +	84	+ +	19	2	+ +	90	+	+	+
22	pT2, G2	+ +	152	+	16	3	+ +	5	(+)	+	+
23	pT2, G1	+ +	30	+	18	4	+	10	(+)	+	+
24	pT3a, g2	+	80	+	17	2	+	250	(+)	+	+
25	pT2, G2	+ +	100	+	35	3	+	4	(+)	+	(+)
chromphi-											
1	pT3b, G3	+	n.d.	(+)	8	3	+	10	+	+	+
2	pT3a, G2	+ +	60	+	15	2	+	50	+	+	+
3	pT2, G2	+ +	n.d.	+	13	5	+ +	10	+	+	+
4	pT2, G1	+ +	10	+ +	36	2	+ +	10	+	+	+

(+): very weak signal; +: weak signal; + +: moderate signal; + + +: strong signal.

IC, immunocytochemistry; ELISA, enzyme linked immunosorbent assay; analysis of protein secretion (pg/ml per 10^7 cells); NB, Northern blot analysis; n.d., not detectable.

[a] Expressed as mean fluorescence intensity.

streptomycin. Our studies were performed with cells from passages 50–80. The mean population doubling time *in vitro* ranged from 27 h (clearCa-11) to 112 h (clearCa-8) and could not be correlated to the histological typing, TNM staging or grading of the original tumours.

2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples of 10 clear cell RCCs and five papillary RCCs were analysed by immunohistochemistry. Because tissue samples of the original tumours from which the cell lines had been derived were not available for further studies, we selected typical representatives of each tumour type. After mild trypsinisation of the deparaffinised sections, the paraffin sections were stained for TGF-α (Ab-2, Oncogene Science), EGF-R (E30, Merck), FGF-2 (Ab-3, Oncogene Science), FGF-3-R (C-15, Santa Cruz) and FGF-4-R (C-16, Santa Cruz) by the avidin–biotin complex peroxidase (ABC) method. Proper negative controls were performed by omitting the primary antibodies. The concentrations of the antibodies used in our immunohistochemical and immunocytochemical analysis are as follows: TGF-α, 1:100; EGF-R, 1:30; FGF-2, 1:50; FGF-3-R, 1:100; FGF-4-R, 1:100.

2.3. Immunocytochemistry

The cultivated RCC cells were seeded on microscopic slides, fixed *in situ* by exposure to methanol (5 min) and acetone (10 s) at −20°C and then air-dried. Primary antibodies of TGF-α, EGF-R, FGF-2, FGF-3-R and FGF-4-R (see above) were applied to the slides and allowed to incubate for 30 min at room temperature in a moist chamber. The visualisation of the primary antibodies was achieved by the ABC method. Proper negative controls were performed by omitting the primary antibodies.

2.4. RNA extraction

Total cellular RNA was isolated by the guanidine–thiocyanate method [28]. RNA concentration was measured by spectrophotometry at 260 nm. The quality of total cellular RNA was verified in an ethidium bromide stained agarose gel.

2.5. Northern blot analysis of FGF-4-R expression

Northern blot analysis was carried out using 20 μg of RNA of each sample under denaturating conditions separated by electrophoresis in a 1% formaldehyde gel. Before transfer of the RNA to the nylon membrane (Hybond N, Amersham, Bucks, UK), the gel was stained with ethidium bromide and the equivalence of RNA samples loaded in each lane was verified under ultraviolet (UV) light and photographed. In a second control step, the complete transfer of the RNA from the gel to the Nylon membranes was again verified under UV light. Afterwards, the RNA was hybridised with a specific FGF-4-R DNA probe obtained from the purified insert of a FGF-4-R plasmid (American Type Culture Collection (ATCC) no: 65790; pGEM-3Zf+; insert: *Eco*RI). The DNA was labelled by incorporation of [^{32}P]-dCTP using the oligo-labelling kit (Pharmacia, Germany). Hybridisation, stringent washing procedures and fluorography were carried out as previously described [14]. All experiments were conducted twice with different Nylon membranes and the results obtained were reproducible. Appropriate positive controls were performed in all blots.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of FGF-4-R expression

Nested RT-PCR was carried out using total RNA isolated as described above. Reverse transcription of 4 μg of total RNA was performed with oligo (dT) primers for 1 h at 37°C using 50 U of molony murine leukaemia virus (MMLV)-reverse transcriptase according to the manufacturer's recommended procedure (Fa. Stratagene, RT-PCR KIT no.: 200420). In each experiment, water was substituted for mRNA as a negative control for contamination. The samples were heated for 5 min at 95°C to inactivate the reverse transcriptase. The first PCR amplification reaction mixture was composed of 2.5 μl first strand cDNA, 10 μl 10× Taq DNA polymerase buffer, 1 μl of dNTPs (100 mM), 0.5 μl Taq DNA polymerase (4 U/μl; Fa. AGS, no.: A007 95 l) and 0.5 μl of each primer: forward: 5′ TGT TGG GAA GTC CAG CTT G 3′, reverse: 5′ TTG CTG GGG GTA ACT GTG C 3′ (size of amplification product is 479 bp). The reaction mixture was overlaid with two drops of mineral oil. After an initial denaturation step at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 1 min were run on a MJ PTC-100 Programmable Thermal Controller. Subsequently, a second amplification step of the first PCR product was performed with two internal primers: forward: 5′ CCT GAG AGC TGT GAG AAG 3′, reverse: 5′ AGA TTC TGC AGG ACG ATC 3′. Ten microlitres of each PCR reaction was analysed by electrophoresis on 1.5% agarose gels and visualised by ethidium bromide staining. As a control, cDNA samples were also amplified with glycerinaldehyd-diphospat-dehydrogenase (GAPDH)-specific primers. All experiments were done twice and the results obtained were reproducible.

RT-PCR analysis of FGF-R-3 expression could not successfully be performed due to a high homology to the FGF-R-4, as well as numerous randomly distributed regions of very high G+C content resulting in multiple 'GC clamps'.

2.7. Enzyme-linked immunosorbent assay (ELISA) analysis of TGF-α and FGF-2 secretion

Supernatants were collected from confluent cultures incubated for 48 h in standard culture medium (DMEM supplemented with 10% FCS). A sandwich enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's recommended procedure (TGF-α: Dianova, no. QIA 05, FRG; FGF-2: R&D Systems, no. DFB 00, Germany). All samples and standards were run in duplicate. Colour intensity was measured at 490 nm (TGF-α) or 450 nm (FGF-2) using a spectrophotometric plate reader and the concentrations were determined by comparison with standard curves. The minimal detectable concentrations were found to be 10 pg/ml (TGF-α) or 1 pg/ml (FGF-2). No TGF-α or FGF-2 could be detected in standard culture medium (DMEM supplemented with 10% FCS).

2.8. FACS analysis of EGF-R protein expression

Tumour cells were harvested in the log growth phase, washed twice 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, which binds to the extracellular domain of EGF-R (clone 528, GRO1L, Dianova; concentration: 0.1 μg/2×10^5 cells) for 1 h at 4°C. It is worth noting the EGF-R antibody used for immunohistochemistry and immunocytochemistry is not recommended for FACS analysis by the manufacturer. Cells were washed twice and incubated with goat anti-mouse dichlorotriazinylfluoreszein (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, Germany). Cells were analysed for cell volume, right-angle light scatter and green fluorescence. Data were acquired in list mode, green fluorescence was collected with logarithmic amplifier and analysed with the software program Immunocount (Ortho Diagnostic Systems). Cells were gated on forward and side-scatter to exclude debris. Anti-EGF-R stained cells were compared with unstained cells and cells stained with the irrelevant mouse IgG as an isotype control. As an 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 conducted twice and the results could be reproduced.

2.9. Assessment of growth properties after exposure to TGF-α or FGF-1

TGF-α (Hermann Biermann, Germany) prepared as a 100 μg/ml stock solution in phosphate buffered saline (PBS) and 0.1% bovine serum albumin (BSA) was added to the cultures to yield a final concentration of 1 and 10 ng/ml, respectively. FGF-1 (Hermann Biermann) prepared as a 10 μg/ml stock solution in 1 mg/ml BSA and 4 mM HCl was added to the cultures to yield a final concentration of 1 and 10 ng/ml, respectively.

Six replicate 25 cm^2 culture flasks were exposed to growth medium containing 1% FCS supplemented with 1 or 10 ng/ml TGF-alpha and 1 or 10 ng/ml FGF-1, respectively. 3×10^5 cells were seeded in each culture flask. As controls, 3×10^5 cells/flask in growth medium containing 1% FCS were added to six replicate 25 cm^2 culture flasks. In each experiment, cells from three culture flasks were harvested separately after 72 h and 168 h. Cells were not refed during this period. The number of cells harvested was determined with the Neubauer haemocytometer chamber. The data were statistically analysed by an analysis of variance with two independent factors.

3. Results

3.1. Expression and function of TGF-α and EGF-R

3.1.1. TGF-α and EGF-R expression in vivo

Immunohistochemical analysis of TGF-α expression in clear cell RCCs demonstrated a moderate to strong cytoplasmic staining in at least 75% of the tumour cells (Fig. 1). Tumour cells with a vacuolated cytoplasm usually exhibited a weaker visible TGF-α staining. Papillary RCCs exhibited moderate TGF-α levels in at least 50% of the tumour cells (Fig. 1).

Antibodies to EGF-R exhibited a staining of the cell membrane in at least 75% of the tumour cells in clear cell RCCs and in at least 50% of the tumour cells in papillary RCCs (Fig. 1). The staining intensities were rather heterogeneous, showing marked intratumoral and intertumoral variation. However, only in the papillary tumours the staining reaction was restricted to the basolateral surface of the cells as was also observed in normal proximal tubule epithelia (Fig. 1).

3.1.2. TGF-alpha and EGF-R expression in vitro

Immunocytochemistry revealed TGF-α staining in at least 75% of the tumour cells of the clear cell and papillary types (Fig. 2; for staining intensities cf. Table 1).

Secretion of TGF-α protein into the supernatant could be demonstrated in 20 out of 24 clear cell RCCs by ELISA, the TGF-α concentrations per 10^7 tumour cells ranged from 17 to 1656 pg/ml (Table 1). In contrast, only low levels of TGF-α secretion (10 and 60 pg/ml/10^7 cells) were observed in two papillary RCCs, whereas no TGF-α secretion could be detected in the two other cell lines of this tumour type. This difference in TGF-α secretion between clear cell and papillary

RCCs proved to be statistically significant using the Mann–Whitney U-test ($P = 0.029$). Moreover, cell lines derived from highly differentiated (G1) RCCs exhibited a significantly lower TGF-α secretion than moderately differentiated (G2) tumours using the Mann–Whitney U-test ($P = 0.008$).

Immunocytochemical analysis of EGF-R expression demonstrated a staining in at least 50% of the tumour cells irrespective of the tumour type (Fig. 2; for staining intensities cf. Table 1).

Flow cytometric analysis revealed EGF-R protein expression in all clear cell and papillary tumours (Table 1), the mean fluorescence intensities varying from 8 (chromphi-1) to 45.0 (clearCa-19).

3.1.3. *Functional analysis of EGF-R* in vitro

Exposure to exogenous TGF-α (10 ng/ml) resulted in a rather heterogeneous response in proliferation (Fig. 3). Eight clear cell RCCs exhibited a significant ($P < 0.05$) stimulation of proliferation after 72 or 168 h. Moreover, in four clear cell RCCs a minor albeit significant ($P < 0.05$) growth inhibition was observed (Fig. 3). The effects of exogenous TGF-α were shown to be concentration-dependent, since 1 ng/ml TGF-α resulted in a weaker, albeit significant ($P < 0.05$) stimulation of proliferation in two cell lines only, i.e. clearCa-10 and -13 (data not shown). In contrast, exogenous TGF-α did not significantly affect the proliferation of any papillary RCC (Fig. 3).

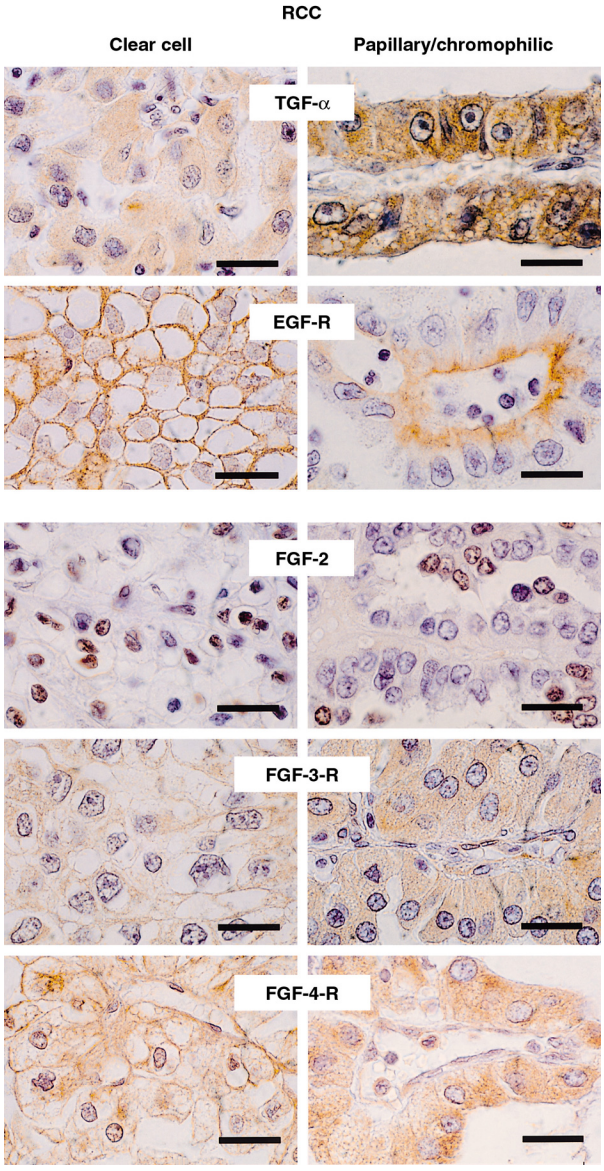

Fig. 1. Immunohistochemical analysis demonstrating expression of TGF-α, EGF-R, FGF-2, FGF-3-R and FGF-4-R in primary RCCs of the clear cell and papillary (=chromophilic) types (scale bar = 20 μM).

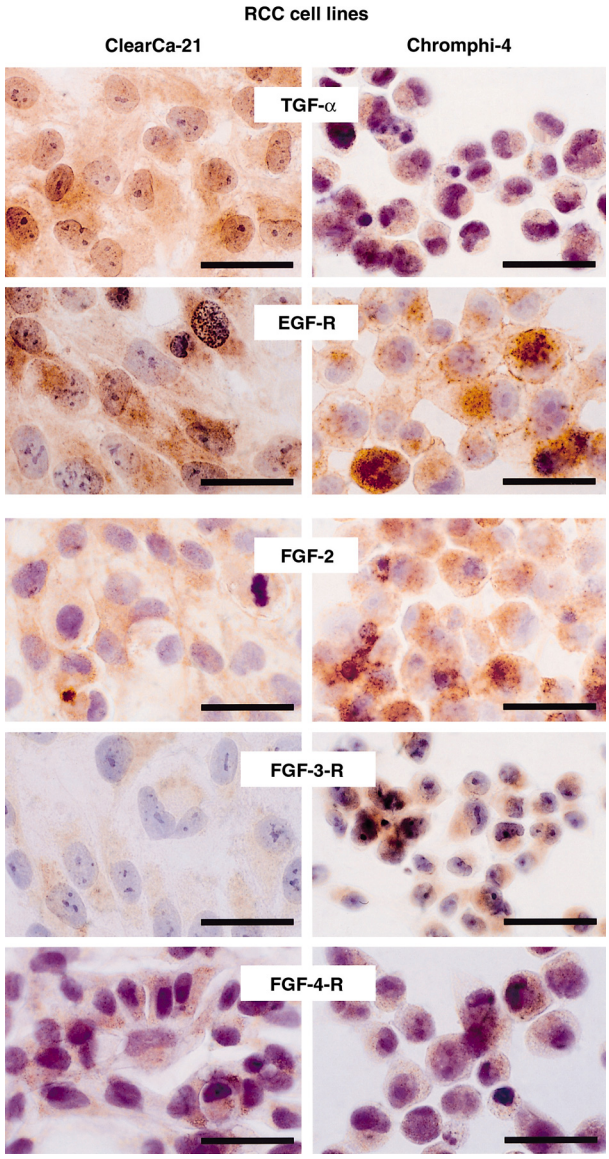

Fig. 2. Immunocytochemical analysis demonstrating expression of TGF-α, EGF-R, FGF-2, FGF-3-R and FGF-4-R in clearCa-21 and chromphi-4 cells *in vitro* (scale bar = 20 μM).

3.2. Expression and function of FGF and FGF receptors

3.2.1. FGF-2 and FGF receptor expression in vivo

Immunohistochemical analysis demonstrated a weak nuclear FGF-2 expression in less than 50% of tumour cells in two out of 10 (20%) clear cell RCCs and in three out of five (60%) papillary RCCs (Fig. 1). All clear cell and papillary RCCs weakly expressed FGF-3-R protein and a weak to moderate FGF-4-R staining was detected in at least 75% of the RCC cells, irrespective of the tumour type (Fig. 1).

3.2.2. FGF-2 and FGF receptor expression in vitro

Immunocytochemistry revealed weak to moderate levels of FGF-2 expression in more than 75% of tumour cells irrespective of the tumour type (Fig. 2), the immunostaining being predominantly located in the cytoplasm. This difference in the location of immunostaining between tumour cells *in vivo* (see above) and *in vitro* has been observed by other groups as well and has not so far been satisfactorily explained [20,29].

Secretion of FGF-2 protein into the supernatant could be demonstrated in all clear cell RCCs by ELISA, the FGF-2 concentrations per 10^7 tumour cells varied considerably from 1 to 250 pg/ml (Table 1). Low levels of FGF-2 secretion (between 10 and 50 pg/ml/10^7 cells) were observed in all papillary RCCs (Table 1). These differences of FGF-2 secretion between clear cell and papillary RCCs, however, were statistically non-significant using the Mann–Whitney U-test.

FGF receptor type 4 mRNA was detected by Northern blot in 14 out of 24 cell lines (58%) of the clear cell type and in two out of four (50%) of papillary RCCs (Fig. 4). To exclude the possibility of type 4-receptor mRNA expression below the detection limits of our Northern blots, RT–PCR analysis was performed, revealing type 4-receptor expression in all those cell lines (Fig. 4). These results were further confirmed by immunocytochemistry

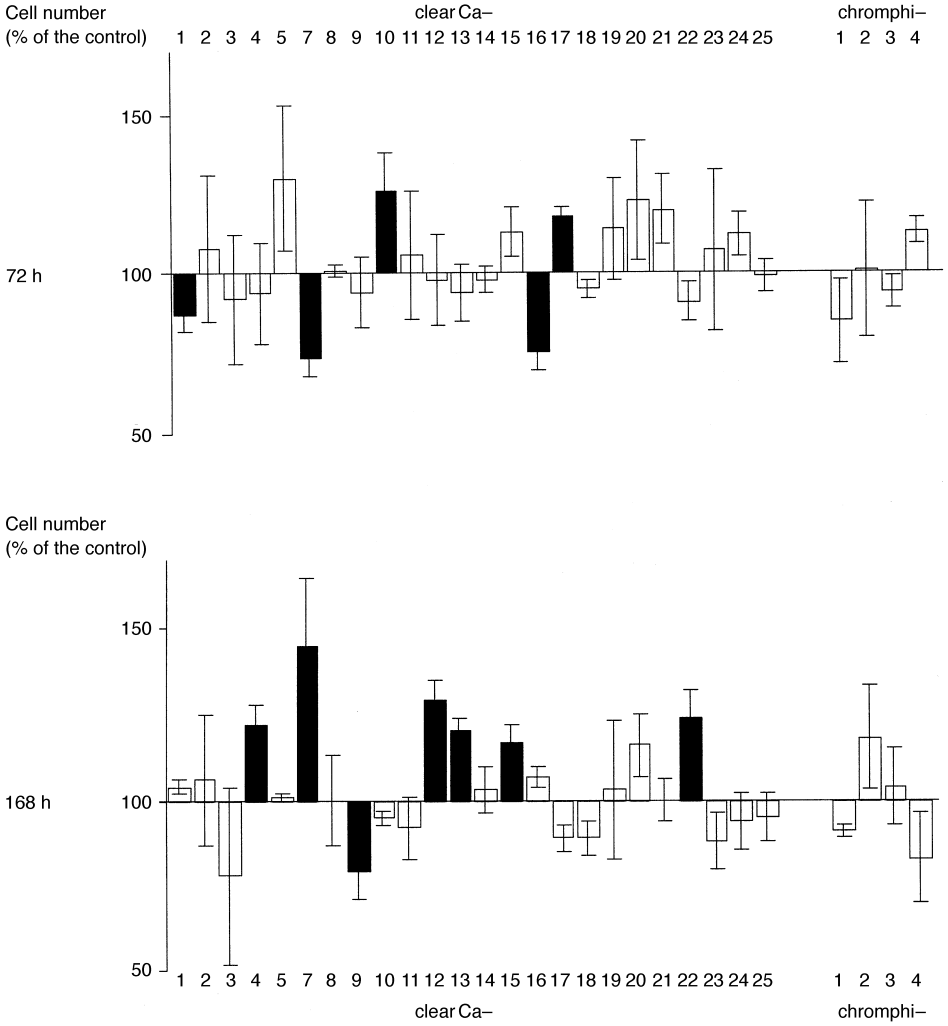

Fig. 3. Effects of exogenous TGF-α (10 ng/ml) on proliferation. Data represent the mean cell number ± standard deviation (S.D.) (in per cent of the control) of three replicate experiments (black box: $P < 0.05$).

demonstrating a FGF receptor type 4 expression in at least 75% of RCC cells, irrespective of the tumour type (Fig. 2; for staining intensities cf. Table 1).

The fact that low levels of receptor mRNA detectable only by RT–PCR are sufficient to produce receptor protein detectable by immunocytochemistry might be explained by different half-times of receptor mRNA and protein.

RT-PCR analysis of FGF receptor type 3 expression could not successfully be performed due to a high homology to FGF receptor type 4 as well as numerous randomly distributed regions of very high G + C content resulting in multiple 'GC clamps'. However, immunocytochemistry demonstrated an expression of FGF receptor type 3 in all RCC cell lines, irrespective of the tumour type (Fig. 2; for staining intensities cf. Table 1).

3.3. *Functional analysis of FGF receptors* in vitro

Functional analysis of FGF receptors type 3 and 4 was performed with FGF-1, which, in contrast to FGF-2, binds to both receptor types with high affinity [30]. Exogenous FGF-1 (10 ng/ml) resulted in a significant ($P < 0.05$) stimulation of proliferation in 11 out of 24 (46%) clear cell RCCs after 72 h and/or 168 h (Fig. 5). After exposure to FGF-1 we could demonstrate an increase in proliferation in clearCa-12 up to $148 \pm 5\%$ (168 h) of the control and in clearCa-16 up to $138 \pm 12\%$ (72 h) of the control. Therefore, both cell lines exhibited a comparable increase in proliferation with a slightly more pronounced effect in clearCa-12. The effects of exogenous FGF-1 were shown to be concentration-dependent since 1 ng/ml FGF-1 resulted in a weaker, albeit significant ($P < 0.05$), stimulation of proliferation in clearCa-16 only, whereas the other cell lines of the clear cell type did not respond (data not shown).

In contrast, no significant growth stimulation became evident in any papillary RCC after exposure to FGF-1 (Fig. 5). This difference in response to FGF-1 between clear cell and papillary RCCs, however, was statistically not significant using the Wilcoxon-test.

4. Discussion

Although RCC had been considered to be a single tumour entity until recently, the histomorphological separation of clear cell and papillary RCCs as different tumour types has been substantiated by recent progress in molecular pathology, demonstrating distinct cytogenetic aberrations [6–8,11,13]. Moreover, clear cell RCCs were shown to exhibit a worse prognosis when compared with papillary RCCs [12,27], suggesting differences in their biological properties as well.

However, comparative investigations into the reasons for this divergent biological behaviour, have not so far been performed, mainly due to the lack of strictly defined RCC cell lines of the papillary type. Therefore, the establishment of four papillary RCC cell lines (named chromphi-1 to -4) by our laboratory [11] provided for the first time the necessary tools for such a comparative investigation. In this report, we present the first data on differences in the functional relevance of TGF-α- and FGF-dependent signalling cascades between clear cell and papillary RCC cell lines.

These differences became evident by functional analysis *in vitro*, but could not conclusively be shown by expression analysis in primary RCCs. Thus, immunohistochemistry revealed elevated levels of TGF-α in both RCC types, when compared with their putative cells of origin, i.e. the proximal tubule epithelia [25,31]. Moreover, an immunohistochemical staining reaction for EGF-R was found in both tumour types, EGF-R expression being restricted to the basolateral cell surface in papillary RCCs. Although this compartimentalisation of EGF-R expression in papillary RCCs corresponds to the localisation of EGF-R in normal proximal tubule epithelia [32], its functional relevance for this tumour type remains elusive. In contrast, no unequivocal differences in the expression of FGF-2 and its corresponding receptors became evident between clear cell and papillary RCCs from the *in vivo* studies.

Data obtained from immunocytochemical and flow cytometric analysis of our RCC cell lines confirmed

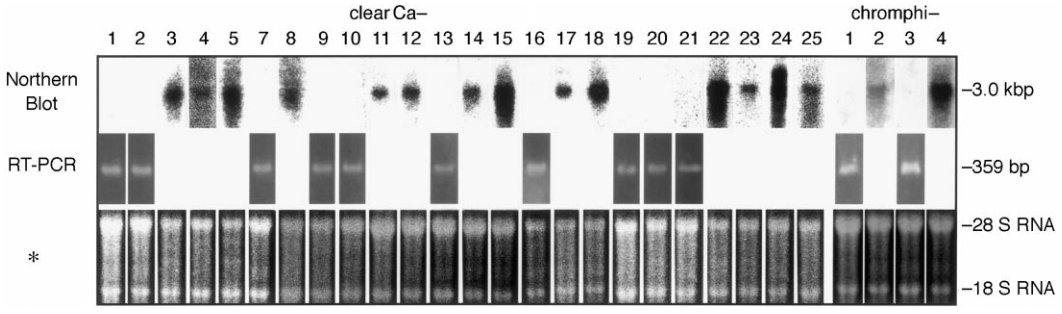

Fig. 4. Northern blot and RT–PCR analysis of FGF receptor type 4 mRNA expression in clearCa-1 to -25 and in chromphi-1 to -4. *Ethidium bromide stained gel showing equal amounts of RNA in each lane. In our RT–PCR analysis, water was substituted for sample as a negative control and negative results were obtained indicating the absence of a contamination (data not shown).

these *in vivo* observations and failed to reveal marked differences between clear cell and papillary RCCs.

However differences in behaviour were observed when the secretion rate of TGF-alpha and FGF-2 was studied. Thus, ELISA analysis revealed TGF-α secretion in almost all clear cell RCCs, but in only two out of four papillary RCCs. Moreover, the mean secretion rate of TGF-α in clear cell RCCs significantly ($P < 0.05$) exceeded that of papillary RCCs. Because TGF-α is known to be generated as a membrane-bound precursor molecule, which has to be processed and enzymatically cleaved before secretion [33], these observations might indicate that defects in the activation and secretion of TGF-α are more prevalent in papillary RCCs. Corresponding observations became evident for the secretion rate of FGF-2, clear cell RCCs again demonstrating a higher mean secretion rate when compared with papillary RCCs. Although these differences in the secretion of FGF-2 did not prove to be statistically significant — possibly due to the low number of papillary RCCs available for our study — we hypothesised that tumour-derived TGF-α and FGF-2 might provide a selective advantage that is more important for clear cell than for papillary RCCs.

This hypothesis was further supported by our experiments, testing the functional activity of the corresponding receptors and their signal transduction cascades in both tumour types. Because the expression of growth factor receptors does not prove their effective operation, we additionally analysed tumour cell proliferation after exposure to exogenously added growth factors. Response to exogenous growth factors would indicate that receptors and their signal transduction pathways are functional and might also respond to endogenous growth factors. These experiments revealed pronounced differences between clear cell and papillary RCC cell lines: whereas papillary RCCs did not significantly respond to either TGF-α or FGF-1 (which interacts with both FGF-3-R and FGF-4-R), eight and 11 out of 24 clear cell RCC cell lines, respectively, responded with

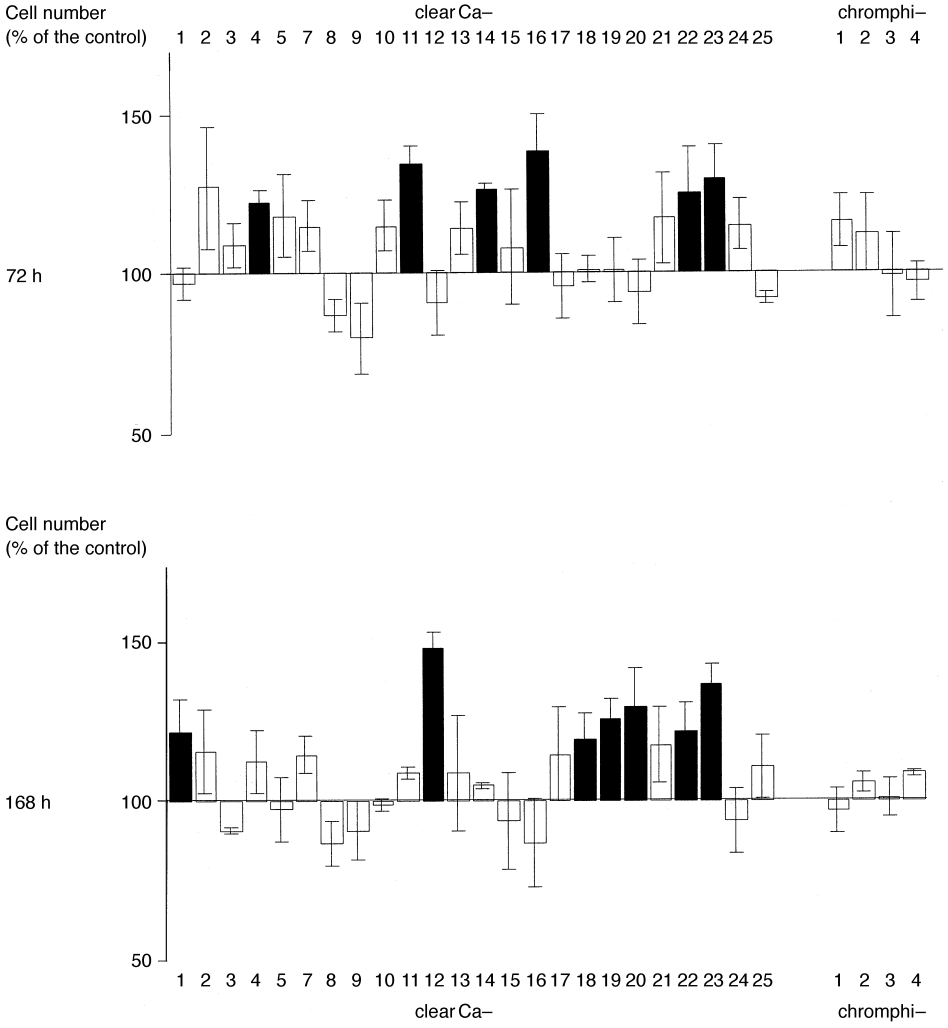

Fig. 5. Effects of exogenous FGF-1 (10 ng/ml) on proliferation. Data represent the mean cell number ± standard deviation (S.D.) (in per cent of the control) of three replicate experiments, (black box: $P < 0.05$).

significant ($P < 0.05$) growth stimulation. These observations suggested that defects or a blockade in the signalling cascades triggered by TGF-α or FGF might be more prevalent in papillary RCCs than in clear cell RCCs, adding further support to our hypothesis of a differential relevance of TGF-α and FGF for these two tumour types.

Of course, it might be argued that the number of four papillary RCC cell lines is too low to draw any conclusions on the relevance of TGF-α and/or FGF for the progression of this tumour type. Functional analysis of more papillary RCC cell lines, however, is not possible to date, because the four cell lines used in our study are, to our knowledge, the only strictly defined *in vitro* models currently available worldwide as permanent cell lines [11]. These results must therefore be considered preliminary and require future confirmation through the analysis of more papillary RCC cell lines. Interestingly in this context, increased serum levels of FGF-2 have recently been suggested to be a prognostic factor in RCCs [23]. Unfortunately, however, this report did not discriminate between clear cell and papillary RCCs. Future investigations will therefore have to show whether FGF-2 or TGF-α and their corresponding receptors might actually be a prognostic factor for clear cell RCCs only, thereby further substantiating our hypothesis.

In conclusion, our investigation presents convincing data indicating that TGF-α and FGFs are functionally involved in the progression of clear cell RCCs, directly stimulating their proliferation by autocrine and/or paracrine actions. In contrast, we could demonstrate for the first time that TGF-α and FGF did not significantly directly stimulate the proliferation of papillary RCCs, thereby suggesting functional defects or a blockade in the corresponding signalling cascades. Further studies, however, are clearly needed to confirm that this differential response observed in our study will actually contribute to the more aggressive behaviour of clear cell RCCs.

Acknowledgements

Our appreciation is expressed to Mrs A. Florange, Mrs H. Balven, Mr Ringler and Mr Rinschede for their excellent technical assistance. We are indebted to Dr Willers for his statistical evaluations.

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