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Hyperplasia of epithelium adjacent to transitional cell carcinoma can be induced by growth factors through paracrine pathways

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Abstract Hyperplasia of transitional cell epithelium adjacent to human transitional cell carcinomas (TCC) is a common finding in pathology. This hyperplasia may be a precancerous aberration. Alternatively, it has been suggested that the hyperplasia is due to paracrine action of tumour-derived growth factors. In this study we tested the latter hypothesis using the mouse tumorigenic TCC cell line NUC-1. Transplantation of NUC-1 tumour cells into the urinary bladder submucosa of syngeneic mice in vivo induced hyperplasia of normal adjacent urothelium in all tested mice. Implantation of normal mouse bladder mucosa did not induce urothelial hyperplasia. In vitro, conditioned medium of NUC-1 cells induced the proliferation of the mouse urothelial cell line g/G, which closely resembles normal urothelial cells. This induction was inhibited by transforming growth factor β_1 (TGFβ_1). Similarly, TGFβ_1 inhibited the fibroblast growth factor-1 (FGF-1) and FGF-2 induced proliferation of g/G cells. Chemico-physical examination, bioassays with conditioned media, and RNA analysis of NUC-1 cells revealed that these cells secreted a growth factor with FGF-like properties. These results indicate that epithelial hyperplasia surrounding carcinomas is not necessarily a precancerous aberration, but may result from direct paracrine action of tumour-derived growth factors.

Key words Growth factors · Hyperplasia
Transitional cell carcinoma · Urothelium
Paracrine regulation

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Introduction

Several morphological aberrations are frequently observed in the human epithelium lining the mucosa adjacent to carcinomas. For example, epithelial hyperplasia represents a rather common finding in a variety of carcinomas, like colon carcinomas [16, 19], oesophageal carcinomas [4], and transitional cell carcinoma (TCC) of the urinary bladder [2, 11]. Its pathogenesis is not clear yet. Two hypotheses have been put forward. According to one hypothesis, the hyperplastic epithelium is a precancerous aberration, which can change into dysplasia and finally to a carcinoma [11]. In the alternative hypothesis, hyperplasia does not represent a precancerous stage, but develops as a reaction to the tumour, possibly because of tumour-derived growth factors acting on the surrounding epithelium [19].

Several data suggest an important role for growth factors in the process of tumour growth in affecting the proliferation or differentiation of both normal and tumour cells (reviewed by Aaronson [1]). Growth factors can be synthesized and secreted by both normal cells and tumour cells, and they regulate physiological cellular processes by an autocrine or paracrine pathway. Some growth factors are present in normal urine (epidermal growth factor (EGF)) [3]. In bladder tumours and in urine of patients with bladder tumours other growth factors including transforming growth factor α (TGFα) and fibroblast growth factors (FGFs), have been found [5, 6, 10, 17]. These growth factors are known to affect the proliferation of both transformed and non-transformed urothelial cells in vitro [7, 14, 22].

Based on experimental work, Sekikawa et al. [19] suggested that hyperplasia of colonic mucosa adjacent to colon carcinoma could be due to tumour-derived growth factors. In this study we investigated whether hyperplasia of normal urothelial cells adjacent to TCC could be induced by TCC-derived growth factors through paracrine action. We first developed an in vivo model to demonstrate the effects in vivo of mouse bladder carcinoma on the surrounding urothelium, by transplanting a mouse

urothelial tumorigenic cell line, designated NUC-1 [7], into the submucosa of the urine bladder of syngeneic mice. Secondly, we determined, in an in vitro model, the presence of tumour-derived growth factors that could enhance the growth or proliferation of normal mouse urothelial cells. We studied the NUC-1 cell line for the presence of growth factors, and their effects on the non-tumorigenic mouse urothelial cell line g/G resembling normal mouse urothelial cells [7].

Materials and methods

Culture media and supplements were purchased from Flow Laboratories (Zwanenburg, NL) and Sigma (St. Louis, USA), respectively. Human FGF-2 and TGFα were obtained from Oncogene Science (Uniondale, USA), human FGF-1 from Boehringer (Almere, NL), and mouse EGF, endothelial cell-derived growth factor (ECGS), and fibronectin from Sigma. Human transforming growth factor β_1 (TGFβ_1) was either obtained from Sigma, or purified from outdated human platelets as described [23]. Tritiated thymidine was purchased from Amersham ('sHertogenbosch, NL).

Mouse urothelial cell lines NUC-1 and g/G were developed as described previously [7]. These cells were cultured routinely in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F10 medium, supplemented with 10% fetal calf serum (FCS), 10 µM HEPES buffer, 100 IU/ml penicillin, 100 µg/ml streptomycin, selenite, transferrin, and 5 µg/ml insulin (standard medium) [7]. Serum-free culture was done in standard medium without FCS, but further supplemented with 0.1% bovine serum albumin (BSA), putrescin, ethanolamine, spermin, and spermidin as described [7].

A431 carcinoma cells were obtained from Dr. P. M. J. J. Berns (Dept. of Biochemistry, Daniel den Hoed Cancer Centre, Rotterdam, NL). These cells were routinely cultured in RPMI 1640 medium, supplemented with 10 µM HEPES buffer, 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. ML-CCL64 mink lung carcinoma cells were cultured routinely in DMEM, buffered with 25 mM BES (N,N-bis[2-hydroxy-ethyl]-2-aminoethane sulfonic acid), and supplemented with 5% FCS, streptomycin and penicillin as outlined above. Fetal bovine heart endothelial (FBHE) cells were routinely cultured in DMEM, supplemented with 10% FCS.

In transplantation studies NUC-1 cells were cultured under routine conditions to confluency. After extensive washing with phosphate buffered saline (PBS) pH 7.2, cells were trypsinized, washed with PBS again and injected (1×10^6 cells in 100 µl PBS) subcutaneously in normal syngeneic mice. After 3 weeks, NUC-1 tumours were excised and minced into 1 mm^3 pieces. One piece was transplanted into the submucosa of the bladder of syngeneic mice via a small incision in the muscular layer of the left side of the bladder dome. As a control, 1 mm^3 pieces of normal bladder from syngeneic mice was transplanted similarly.

To collect conditioned medium NUC-1 cells were grown in 167 cm^2 culture flasks (Falcon, Lincoln Park, USA) to subconfluency in standard medium. The cells were then washed with PBS, incubated during one hour with serum-free medium, washed again with PBS, and finally incubated during 24 or 48 h with 35 ml of serum-free medium. After collection, all media were filtered through a 0.2 µm filter, and frozen at –20° C until use.

Heat treatment of medium was carried out at 65° C for 30 min. Acid treatment was done with 1.0 M hydrochloric acid for 1 h, followed by neutralisation with 0.1 M sodium hydroxide. Reduction of medium components was performed with 0.1 M dithiothreitol (DTT, Boehringer, Almere, NL) for 1 h, followed by dialysis of the medium twice against serum-free medium, and a final filtration through a 0.2 µm filter. Enzymatic digestion of medium proteins was done with 10 µg/ml trypsin for 1 h. The trypsin was inactivated by addition of 40 µg/ml trypsin inhibitor. Heparin-binding capacity of the conditioned medium was tested by incubation with heparin-sepharose (Pharmacia, Woerden, NL) during 2 h at 4° C. The medium was collected by centrifugation. The heparin-sepharose beads were washed firstly with water and subsequently treated with stepwise increases in the sodium chloride concentration ranging from 0.15 M to 2.0 M. The eluted fractions were collected by centrifugation at 4° C after each treatment and tested for their ability to induce the proliferation of g/G cells.

Testing effects of growth factors on the proliferation of g/G cells was performed as previously described [7]. In brief, cells were first washed with PBS, then incubated during 1 h in serum-free medium, washed again, and trypsinized. Fibronectin-precoated wells in a 96-multiwell dish (NUNC, Roskilde, DK) were filled with 10000 viable cells per well in 150 µl serum-free medium. After 16 h, conditioned medium fractions to be tested were added (50 µl/well). After another 72 h, cultures were terminated.

The presence of growth factors in NUC-1 conditioned medium was assessed in bioassays with different cell lines. The proliferation of these cell lines is modulated by specific growth factors under defined culture conditions [24]. The proliferation of ML-CCL64 and A431 cells is specifically inhibited by members of the TGFβ family and the EGF family, respectively. The proliferation of FBHE cells is specifically enhanced by members of the FGF family.

Bioassays with ML-CCL64 cells, A431 cells, and FBHE cells were performed as described previously [24]. In brief, A431 cells were grown to subconfluency, rinsed with PBS, trypsinized, counted, and seeded at 2500 viable cells per well in a 96-multiwell dish in 150 µl/well DMEM medium with 5% FCS. After 16 h, 50 µl of fractions to be tested were added. Cells were then incubated for another 72 h. ML-CCL64 and FBHE cells were seeded at 5000 viable cells per well of a 24-well dish in 1 ml of DMEM with FCS (ML-CCL64) or without FCS (FBHE). After 3 h, 200 µl of the fractions that were to be tested, was added per well. Cells were then cultured for another 68 h. In experiments with neutralizing antibodies against TGFβ_1 or TGFβ_2 (British Biotechnology, Oxford, GB) the fractions were pre-incubated with 2.0 µg of antibody/ml medium at 37° C before addition to ML-CCL64 cells.

Proliferation was assessed in vitro by [^{3}H]-thymidine labelling using scintillation counting, and in vivo by bromodeoxyuridine (BrdU) labelling for the determination of the labelling index (L.I.) by immunocytochemistry.

For assessing the proliferation in vitro, 0.5 µCi [^{3}H]-thymidine per well was added 16 h before termination. Cells in 96-multiwell dishes were rinsed with PBS, trypsinized, and harvested. Tritiated thymidine was counted using a BetaPlate scintillation counter (Wallac-Pharmacia, Woerden, NL). Alternatively, cells in 24-wells dishes were rinsed with PBS, fixed with methanol, lysed in 0.1 M sodium hydroxide, and radioactivity in cell lysates was measured in a liquid α, β scintillation counter. Radioactivity was expressed as counts per minute.

For assessing the proliferation in vivo, mice were injected i.p. with bromodeoxyuridine (BrdU) in PBS (30 mg/kg body weight) 1 h before sacrifice. Immunohistochemistry on paraffin-embedded tissue was performed as described previously [18], using the primary anti-BrdU antibody IIB5 (kindly donated by Prof. Dr. F. C. S. Ramaekers, University of Limburg, Maastricht, NL). The L.I. was calculated as the relative number of BrdU positive cells per 100 adjacent cells in the basal urothelial cell layer (normal) or 200 adjacent cells in the first two basal cell layers (hyperplastic). The L.I. is given as the mean L.I±standard deviation.

Results

In vivo model

After 1, 2, and 3 weeks, 5 mice were sacrificed and urine bladders were examined. No tumours were found in mice in which normal urine bladder was transplanted. The transplanted normal bladder had largely degenerated

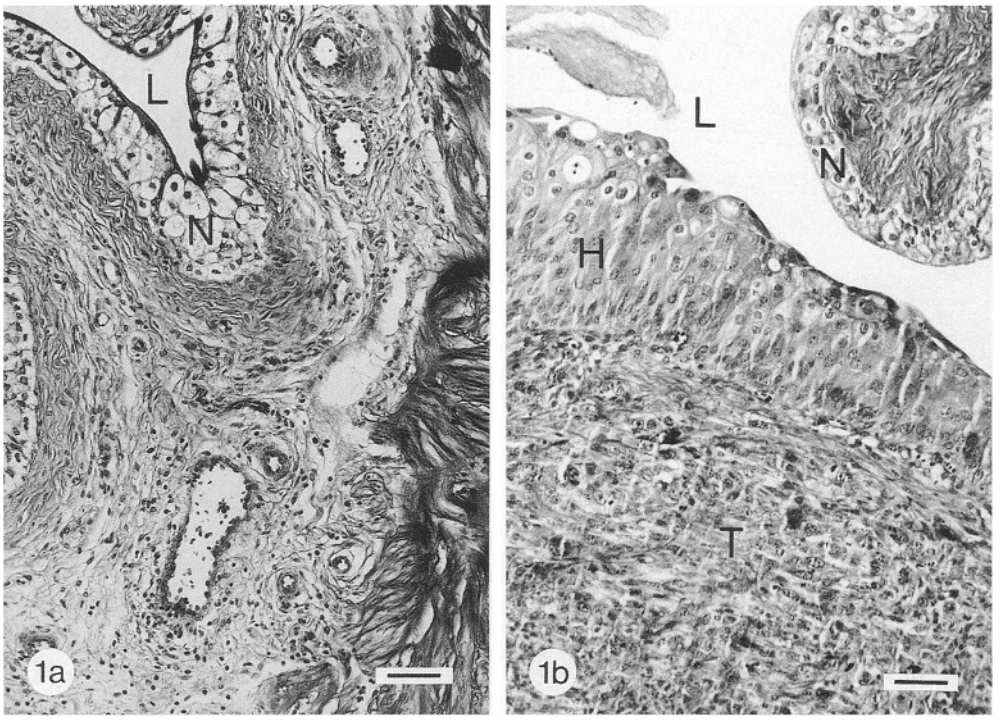

Fig. 1 Photomicrograph of haematoxylin-stained paraffin-embedded sections of (**A**) normal mouse urothelium and (**B**) a tumour of NUC-1 mouse urothelial cells 1 week after transplantation into the bladder submucosa of a syngeneic mouse. **A** Normal, non-hyperplastic urothelium adjacent to the region of degenerated, transplanted normal bladder. **B** Hyperplastic urothelium overlying the NUC-1 tumour, and non-hyperplastic urothelium at the opposed bladder wall. *H*, hyperplastic urothelium; *N*, normal urothelium; *L*, lumen of bladder; *T*, NUC-1 tumour. Magnification=200×; bar=50 μm

Table 1 Occurrence of hyperplasia in the in vivo transplantation model. In vivo data on transplanted tissue into bladder submucosa. The number of tumours in 5 animals is given at the indicated time interval after transplantation (weeks). The number of mice in which hyperplasia adjacent to the tumour was observed, is also given, as well as the number of cell layers in the hyperplastic urothelium. The proliferation was expressed as the mean labelling index±standard deviation (n=5)

Weeks	Tumours	Hyperplasia	Number of cell layers	Labelling index (%)	
				Hyperplastic	Normal
Transplanted NUC-1 tumours					
1	5	5	6–10	9.6±5.6	0.7±0.7
2	5	3[a]	5	20.7±8.5	1.6±0.1
3	5	4[b]	5–10	11.5±4.7	4.4±0.5
Transplanted normal bladder					
1	0	0	3		0.6±0.6
2	0	0	3		0.2±0.2
3	0	0	3		0.5±0.5

Overlying urothelium completely replaced by tumour an cells in
[a] 2 out of 5 mice
[b] 1 out of 5 mice

within one week. Hyperplasia of the urothelium was not noted (Fig. 1A) and BrdU incorporation was low in the overlying urothelium (Table 1). In contrast, at all three time intervals after transplantation of NUC-1 tumour pieces, the urothelium overlying the transplants was hyperplastic, with 5 or more cell layers (Fig. 1B). The hyperplastic urothelium also showed high BrdU incorporation, particularly in basal cell layers (Table 1). No nuclear atypia was noted in the hyperplastic urothelium. The urothelium lining the bladder lumen opposite to the hyperplastic urothelium exhibited a normal appearance in 5 out of 5 mice after one week (Table 1), and showed a normal L.I.

Chemico-physical analysis and bioassays

The next step was to find out if and which NUC-1 derived growth factors might be involved in the development of hyperplasia. First, we examined whether NUC-1 cells produced and secreted growth stimulatory factors. Therefore, a chemico-physical analysis of serum-free conditioned medium of NUC-1 cells was performed. The conditioned medium was tested in bioassays with g/G cells. As presented in Fig. 2A, conditioned medium of NUC-1 induced the proliferation of g/G cells, which could be inhibited by exogenous addition of TGFβ_1. Treatment with trypsin or acid completely abolished the

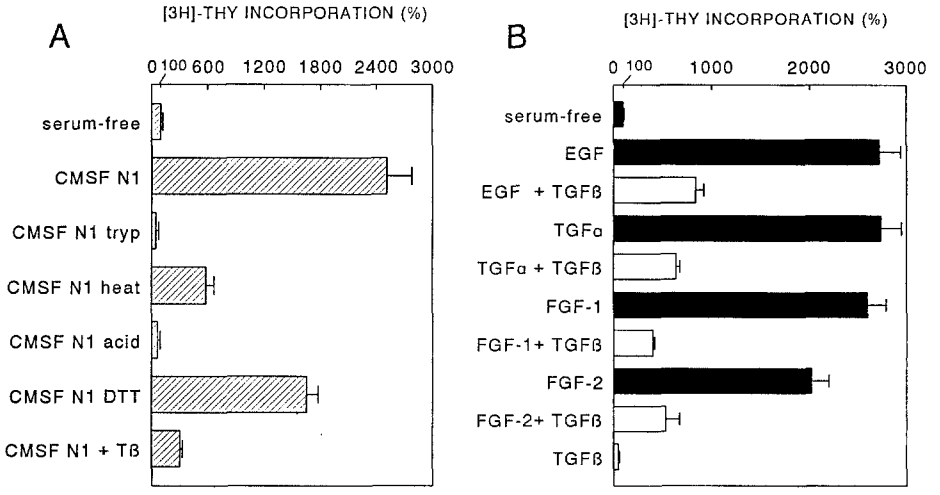

Fig. 2 [^{3}H]-thymidine incorporation of g/G cells to (**A**) serum-free conditioned medium of NUC-1 cells and (**B**) growth factors in serum-free medium. **A** Proliferation in the presence of serum-free conditioned medium of NUC-1 with or without 1 ng/ml TGFβ_1, or pretreated under the indicated conditions. Incorporation in serum-free medium alone was taken as 100%. *Tryp*, trypsin-treated; *heat*, heat-treated; *acid*, acid-treated; *DTT*, DTT-treated; *T*β=TGFβ_1. **B** Proliferation in the presence of the indicated growth factors alone (*closed bars*) or in the presence of 1 ng/ml TGFβ_1 (*open bars*). Incorporation in serum-free medium alone was taken as 100%. Growth factor concentrations: 1 ng/ml (EGF, TGFα, TGFβ_1) or 10 ng/ml (FGF-1, FGF-2)

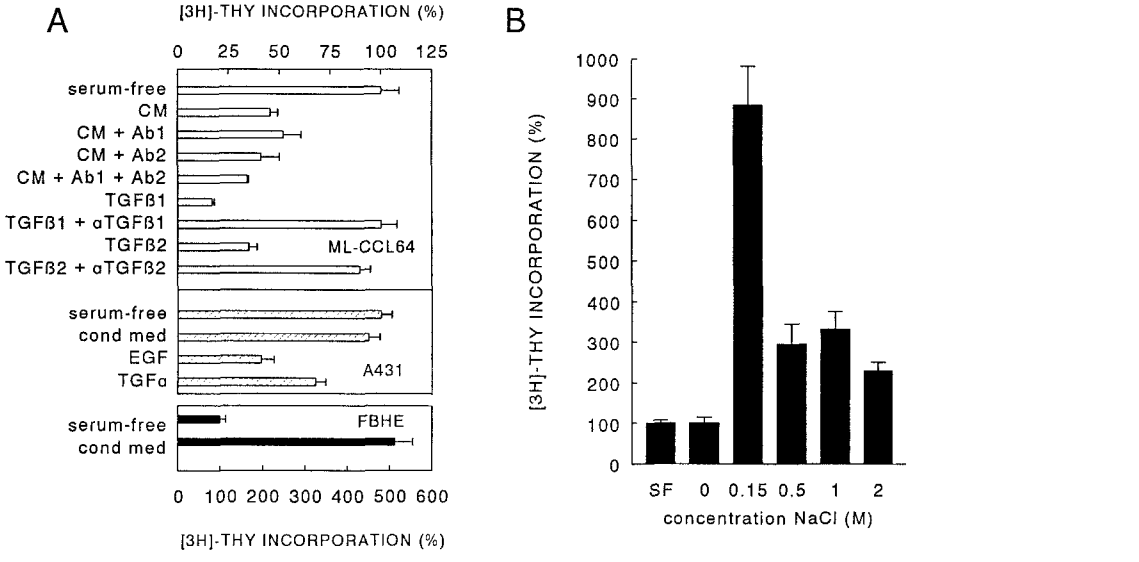

Fig. 3 Bioassays with ML-CCL64, A431, FBHE, or g/G cells. **A** Incorporation of tritiated thymidine into the indicated cell lines in the presence of serum-free conditioned medium of NUC-1 (CM), or in the presence of 0.88 ng/ml TGFβ_1 or TGFβ_2 pretreated or not with antibody against TGFβ_1 (Ab1) or TGFβ_2 (Ab2) (ML-CCL64 assay), 1 ng/ml EGF or TGFα (A431 assay), or 15 µg/ml ECGS (FBHE assay). Conditioned medium of NUC-1 cells was heat-pretreated before use in the bioassay with ML-CCL64 cells; for use in the other bioassays, the NUC-1 conditioned medium was not pretreated. Proliferation of ML-CCL64 and A431 cells are on the upper axis, and of FBHE cells on the lower axis. Incorporation in the presence of non-conditioned serum-free medium alone was taken as 100%. ECGS (15 µg/ml) induced the proliferation of FBHE cells approximately 20 times. **B** Incorporation of [^{3}H]-thymidine into g/G cells in the presence of heparin-sepharose treated serum-free conditioned medium of NUC-1 cells fractionated with increasing salt concentrations. Final dilution of conditioned medium fractions as tested on g/G cells was 1:8. Incorporation in the presence of serum-free medium alone (SF) was taken as 100%

activity of the NUC-1 derived conditioned medium, and treatment with heat partially reduced the enhanced proliferation. Treatment with DTT only slightly affected the enhanced proliferation of g/G cells. This slight decrease in proliferation might be due to the dialysis step following DTT treatment. The results indicate that at least a DTT-stable polypeptide growth factor is present in the NUC-1 conditioned medium. The sensitivity to heat and acid treatment might indicate that the proliferation inducing activity is a heat and acid labile protein. However, the possibility that latent NUC-1 derived TGFβ is activated upon heat or acid treatment, resulting in an inhibition of proliferation, cannot be excluded.

Next, we investigated the effect of different growth factors on proliferation of g/G cells under serum-free conditions. Figure 2B shows that EGF, TGFα, FGF-1,

and FGF-2 could induce the proliferation of g/G cells. This growth factor induced proliferation could be inhibited by addition of TGFβ_1.

A further characterization of the proliferation-inducing activity was made using a series of growth factor specific bioassays. Bioassays with ML-CCL64 cells revealed TGFβ-like activity in NUC-1 derived conditioned medium that was heat-pretreated for activation of TGFβ, but this activity could not be neutralized by specific antibodies against TGFβ_1 or TGFβ_2, nor by combinations of these antibodies (Fig. 3A). Untreated NUC-1 conditioned medium was not active in the ML-CCL64 bioassay (data not shown). Bioassays with A431 indicated that the serum-free conditioned medium of NUC-1 cells did not contain detectable EGF-like growth factors (Fig. 3A). In particular, the resistance of DTT treatment excludes the involvement of growth factors of which the biological activity is dependent on intact disulphide bonds. In order to test the possibility that the NUC-1 derived growth factor polypeptide was an FGF-like protein which is DTT stable, FGF-specific bioassays with FBHE cells were performed and the heparin-binding capacity was determined. Figure 3A shows that untreated conditioned medium of NUC-1 stimulated the proliferation of FBHE cells by five times. If the conditioned medium of NUC-1 was treated with heparin-sepharose, then washed with water, and subsequently eluted with increasing salt concentrations, the stimulatory protein was mainly contained in the 0.15 M sodium chloride fraction (Fig. 3B). The first fraction which was eluted in the absence of salt, did not induce the proliferation (Fig. 3B). This indicates the presence of a protein in conditioned medium of NUC-1 that binds specifically to heparin though with low affinity. Analysis of Western blots did not reveal any FGF-2-like molecule in NUC-1 conditioned medium. In line with this result, Northern blot analysis of poly A$^+$ RNA from NUC-1 cells did not demonstrate detectable FGF-2 expression. In addition, we failed to detect expression of FGF-1, FGF-3, and FGF-4 RNA in NUC-1 cells (data not shown).

Discussion

Several studies on human transitional cell carcinomas (TCC) have revealed hyperplasia of the normal urothelium adjacent to the TCC [2, 11]. This hyperplasia has also been observed in experimentally induced urothelial carcinomas in the rat [12]. It has been suggested that this hyperplasia represented a precancerous stage in urothelium [11]. Similarly, several authors have considered hyperplasia adjacent to a colon carcinoma as a precancerous change [9, 16], an hypothesis disputed by Sekikawa et al. [19]. Using transplantations of colon carcinoma cells in mouse colon in vivo, they demonstrated alterations in mucin expression, morphology, and BrdU incorporation in the mucosa overlying the tumour, but not in the remote mucosa or in their control animals transplanted with fibroblasts. They suggested an involvement of tumour-derived growth factors in the development of this hyperplasia.

Our data demonstrated that only epithelium overlying the tumour was hyperplastic and showed a marked proliferation. Urothelium opposite or remote from the tumour, as well as urothelium of control transplanted mice did not show hyperplasia or significant proliferation. Bioassays with A431 cells, the lack of detectable TGFα RNA and EGF protein expression in NUC-1 cells (data not shown), and the DTT stability excluded the identification of the NUC-1-derived, growth stimulatory protein as an EGF-like growth factor. Although bioassays with ML-CCL64 cells indicated that NUC-1 cells secreted a TGFβ-like factor, this activity could not be neutralized by specific antibodies against TGFβ_1 or TGFβ_2. However, other members of the TGFβ family, like TGFβ_3, could also be produced by NUC-1 cells. The data from Fig. 2A indicate that the NUC-1 stimulating activity disappears after heat or acid treatment of conditioned medium. This might be due to inactivation of the stimulatory protein, or to activation of TGFβ_3. Because neutralizing antibodies against TGFβ_3 were not available, we were unable to check for the presence of TGFβ_3 in the NUC-1 derived conditioned medium. The data of the bioassays with g/G cells indicated that the growth stimulating effect of the NUC-1-derived factor can be inhibited by TGFβ, as is the case with the growth stimulating factors EGF and FGF (Fig. 2). The heparin-binding data indicate that the major fraction of growth stimulating factors binds heparin with low affinity. RNA analysis of NUC-1 cells failed to detect FGF-1, FGF-2, FGF-3, and FGF-4. Of other FGFs, FGF-5 binds heparin with high affinity [25], and FGF-7 is a non-epithelial cell derived FGF [8]. These data may exclude the identification of our growth stimulating factor as one of the classical FGFs. Little is known yet about FGF-6, FGF-8, and FGF-9 with respect to epithelial cells.

Several data support the role of TCC-derived growth factors in hyperplastic development. Both TGFα and FGF-like (FGF-1 or FGF-2) growth factors have been detected in the urine of patients with TCC [5, 6, 10]. Other studies have demonstrated an enhanced expression of FGF-1 proteins [17], FGF-5 RNA expression [25], and co-amplification of FGF-3 and FGF-4 genes [20] in human TCCs. Normal urine and urothelium contains EGF in high concentrations and only small amounts of FGF-1 or FGF-2 [3, 5, 6, 17]. The present study and previous studies showed that these EGF-like and FGF-like growth factors could induce proliferation of non-tumorigenic transitional epithelial cells in vitro and in vivo [7, 13]. Supporting evidence for the involvement of FGF-like factors in hyperplasia of epithelium was given by Muller et al. [15], who showed that expression of the FGF-3 gene in prostate or mammary gland epithelium of FGF-3 transgenic adult mice resulted in epithelial hyperplasia in these organs. Hence, the observed hyperplasia of urothelium adjacent to TCC in vivo may be due to a direct, paracrine effect of TCC-derived, FGF-like growth factors on the normal urothelial cells. However, we do

not know yet whether the NUC-1 derived factor is indeed FGF-like.

Hyperplasia of epithelium adjacent to primary carcinomas has also been found in the oesophagus [4]. A striking finding is that approximately 50% of all primary oesophageal carcinomas have a co-amplification of the FGF-3 and FGF-4 genes [21]. This indicates a more general involvement of FGF-like growth factors in the development of epithelial hyperplasia adjacent to carcinomas.

In conclusion, in this study we obtained evidence for the induction of hyperplasia of urothelium surrounding tumorigenic urothelial NUC-1 cells transplanted into the bladder wall. We also demonstrated that mouse tumorigenic urothelial NUC-1 cells produced and secreted a protein in vitro which was DTT stable, bound to heparin, and stimulated the proliferation of FBHE cells, which respond specifically to FGFs. The present data support the hypothesis that hyperplasia in bladder biopsies of patients with TCC does not necessarily reflect a precancerous lesion prone to the development of a new TCC. The observed hyperplasia may be due to tumour-derived growth factors which have some chemico-physical and biological properties of FGFs. A full characterization of the TCC-derived growth stimulating factor(s) awaits further study.

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