Altered expression of ß1 integrins in renal carcinoma cell lines exposed to the differentiation inducer valproic acid

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Abstract. Renal cell carcinoma (RCC) is the most common malignant tumor of the kidney. Adhesion receptors of the ß1 integrin family are assumed to be involved in carcinogenesis, but it is not clear how they contribute to RCC progression. In an *in vitro* model, we evaluated growth and adhesion capacity of Caki-I and KTC-26 kidney carcinoma cell lines compared to normal renal proximal tubular epithelial cells (PTC). α1-α6ß1 integrin subunits in malignant and non-malignant cells were evaluated by Western blotting and RT-PCR, integrin surface expression was measured by flow cytometry and confocal microscopy. Additionally, tumor cells were allowed to re-differentiate in the presence of valproic acid (VPA) and dynamic alterations of the integrin profile were analyzed. Caki-I and KTC-26 were characterized by accelerated proliferation and adhesion to an endothelial cell monolayer, compared to PTC cells. The integrin ß1 repertoire in RCC cell lines was significantly different from that detected in PTC, and included down-regulated α2 and α6, but up-regulated α1, α3 and α5 proteins. VPA application reduced tumor malignancy which was evidenced by reduced cell growth and adhesion capacity. The reduction in tumor malignancy was paralleled by the integrin expression profile of renal tumor cells 'matching' the pattern seen in PTC. We assume that a sensitive integrin balance exists in normal renal epithelial cells, and that dysregulation of the 'physiological' receptor equipment drives these cells towards malignancy. VPA acted on all investigated integrin subtypes and restored the receptor pattern typical for non-malignant cells. Therefore, VPA may represent a novel therapeutic option in RCC treatment.

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Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor of the kidney. It is characterized by a high potential for metastasis and poor prognosis, however, the mechanistic background leading to tumor dissemination has not been sufficiently evaluated. Receptors of the integrin family are known to be important mediators of tumor cell de-differentiation, tumor cell attachment to the vessel wall and tumor cell proliferation (1). Integrins are structurally organized into heterodimeric transmembrane complexes, variously assembled through the non-covalent association between an α and a ß subunit. The integrin family is divided into subfamilies which share the ß subunit. In particular, integrins of the ß1 family are thought to influence the metastatic potential of cancer cells, making them attractive targets for future therapeutic strategies.

Although there is no doubt that ß1 integrins are involved in carcinogenesis, it is not clear how they contribute to RCC progression. Down-regulation of ß1 integrins is associated with reduced proliferation of kidney carcinoma cells, but does not influence migration (2). In contrast, blocking the α3 subunit inhibits both attachment and migration of kidney carcinoma cells (3). Studies conducted by Rissanen and colleagues point to the α3ß1 receptor responsible for increased malignancy (4), although others have observed reduced α3 expression in metastatic cells (5). There is also evidence that α6 levels significantly decrease in patients with high-grade RCC (6). Tomita *et al* have hypothesized that α4 plays a crucial role in hematogenous metastasis of RCC (7), a subunit which was not detected by immunohistochemical analysis of tissue specimen (8). Rather, all tumors positive for α2 integrin are characterized by invasion beyond the renal capsule or metastases (8,9).

These results suggest that RCC may be influenced by ß1 integrins during metastasis. However, a single integrin subtype might not be the sole trigger initiating the complex program of tumor dissemination. We hypothesized that the balance of tumoral integrin production and alterations of the integrin profile might determine whether a tumor cell will penetrate blood vessels to grow as a secondary tumor. In the present study, the integrin α1-α6 coding mRNA was analyzed, using two different kidney carcinoma cell lines and normal renal

proximal tubular epithelial cells derived from human kidneys not involved in renal cell carcinoma. α1 - α6ß1 integrin subunits were further evaluated by Western blotting, integrin surface expression by flow cytometry and confocal microscopy. The expression pattern was correlated to tumor cell growth and adhesion to an endothelial cell monolayer. To investigate whether alterations of the integrin expression profile contributed to an altered malignant behaviour, tumor cells were treated with the differentiation inducing agent valproic acid (VPA) and modifications of the integrin subunits were evaluated. Growth and adhesion characteristics of treated versus non-treated cells were also analyzed.

Table I. The sequences of the primers used for RT-PCR.

mRNA	Sense primer sequence	Antisense primer sequence	bp
GAPDH	atcttccaggagcgagatcc	accactgacacgttggcagt	509
α1ß1	catgcgctcgttttggaa	cggccacatctcgggaccaga	309
α2ß1	gcatctcagaagtctgttgcc	cctgttgttaccttcagggag	335
α3ß1	tacgtgcgaggcaatgaccta	tttgggggtgcaggatgaagct	306
α6ß1	tggaggtacagttgttggcg	ctccgttaggttcagggagt	253

Materials and methods

Cell cultures. Kidney carcinoma Caki-I and KTC-26 cells were purchased from LGC Promochem (Wesel, Germany). Tumor cells were grown and subcultured in RPMI-1640 medium (Seromed, Berlin, Germany) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified, 5% CO_2 incubator.

Human renal proximal tubular epithelial cells (PTC) were isolated with antibody-coated magnetic beads as previously described (10). Briefly, cells were prepared after tumor nephrectomy from portions of the human kidney not involved in renal cell carcinoma. Tissue fragments were digested with collagenase/dispase (1 mg/ml; Boehringer Mannheim, Mannheim, Germany), collagenase IV (1 mg/ml; Gibco, Eggenstein, Germany), and DNase (100 μg/ml; Boehringer Mannheim). After Percoll density gradient centrifugation, cells were preincubated with human immunoglobulin G (2.5 mg/ml; Biotest, Dreieich, Germany), and then incubated with the primary antibody. PTC were purified with an mAb against aminopeptidase M (CD13; Cymbus Biotechnology, Chandlers Ford, UK), specific for the proximal tubule. Cells were incubated with a microbead-conjugated anti-IgG1 mAb (Miltenyi Biotec, Bergisch-Gladbach, Germany), and isolated by immunomagnetic separation applying a Mini-MACS system (Miltenyi Biotec). PTC were seeded in 6-well plates precoated with human collagen IV (20 μg/ml; Boehringer Mannheim) and grown in medium 199 (Gibco) with 10% FCS (PAA, Cölbe, Germany) at 37˚C and 5% CO_2 in a humidified atmosphere. Preservation of differentiation and function of cultured tubular cells were tested as previously described (11). Passages 3-5 of PTC were used for our experiments.

Endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVEC were grown in medium 199 (Biozol, Munich, Germany), 10% fetal calf serum (FCS; Gibco, Karlsruhe, Germany), 10% pooled human serum (Blood Bank of The German Red Cross, Frankfurt am Main, Germany), 20 μg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin (Roche, Basel, Switzerland), 100 ng/ml gentamycin (Gibco) and 2% 1 M HEPES-buffer (Seromed, Berlin, Germany). To control the purity of HUVEC cultures, cells were stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against Factor VIII-associated antigen (Von Willebrand factor; clone F8/86; Dako, Hamburg, Germany) and analyzed microscopically or by FACscan (Becton Dickinson, Heidelberg, Germany; FL-1H (log) channel histogram analysis; $1x10^4$ cells/scan). Cell cultures with a purity >95% were serially passaged. Subcultures from passages 2-4 were selected for experimental use.

Tumor cell growth. Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Roche Diagnostics, Penzberg, Germany). Caki-I and KTC-26 or PTC cells (100 μl, $1x10^4$ cells/ml) were seeded onto 96-well tissue culture plates and incubated as described above. After 24 h, MTT (0.5 mg/ml) was added for an additional 4 h. Thereafter, cells were lysed in a buffer containing 10% SDS in 0.01 M HCl. The plates were allowed to stand overnight at 37˚C, 5% CO_2. Absorbance at 570 nm was determined for each well using a microplate ELISA reader. Each experiment was done in triplicate. After subtracting background absorbance, results were expressed as mean cell number.

Monolayer adhesion assay. HUVEC were transferred to 6-well multiplates (Falcon Primaria; Becton Dickinson) in complete HUVEC-medium. When confluency was reached, $0.5x10^6$ tumor cells/well were carefully added to the HUVEC monolayer for different time periods. Subsequently, non-adherent tumor cells were washed off using warmed (37˚C) medium 199. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields (5x0.25 mm^2) using a phase contrast microscope (20x objective) to calculate the mean cellular adhesion rate.

Evaluation of integrin surface expression. Tumor cells were washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min at 4˚C with the FITC-conjugated monoclonal antibody anti-α2ß1 (Becton Dickinson; clone AK-7), anti-α4ß1 (Cymbus Biotechnology, Hofheim, Germany; clone HP2I1), anti-α5ß1 (Cymbus Biotechnology; clone SAM-1), anti-α6ß1 (Becton Dickinson; clone GOH3), or with the PE-conjugated monoclonal antibody anti-α1ß1 (Becton Dickinson; clone SR84), or anti-α3ß1 (Becton Dickinson; clone C3II1). Integrin expression of tumor cells was then measured using a FACscan [Becton Dickinson; FL-1H or FL-2H (log) channel histogram analysis; $1x10^4$ cells/scan] and expressed as mean fluorescence units (MFU). A mouse IgG1-FITC was used as an isotype control for FITC conjugated antibodies. To evaluate background staining of PE conjugated antibodies, goat anti-mouse IgG-PE was used (all: Cymbus Biotechnology).

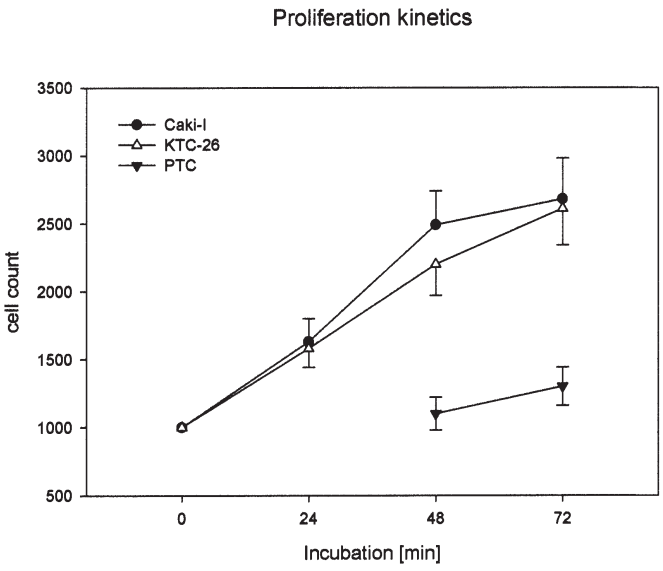

Figure 1. Proliferation kinetics of Caki-I and KTC-26 tumor cells versus normal kidney epithelial PTC cells. Cells were seeded on 96-well culture plates and cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Each data point depicts the mean value ± SD of three wells. One representative of six experiments is shown.

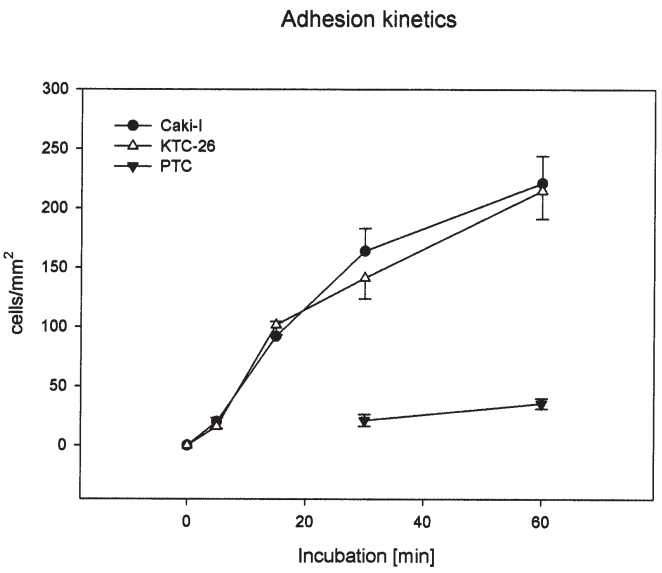

Figure 2. Adhesion capacity of Caki-I and KTC-26 tumor cells versus normal kidney epithelial PTC cells. Each cell type was added to human umbilical vein endothelial cell monolayers for different time periods. Non-adherent cells were washed off and the remaining cells were fixed and counted in five different fields (5x0.25 mm^2) using a phase contrast microscope. Adhesion capacity is depicted as the number of bound cells/mm^2 (mean ± SD). One representative of six experiments is shown.

To analyze integrin ß1 distribution on the cell membrane, tumor cells were transferred to round cover slips (pretreated with 2% 3-aminopropyl-triethoxysilan) placed in a 24-well multiplate. Upon reaching confluency, cell cultures were washed and fixed in cold (-20˚C) methanol/acetone (60/40 v/v). Subsequently, cells were incubated for 60 min with unconjugated anti-integrin monoclonal antibodies. Indocarbocyanine (Cy 3™; Dianova; working dilution: 1:50) conjugated goat anti-mouse IgG was then added as the secondary antibody. To prevent photobleaching of the fluorescent dye, cover glasses with stained cells were taken out of the wells and the residual liquid was removed. These were then embedded in an antifade reagent/mounting medium mixture (ProLong™ antifade kit, MoBiTec, Göttingen, Germany) and mounted on slides. The slides were viewed using a confocal laser scanning microscope (LSM 10; Zeiss, Jena, Germany) with a plan-neofluar x100/1.3 oil immersion objective.

mRNA expression of ß1 integrins. mRNA expression of ß1 integrins was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Tumor cells were seeded in 50-ml culture flasks (25 cm^2 growth area; Falcon Primaria, Becton Dickinson) and cultured with or without MMF. Total RNA was extracted by using RNeasy kit (Qiagen, Hilden, Germany) and RNA samples were then treated with 80 U/ml of RNAse-free DNAse I (Boehringer Mannheim) for 60 min at 37˚C, to eliminate amplifiable contaminating genomic DNA. Subsequently, samples were incubated for 10 min at 65˚C to inactivate DNAse. Complementary DNA was synthesized from 1 μg of total RNA per sample with a 60-min incubation at 42˚C, using the Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany) and oligo-(dT) priming (Boehringer Mannheim). Amplification was carried out using gene-specific primers and Platinum-Taq polymerase (Invitrogen) in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Reactions were performed in the presence of 0.5 μl cDNA, with an initial incubation step at 95˚C for 2 min. Cycling conditions consisted of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec over a total of 30 cycles. The reaction was completed by another 10-min incubation step at 72˚C. The specific sequences for sense and anti-sense primers are shown in Table I. The PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by ethidium bromide.

Western blot analysis. Total ß1 integrin content in Caki-I and KTC-26 or PTC cells was evaluated by Western blot analysis: tumor cell lysates were applied to a 7% polyacrylamide gel and electrophoresed for 90 min at 100 V. The protein was then transferred to nitrocellulose membranes. After blocking with non-fat dry milk for 1 h, the membranes were incubated overnight with an anti-α1, anti-α2, anti-α3 or anti-α6 monoclonal antibody (dilution 1:100; all: Santa Cruz Biotechnology). HRP-conjugated goat anti-rabbit IgG or mouse anti-goat IgG (Biozol, München, Germany; dilution 1:5000) served as the secondary antibody. The membranes were briefly incubated with ECL detection reagent (ECLTM, Amersham) to visualize the proteins and exposed to X-ray film (Hyperfilm™ ECTM, Amersham).

Differentiation induction by valproic acid. Tumor cells were treated with the differentiation inducing agent valproic acid (VPA; Sigma, München, Germany) at a final concentration of 1 mM for 3 or 5 days. The ß1 integrin coding mRNA, ß1 integrin protein level, and ß1 integrin surface expression were then measured in VPA-treated cells. Results were compared to untreated controls. Viability of tumor cells in the presence of VPA was assessed by propidium iodide

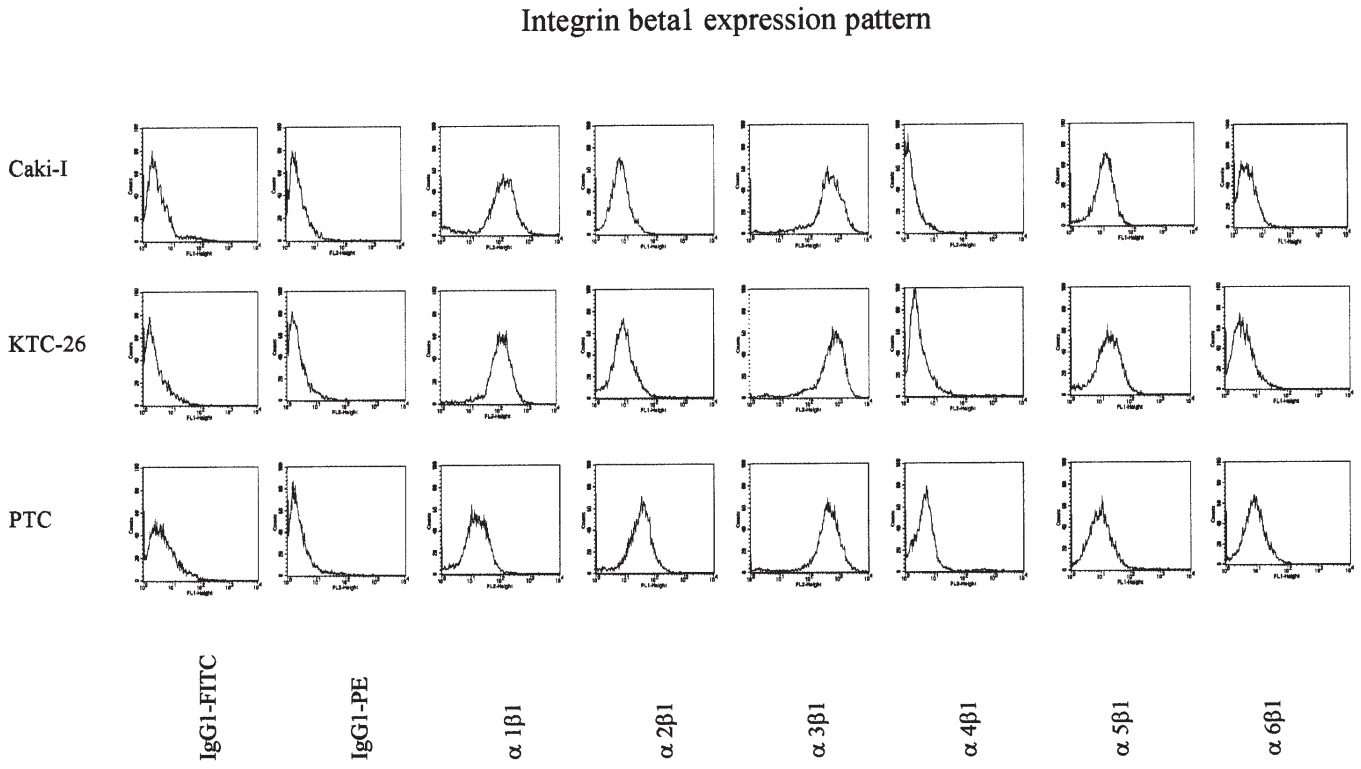

Figure 3. FACS analysis of ß1 integrin surface expression on Caki-I and KTC-26 tumor cells versus normal kidney epithelial PTC cells. Cells were washed in blocking solution and then stained with specific monoclonal antibodies as listed in Materials and methods. A mouse IgG1-FITC was used as an isotype control for FITC conjugated antibodies. To evaluate background staining of PE conjugated antibodies, goat anti-mouse IgG-PE was used. Fluorescence was analyzed using a FACScan flow cytometer, and a histogram plot was generated to show FITC/PE-fluorescence.

dsDNA-intercalation or quantitative fluorescence analysis of enzyme-catalyzed fluorescein-diacetate metabolism.

Statistical analysis. All experiments were performed 3-6 times. Statistical significance was investigated by the Wilcoxon Mann-Whitney U test. Differences were considered statistically significant at $p<0.05$.

Results

Growth capacity of tumor cells. Proliferation analysis revealed rapid cell growth of both Caki-I and KTC-26 tumor cells with doubling every 48 h. In contrast, non-malignant PTC cells exhibited significantly lower growth activity (Fig. 1).

Tumor cell adhesion. Adhesion capacity of tumor cells to an endothelial cell monolayer was evaluated. The 60-min adhesion rates of tumor cells to HUVEC were 221±23 cells/mm^2 (Caki-I) or 215±24 cells/mm^2 (KTC-26) (Fig. 2). The 60-min adhesion capacity of PTC cells was significantly reduced, compared to Caki-I and KTC-26 (35±4 PTC cells/mm^2).

Integrin ß1 surface expression. Malignant Caki-I and KTC-26 cells were characterized by a high adhesion rate and growth capacity, whereas non-malignant PTC cells grew slowly and attached to endothelial cells to a very low extent. In a next step, integrin surface expression was evaluated on malignant versus non-malignant cells by flow cytometry. The same integrin pattern was detected on Caki-I and KTC-26 cells, which however differed distinctly from the integrin pattern detected on PTC cells (Fig. 3). In this context, elevated amounts of α1ß1 were measured on Caki-I (110.8±45.7 MFU; n=6) and KTC-26 (109.6±34.3 MFU; n=6), in comparison to PTC cells whose receptor was drastically reduced (21.5±1.2 MFU; n=6). α2ß1 expression level was lowered in Caki-I (9.4±3.2 MFU; n=6) and KTC-26 (12.1±2.3 MFU; n=6) when compared to the α2ß1 level of PTC cells (43.2±12.7 MFU; n=6). Maximum fluorescence was measured with respect to the α3ß1 receptor (Caki-I: 688.4±162.9 MFU; KTC-26: 737.8±142.0 MFU) which was diminished in PTC cells (537.8±71.1 MFU; n=6). Neither the malignant cell lines nor PTC cells expressed α4ß1. A higher α5ß1 level was seen on Caki-I (21.2±8.8 MFU; n=6) and on KTC-26 (28.1±7.6 MFU; n=6) than on PTC cells (13.2±3.2 MFU; n=6). Conversely, α6ß1 was expressed on PTC cells (14.3±4.9 MFU; n=6) but reduced to nearly zero on Caki-I and KTC-26.

Confocal analysis confirmed that α1ß1 and α3ß1 receptors, both of which were clearly detected by flow cytometry, are distributed along the cell surface of the tumor cells (Fig. 4).

Integrin ß1 protein level. Ongoing studies concentrated on the intracellular integrin protein content. In good accordance to the facs-data, α3ß1 proteins accumulated in both Caki-I and KTC-26 cells (Fig. 5). The α1ß1 proteins were also detected, although to a lesser extent than α3 subtypes. Slight protein bands were visualized with respect to α2ß1 integrins, whereas α4ß1 proteins were not detected.

Integrin ß1 mRNA expression pattern. Comparative analysis of mRNA coding for the ß1 integrin family was carried out by

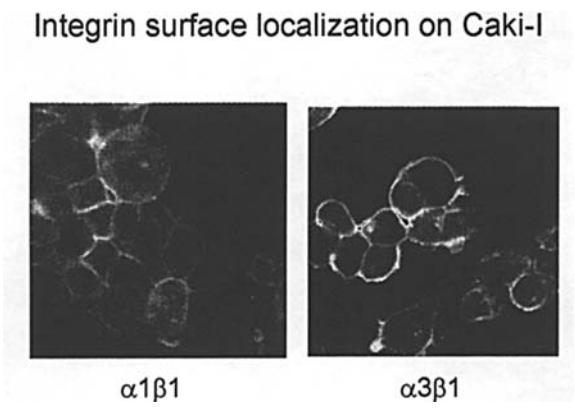

Figure 4: Confocal images of the distribution pattern of ß1 integrin receptor molecules on Caki-I tumor cells. Integrin subtypes which were predominantly expressed on the respective tumor cell lines are shown. Magnification x100.

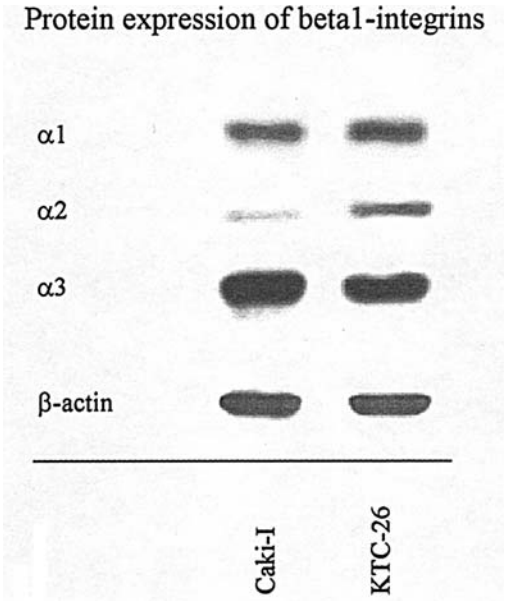

Figure 5. Western blot analysis of integrin ß1 subtypes in Caki-I and KTC-26 tumor cells. The monoclonal antibody clones which were used to recognize α1, α2 and α3 subtypes are listed in Materials and methods. ß-actin served as the internal control. One representative experiment of three is shown.

RT-PCR. Fig. 6 demonstrates close similarity between Caki-I and KTC-26 integrin expression pattern. In both cell lines, α1 and α3 coding mRNA were mostly active (α3 > α1), in contrast to α2 and α6 mRNA which were detected only moderately. PTC cells were characterized by a similar mRNA pattern for all integrin subtypes investigated.

Tumor cell differentiation by VPA. Based on the above observations, it was hypothesized that differentiation and malignancy of RCC might be characterized by specific alterations of integrin synthesis and membrane presentation. To elucidate this, Caki-I cells were treated with the differentiation inducing compound VPA whereby the integrin ß1 mRNA and surface level were evaluated and compared to the integrin expression in non-malignant PTC cells.

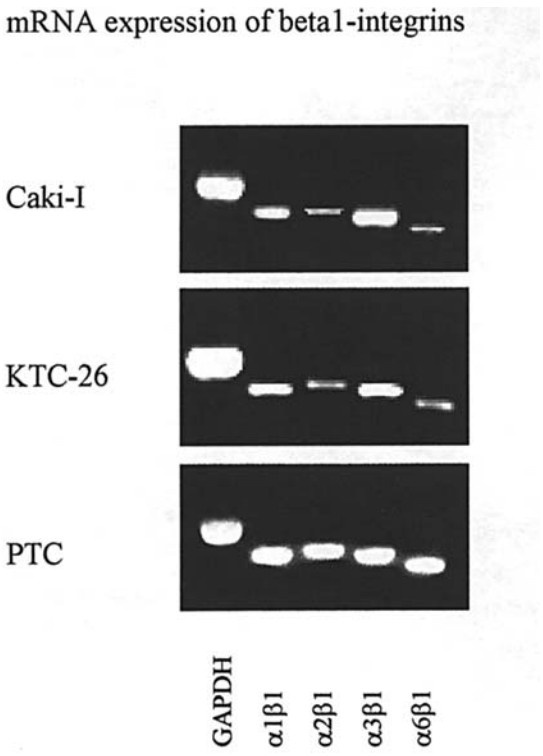

Figure 6. Semiquantitative RT-PCR analysis, carried out on Caki-I and KTC-26 tumor cells versus normal kidney epithelial PTC cells. The figure demonstrates analysis of mRNA coding for α1, α2, α3, or α6 integrins. RNA was extracted, reverse-transcribed and submitted to semiquantitative reverse transcription-PCR using gene-specific primers as described in Materials and methods. The internal control for the RT-PCR reaction was performed by running parallel reaction mixtures with the housekeeping gene GAPDH. One representative of three separate experiments is shown.

VPA significantly blocked tumor cell proliferation (Fig. 7), as well as tumor cell attachment to human endothelial cells (Fig. 8). A 5-day VPA-incubation evoked stronger effects on Caki-I than a 3-day incubation period. VPA did not exert any toxic effects on the cell cultures which might contribute to this phenomenon. Reduced tumor malignancy, evidenced by reduced cell growth and binding, was paralleled by 'matching' the Caki-I integrin expression profile to the pattern seen in PTC cells (Fig. 9). α2 and α6 subtypes were increased, α3 and α5 subtypes were decreased in the presence of VPA, ultimately leading to the same fluorescence level as measured in PTC cells. The α1ß1 integrins did not follow this rule. Rather, this subtype, which was already enhanced in Caki-I cells when compared to PTC cells, further increased when Caki-I were exposed to VPA for 3 or 5 days.

The data, presented in Fig. 10, demonstrate VPA triggered modifications of the integrin ß1 coding mRNA. In good accordance with our hypothesis, both the α2 and α6 mRNA level were elevated by the differentiation inducing agent. Five days after VPA application, the mRNA pattern of Caki-I cells exactly matched the mRNA pattern identified in PTC cells.

Discussion

Based on a xenograft model, Davidowitz and coworkers recently hypothesized that alterations of integrins might

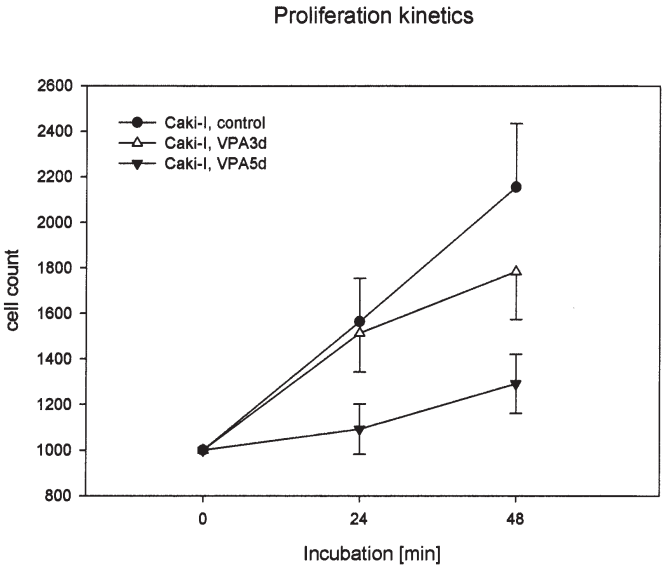

Figure 7. Dose-response analysis. Caki-I cells were pretreated with 1 mM VPA for 3 or 5 days. Controls are represented by non-treated cells. Proliferation (24 and 48 h) was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Each data point depicts the mean value ± SD of three wells. One representative of six separate experiments is shown.

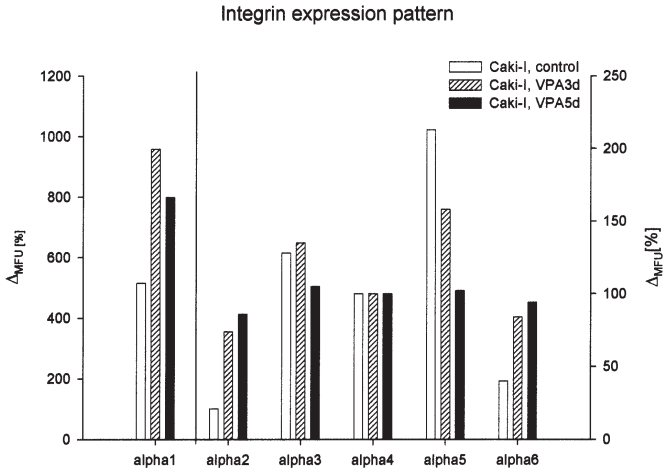

Figure 9. FACS analysis of VPA-induced changes of ß1 integrin surface expression. Caki-I cells were pretreated with VPA (1 mM) for 3 or 5 days, washed in blocking solution and then stained with specific monoclonal antibodies as listed in Materials and methods. Controls remained untreated. To clearly demonstrate the 'matching' effect of VPA, integrin subtypes were also evaluated on PTC cells and MFU data of each integrin were set to 100%. Caki-I control bars as well as bars representing VPA treated cells depict the difference to the 100% values. Please note that α2-α6 subtype presentation is related to the right y-axis, α1 subtype presentation is related to the left y-axis. One representative of six experiments is shown ($SD_{intraassay}$ <20%).

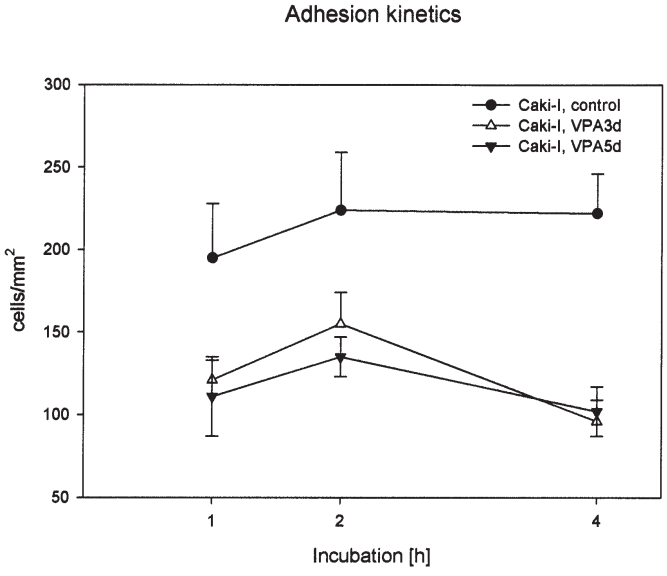

Figure 8. Adhesion analysis of VPA treated Caki-I cells. Tumor cells were pretreated with 1 mM VPA for 3 or 5 days (controls remained untreated) and adhesion capacity measured after 1, 2, or 4 h. Adhesion capacity is depicted as the number of bound cells/mm^2 (mean ± SD). One representative of six experiments is shown.

contribute to the development of RCC (12). We now present evidence that changes of the integrin ß1 repertoire in renal cells determine their differentiation state and drive 'normal' cells to become malignant. Surprisingly, modification of each integrin subtype was not the same. Down-regulated α2 and α6, but up-regulated α1, α3 and α5 proteins were seen in Caki-I and KTC-26, compared to non-malignant PTC cells. It appears that a sensitive integrin balance prevails in renal epithelial cells which might guarantee adequate cell growth and adhesion capacity. Disturbing this balance may modify downstream targets and subsequently displace the fine tuned intracellular signalling network.

Tumor cell proliferation and binding capacity dropped in the presence of the differentiation inducing compound VPA, a process which was accompanied by matching the integrins to the pattern measured in normal PTC cells. According to this, differentiation of human colonic Caco-2 cells induced by the short-chain fatty acid butyrate was associated with alterations in the ß1 integrin-mediated signaling pathway (13). Although the expression of integrin ß1 subtypes was not evaluated in this study, the authors hypothesized that changes of ß1 receptors might lead to perturbation of downstream signal transduction cascades and loss of cell growth control.

Recently, a novel orthotopic mouse tumor model of RCC was developed and genes involved in metastasis evaluated. In good accordance with our data, α3 integrins were demonstrated to be up-regulated in tumors grown in kidneys. In the same study, blocking α3 integrin inhibited tumor cell attachment and migration, supporting integrin driven tumor dissemination (3). Rissanen *et al* observed that adhesion of normal human renal epithelial cells and RCC cells from a G1 tumor was mediated mainly by α6ß1 integrins, while cells from a G3 tumor mainly used the α3ß1 integrin complex (4). Hypothetically, tumor cells might use distinct sets of integrin receptors, depending on their malignant potential. Based on the study of Rissanen *et al*, it appears that α6ß1-mediated adhesion, which is also a prerequisite for the formation of polarized renal tubular epithelium (14), is retained in cells from G1 tumors, whereas cells from more poorly differentiated G3 tumors have replaced α6ß1 by an α3ß1 dependent adhesion mechanism.

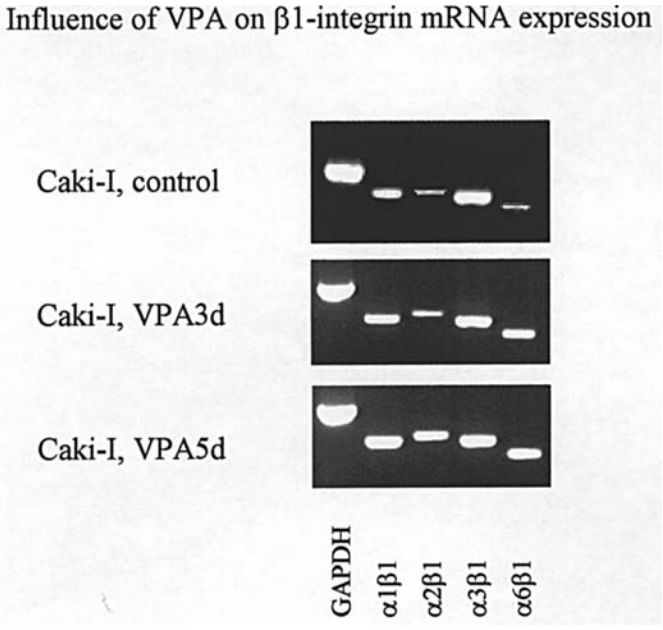

Figure 10. Semiquantitative RT-PCR analysis, carried out on VPA treated versus non-treated Caki-I tumor cells. mRNA coding for α1, α2, α3, or α6 integrins was analyzed. RNA were extracted, reverse-transcribed and submitted to semiquantitative reverse transcription-PCR using gene-specific primers as described in Materials and methods. The internal control for the RT-PCR reaction was performed by running parallel reaction mixtures with the housekeeping gene GAPDH. One representative of three separate experiments is shown.

In accordance with this statement, we found reduced α6 proteins in the malignant RCC cell lines, compared to the normal human renal epithelial cells. Nevertheless, the role of ß1 integrins in tumor development might be more complex than initially thought.

Our differentiation experiments revealed that a particular integrin subtype was not simply replaced by another. Rather, both up- and down-regulation of a cohort of receptor proteins took place. Although integrin receptors undoubtly play a dominant role during tumor cell-endothelial cell interaction, the biphasic alterations verified in our study rule out that integrins exclusively serve as mechanistic adhesion elements. We have presented evidence in an earlier study that adhesion blockade of DU-145 prostate tumor cells is accompanied by a dose-dependent up-regulation of integrin ß1 subtypes (15). Consequently, down-regulation of ß1 integrin family members allowed prostate tumor cells to become more invasive and led to an increased propensity for metastasis (16). A similar situation has been observed with the RCC cell line CCF-RC1, which displayed reduced proliferation but not reduced migration properties after down-regulating ß1 integrins (2).

Therefore, ß1 integrins may play a more general promoting role in the progression of cancer. Up-regulation of the same integrin type might lead either to enhanced cell adhesion, by coupling the receptor to its ligand, or to reduced cell adhesion, by activating integrin driven differentiation signals. The fact that integrin up-regulation might not always stimulate cell adhesion has been shown recently: forced expression of α5ß1 in transformed Chinese hamster ovary cells suppressed growth in soft agar and tumor formation in immunodeficient mice, caused by increased expression of the tumor suppressor p16INK4a (17). Similarly, enhanced expression of integrin α5ß1 in colon cancer cells resulted in the post-transcriptional down-regulation of the HER-2 oncoprotein which then led to diminished proliferation and tumorigenicity (18).

Based upon our *in vitro* assay, we conclude that tumor development might be caused by dysregulation of the 'physiological' integrin ß1 balance, which includes both enhanced expression as well as the loss of specific ß1 subtypes. Enhancement of integrin ß1 subtypes might directly contribute to accelerated adhesion, whereas receptor reduction might cause de-differentiation of RCC cells towards a highly adhesive phenotype with accelerated cell growth. However, it still remains to be determined if integrin up-regulation, seen in this cell system, also induces de-differentiation by selective activation of tumor-promoting pathways.

The data presented here are based on Caki-I and KTC-26 cells, therefore applying particularly to RCC. The role of integrins may be quite different for other tumor types. In fact, comparative analysis of pancreatic, colon and prostate carcinoma cell lines have revealed that ß1 integrins are not involved in the same way in tumor cell adhesion (14). Pancreatic cells have shown reduced cell adhesion to an endothelial cell monolayer, coupled with reduced α3ß1 integrin surface expression. Prostate carcinoma cells have shown enhanced α3ß1 integrin surface expression. Why the malignant process seems to be triggered by integrin up-regulation in one tumor entity but down-regulation in another entity is not clear. Possibly, different tumor cells are equipped with different enzyme systems, and the intracellular signaling cascade responds to different signals.

When discussing the dynamic alterations of the integrin pattern during tumor cell de-differentiation one event did not follow the rule of protein re-balancing. Flow cytometry (but not mRNA analysis) indicated that α1 surface level on Caki-I cells further increased in the presence of VPA, although this subtype was already elevated in non-treated malignant cells, compared to PTC. The interpretation of this peculiarity is difficult because the effect was not seen at the transcriptional level. Information about the function of α1ß1 integrins in renal cancer are sparse and the extent to which such alterations affect the onset and outcome of the disease is not known. Correlation analysis of scored integrin levels with histopathological parameters and TNM status performed on RCC showed that α1 was present in all specimens studied (6). This observation might exclude α1ß1 integrins from being a highly sensitive differentiation element. Slight aberrations of the α1 integrin surface level might not necessarily lead to distinct alterations of the cellular differentiation state. VPA evoked matching of α2, α3, α5 and α6 integrins may be sufficient to revert neoplastic transformation and counteract the 'irregular' expression of α1 integrins.

Nevertheless, another possibility should be considered. VPA did not completely block proliferation and adhesion of Caki-I cells, and some slight differences remained between the biological activity of treated tumor cells and the non-malignant PTC controls. Therefore, perfect integrin matching between treated tumor cells and non-malignant PTC controls probably cannot be expected.

From a clinical viewpoint, anti-integrin therapy is an attractive approach to treating RCC. However, considering

the roles of tumor cell integrins in the experiments presented here, and remembering that RCC tumor cells seem to gain and lose specific integrins during the course of tumor progression, simply blocking integrin function is probably not the optimum cancer treatment. A strategy should be pursued which stabilizes and/or re-establishes the 'physiological' integrin balance of normal epithelial cells. VPA did influence all integrin subtypes in our experiments and restored the receptor pattern representative for non-malignant cells. VPA has been demonstrated to overcome the epigenetic barriers to transcription of a prototypical silenced tumor suppressor gene in human breast cancer cells (19). VPA is also highly effective at suppressing growth in poorly differentiated thyroid cancer cell lines (20). It down-regulates oncoprotein expression, induces differentiation and inhibits *in vivo* growth of medulloblastoma in immunodeficient mice (21).

Valproic acid may present a novel therapeutic option in cancer treatment. Further studies are underway to explore the differentiation inducing properties of VPA in RCC.

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