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Regulation of prostate cell collagen receptors by malignant transformation

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Cell adhesion receptors, including the integrin-type collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$) participate in cancer progression and invasion. Quantitative RT-PCR indicated that all 4 receptors are abundantly expressed in sarcoma-derived cell lines, whereas most carcinoma-derived cells express $\alpha_1\beta_1$ and $\alpha_2\beta_1$ only. This was surprising because $\alpha_{11}\beta_1$ has been connected previously to the progression of lung adenocarcinomas. To test the hypothesis that α_{11} expression may not persist in cultured cancer cells we analyzed fresh tissue samples of 104 total prostatectomies, keeping in mind that prostate cancer cell lines showed negligible α_{11} mRNA levels. In prostate α_2 expression was significantly lower in poorly differentiated carcinomas when compared to benign lesions ($p = 0.0331$). In immunohistochemistry the protein levels of α_2 integrin decreased significantly ($p = 0.0001$) and the protein levels of α_{11} subunit increased significantly ($p = 0.029$) with the increasing grade of carcinoma. Thus $\alpha_{11}\beta_1$ may replace $\alpha_2\beta_1$ during tumor progression. Our observations support the idea that $\alpha_{11}\beta_1$ may be expressed in tumors but the corresponding cell lines may lose the expression of this integrin. Previous studies have shown that in cell culture androgen receptor (AR) controls $\alpha_2\beta_1$ expression. We measured AR mRNA levels and the number of AR positive nuclei in the prostate samples and the results showed a significant correlation between $\alpha_2\beta_1$ and AR. Androgen receptors may control the mechanisms regulating integrin expression in prostate.
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Integrins are adhesion molecules that act in cell–cell and cell–extracellular matrix interactions. They comprise of 2 subunits called α and β, which can form at least 24 different non-covalently linked α/β-heterodimers.[1] Four collagen receptors form a specific subclass of integrins because they all contain an inserted domain (I domain) in their α subunit. These α subunits form heterodimers with β_1 subunit only.

The most abundant integrin-type collagen receptors are $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Integrin $\alpha_1\beta_1$ is predominantly expressed in mesenchyme and the highest expression levels are on smooth muscle cells.[2,3] Integrin $\alpha_2\beta_1$ is expressed abundantly on epithelial cells but it is also present on many mesenchymal cells, such as fibroblasts, chondrocytes and osteoblasts.[4–6] The newest members of the collagen receptor integrins, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, were found more recently[7,8] and their expression patterns are less well-known.[9,10]

Integrins have been shown to regulate cell growth, differentiation and survival, as well as malignant transformation, cancer cell invasion and metastasis.[11] When malignant tumors and cancer cells have been analyzed, the expression and function of $\alpha_2\beta_1$ has been connected to invasion of malignant melanoma[12] as well as to prostatic, gastric and ovarian carcinomas,[13–15] whereas $\alpha_1\beta_1$ integrin is the collagen receptor of T cell derived lymphoma cells.[16] α_{11} integrin is recently found to be a tumor marker in non-small cell lung cancer.[17]

The collagen receptors have also differences in their ability to bind distinct collagen subtypes. $\alpha_1\beta_1$ integrin has highest affinity to collagen types IV, VI and XIII[18–20] and it is also a receptor for laminins.[21,22] $\alpha_2\beta_1$ integrin is a receptor for fibril-forming collagens[18,19,23] and it also recognizes laminins[24–26] and tenascin 1.[27] $\alpha_{10}\beta_1$ integrin resembles $\alpha_1\beta_1$ in its selectivity to different collagen subtypes and $\alpha_{11}\beta_1$ integrin mainly binds to fibril-forming collagens.[19,28] In addition the 4 collagen receptors have distinct signaling functions.[29] Thus the cells can behave in a different way depending on which one of one of the collagen receptor they use to interact with extracellular matrix.

Prostate cancer is the most common malignancy among men in western world and it frequently leads to the formation of bone metastasis. The importance of cell–collagen interaction has been emphasized in cancer progression and especially in the process of cancer spread into bones. There also is a putative association between collagen receptors and androgens. Androgens mediate their physiological effects on prostate epithelial cells *via* the androgen receptor (AR). In cancer cell culture AR-transfection have been shown to increase $\alpha_2\beta_1$ integrin expression and cell adhesion to collagen I.[30]

Our aim was to analyze the expression of collagen receptor integrins in different carcinoma- and sarcoma-derived cell lines. Based on the results on cell lines we focused the analyses to prostate cancer progression in clinical samples of different grade prostatic lesions. The expression pattern of α_2 and α_{11} integrins was analyzed in prostate tissue with specific antibodies and real-time RT-PCR. The hypothesis that AR may regulate the expression of integrins *in vivo* was also studied. Our findings indicate that $\alpha_2\beta_1$ integrin can be replaced by another collagen receptor, namely $\alpha_{11}\beta_1$, during the progression of prostate adenocarcinomas. This switch may have important consequences for the behavior of cancer cells. Although α_2 mRNA levels decreased when prostate cancer progressed, AR mRNA levels did not change, suggesting that regulation of $\alpha_2\beta_1$ expression by malignant transformation is independent of AR transcription. In normal and transformed cells (Grades 4–5) AR mRNA expression had a strong correlation to integrin expression, suggesting that it may still be one of the mechanisms regulating integrins in prostate cells. The comparison of α_2 and AR protein expression in prostate samples also proposed a linkage between AR and integrins.

Material and methods

Cell lines

WM-115, A2058, SK-MEL-5, MG-63, KHOS-240, HT-1080, SK-LMS-1, SAOS, HT-29, SW-620, HeLa, PC-3, DU145 and MDA-MB-231 human cancer cell lines were cultured in DMEM

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TABLE 1 – MEAN COLLAGEN RECEPTOR INTEGRIN MRNA LEVELS IN DIFFERENT ORIGIN CELL LINES

Cell line	Origin	α_1 mRNA	α_2 mRNA	α_{10} mRNA	α_{11} mRNA
Melanomas					
WM-115	Skin melanoma	0	0.6	0.2	0
A2058	Melanoma, lymph node metastasis	3.0	5.2	0.2	0
SK-MEL-5	Melanoma, lymph node metastasis	5.7	1.5	0.1	1.9
Sarcomas					
MG-63	Osteosarcoma, bone	1.2	28.3	0	3.2
KHOS-240	Osteosarcoma, bone	1.4	1.9	0.2	1.5
HT-1080	Fibrosarcoma, connective tissue	1.5	41.6	4.3	1.5
SK-LMS-1	Leiomyosarcoma, vulva	0.5	8.6	0.1	0.6
SAOS	Osteosarcoma, bone	6.5	0	0.3	9.5
Carcinomas					
UT-SCC-10	Squamocellular ca, head and neck region	0.6	25.5	0	0
UT-SCC-15	Squamocellular ca, head and neck region	0.6	9.9	0	0
NCI-H2170	Squamocellular ca, lung	0	3.2	0	0
NCI-H520	Squamocellular ca, lung	0	1.2	0	0
NCI-H1563	Non-small cell adenoca, lung	0.7	1.4	0	1.0
NCI-H2228	Non-small cell adenoca, lung	1.8	34.1	0	0.2
HT-29	Adenocarcinoma, colon	1.9	3.1	0	0
SW-620	Adenocarcinoma, colon	0.2	1.7	0	0
HeLa	Adenocarcinoma, uterine cervix	7.4	3.0	0	0
PC-3	Prostate ca, bone metastasis	7.7	18.4	0.6	0
DU145	Prostate ca, brain metastasis	1.2	17.0	0.1	0.1
LNCaP	Prostate ca, lymph node metastasis	0.8	1.4	0.01	0.02
MDA-MB-231	Breast ca, pleural metastasis	3.5	25.8	0.8	0
T47-D	Breast ca, pleural metastasis	0	4.4	0.4	0
Embryonal carcinomas					
Tera-1		0.8	1.7	0	0.2
Tera-2		3.4	2.2	0	3.0

Values are presented as percentage of control mRNA (β-actin).

supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin-G and 100 μg/ml of streptomycin. LNCaP, T47-D, NCI-H2170, NCI-H520, NCI-H1563, NCI-H2228, Tera-1 and Tera-2 human cancer cell lines were cultured in RPMI containing 10 mM HEPES and 1 mM sodium pyruvate (Table I) All the cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) except the 2 head and neck region squamocellular carcinoma cell lines, UT-SCC-10 and UT-SCC-15 (cultured in DMEM), which were a kind gift from Prof. R. Grenman (University of Turku).

Immunoprecipitation

Specific antisera against human β_1, α_2 and α_{11} integrin subunits were used in immunoprecipitation assays of MG-63 cells.[8,31,32] Cell cultures were metabolically labeled with 50 μCi/ml of [^{35}S] methionine (Tran^{35}S-label, ICN Biomedicals Inc., Irvine, CA) for 18 hr in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM Tris-HCl (pH 7.4) and then detached by scraping. Cell pellets obtained by centrifugation at 500*g* for 5 min were solubilized in 200 μl of the same buffer containing 100 mM *n*-octyl-β-D-glucopyranoside (Sigma, St. Louis, MO) on ice with occasional vortexing. Insoluble material was removed by centrifugation 10,000*g* for 5 min at 4°C. Radioactivity in cell lysates was counted, and an equal amount of radioactivity was used in immunoprecipitation assays. Triton X-100 (0.5% v/v) and BSA (0.5 mg/ml) were added to the supernatants, which were then pre-cleared by incubation with 50 μl of packed protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Supernatants were immunoprecipitated with anti-integrin antibodies for 12 hr at 4°C. Immunocomplexes were recovered by binding to protein A-Sepharose and washing the beads 4 times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml BSA and twice with 0.5 M NaCl and 25 mM Tris-HCl (pH 7.4). The immunoprecipitates were analyzed by SDS-PAGE, containing 6% polyacrylamide, under non-reducing conditions followed by fluorography.

Clinical tissue samples

Prostate tissue material consisted of specimens freshly collected from 104 prostate cancer patients operated at the Turku University Central Hospital during the years 1999–2002. Each patient gave an informed consent and the study protocol was approved by the ethical committee of the University of Turku. A sample from both sides of the prostate was taken under palpation control and stored in −80°C in solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate dehydrate, 0.5% (w/v) sodium lauryl sarcosinate and 0.7% (v/v) 2-mercaptoethanol until the RNA isolation.[33] Adjacent samples were frozen for IHC analysis and pathological diagnosis.

RNA extraction and cDNA synthesis

Total RNA was extracted from clinical samples using modified guanidium-thiocyanate-phenol-chloroform extraction method as previously described.[34,35] RNA was extracted from cultured cells using either Nucleospin RNA II total RNA isolation kit (Macherey-Nagel Inc., Easton, PA) or RNAzol B reagent (Tel-Test Inc., Friendswood, TX) according to manufacturer's instructions. RNA samples were treated with 5 U of DNase I (Roche) to eliminate possible contaminating DNA.

cDNA was synthesized from 1 μg of total RNA in a reaction containing 0.5 μg of random hexamer primers and 200 U of Moloney murine leukemia virus RNase H minus reverse transcriptase, in a total volume of 25 μl according to manufacturer's protocol (Promega, Madison, WI). A similar reaction was carried out in the absence of reverse transcriptase enzyme to control for false positive amplification resulting from chromosomal DNA.

Real-time quantitative PCR

Specific primers and fluorescent probes for real-time PCR analysis of cDNA samples were designed using, Primer Express v1.5 software (Perkin-Elmer Applied Biosystems, Foster City, CA). The primer and probe sequences for integrin analyses were synthesized by Eurogentec S.A. (Liege, Belgium) based on the unconserved regions of integrins α_1, α_2, α_{10} and α_{11}. The AR primer and probe sequences were based on the information published previously [36] and synthesized by Applied Biosystems (Foster City, CA). Primers and probe to β-actin mRNA were used as an internal control (Table II).

To control the linearity of the PCR reaction at any template mRNA concentration in the original samples, standard curves

TABLE II – SEQUENCES OF PRIMERS AND PROBES USED IN QRT-PCR

Template mRNA	Primer/probe	Sequence
α_1 integrin	Forw primer	5′-GCTCTCAATCAGACAAGGTTTGAA-3′
	Rev primer	5′-AATTGTGCTGCCGAGATGAAC-3′
	Probe	5′-CACGTCTGCTTAATAGGTTCCAGGCTCATT-3′
α_2 integrin	Forw primer	5′-TGGATTTGCGTGTGGACATC-3′
	Rev primer	5′-GGCAGTTCTAGAATAGGCTTCAA-3′
	Probe	5′-TCTGGAAAACCCTGGCACTAGCCCTG-3′
α_{10} integrin	Forw primer	5′-GGATTGCTGCTGCCTCCAT-3′
	Rev primer	5′-AGATCTAGCCGACCATCCACACT-3′
	Probe	5′-CCACATGCCCTCAGCTACTTTGGCC-3′
α_{11} integrin	Forw primer	5′-CGGTCCTAAATATCTCGCAGTCA-3′
	Rev primer	5′-ATGCTACCGTCTGAGTCCTCCTT-3′
	Probe	5′-CAAACCTGCAGTTTGCCAGCTTGATCC-3′
AR	Forw primer	5′-AAGGCTATGAATGTCAGCCCA-3′
	Rev primer	5′-CATTGAGGCTAGAGAGCAAGGC-3′
	Probe	5′-TGTGTGCTGGACACGACAACAACC-3′
β-actin	Forw primer	5′-TCACCCACACTGTGCCCATCTACGC-3′
	REV primer	5′-CAGCGGAACCGCTCATTGCCAATGG-3′
	Probe	5′-ATGCCCTCCCCCATGCCATCCTGCGT-3′

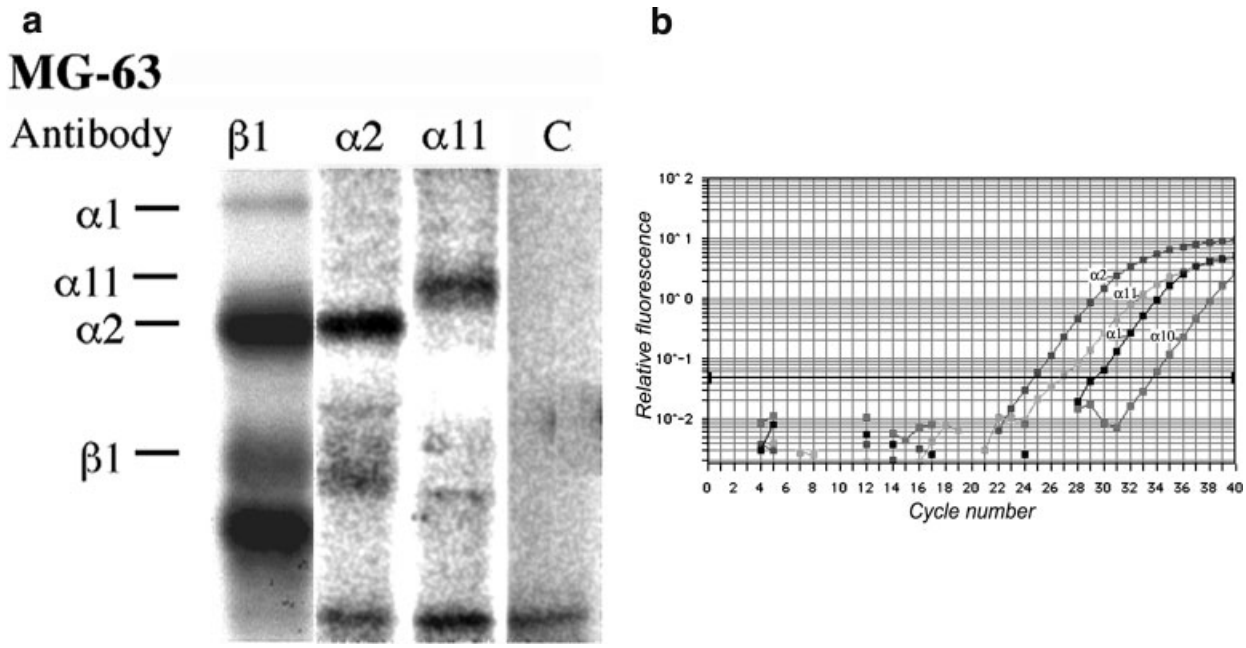

FIGURE 1 – Collagen receptor integrin expression of MG-63 osteosarcoma cells. (*a*) The integrins were immunoprecipitated with specific antisera and analyzed by electrophoresis and fluorography. C, control serum. (*b*) mRNA levels were measured with quantitative RT-PCR analysis. Amplification curves were generated using specific primers for each α-subunit. The C_T-value is the cycle number at which the signal reaches the threshold level of 0.05 on a relative fluorescence scale.

were generated from real-time PCR analyses using 2-fold dilution series of cDNA generated from total RNA of KHOS-240 cells. KHOS-240 cells express all collagen receptor integrins at mRNA level (Nissinen *et al*., unpublished results; see also Table I). In the case of AR, the standard curve was generated using cDNA from LNCaP cells, which express AR endogenously (16.7% of β-actin, data not shown). PCR reactions were carried out in a solution containing 300 nM of primers and 200 nM of probe, 5 μl of TaqMan universal PCR Master Mix (Perkin-Elmer Applied Biosystems) and 2 μl of template cDNA, diluted 1:10, in a final volume of 10 μl. Thermal cycling was carried out in an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) with a 2 min initiation at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

The specific PCR products were detected real-time as an increase in fluorescence. C_T-values were determined as cycle number at which the fluorescence signal exceeded a relative value of 0.05 fluorescence units (Fig. 1). Each determination of a C_T-value was normalized with the C_T-values of simultaneous β-actin measurements from the same samples. The SEM in 3 parallel measurements was <5% in all cases that were included in the final analysis. The relative expression of the analyzed gene against the β-actin expression was counted using the formula: relative expression = $2^{-\Delta CT}$, where $\Delta C_T = C_T$ (target gene) − C_T (β-actin).

Immunohistochemistry

α_{11} *IHC staining*. Frozen sections (6 μm thick), cut to silane coated glasses and dried in room temperature for 30 min, were fixed in −20°C temperature with acetone for 8 min and dried for 10 min. Slides were washed in TBS (Tris-buffered saline, pH 7.4–7.6) for 3 × 10 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in TBS for 20 min. Slides were washed again in TBS for 3 × 5 min. To prevent unspecific staining, slides were incubated in goat normal serum (15 μl goat normal serum in 1 ml TBS) for 1 hr in a moist chamber. Affinity purified IgG to human α_{11}[8] was diluted 1:500 with TBS-buffered 1% bovine albu-

min and the slides were incubated with the primary antibody at room temperature for 30 min in a moist chamber. After washing with TBS, the slides were incubated with biotinylated goat anti-rabbit secondary antibody at room temperature in a moist chamber for 30 min. The slides were again washed with TBS and incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) at room temperature in a moist chamber for 30 min. After another wash with TBS the slides were stained with diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated, treated with xylene and mounted. As a positive control, a sample of fresh normal human uterine muscle was obtained from routine hysterectomy.

For α_2 IHC staining sections were stained similarly to α_{11} staining except the primary antibody was mouse monoclonal antihuman α_2 integrin MAB1950/P1E6 (Chemicon, Temecula, CA) diluted 1:1,000. Human epidermal skin section served as a positive control for α_2 staining. No specific immunostaining was seen in either integrin staining when the primary antibody was omitted from the protocol.

The IHC staining protocol for AR was similar to integrin protocol with few exceptions. Affinity purified rabbit polyclonal primary antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, California) was diluted 1:1,000 with TBS-buffered 3% bovine albumin containing 0.05% Tween 20. Slides were incubated with the primary antibody at +4°C overnight. After washings, sections were incubated with DAKO EnVision/HRP ready-to-use detection reagent (DakoCytomation Denmark A/S, Glostrup, Denmark) at room temperature for 30 min. After another wash, the slides were stained with diaminobenzidine, counterstained, dehydrated, treated with xylene and mounted.

α_2 and α_{11} staining intensity and percentage of stained epithelial cells were estimated in different areas to obtain an IHC score of individual foci as described previously.[37] Strong intensity gave a value of 3, moderate 2, weak 1 and negative 0. The sum of the percentage of cells staining at each intensity level was calculated for each sample, to get the IHC score. Integrin α_2 IHC score ranged from 101.9–300.0 in benign cases, including low grade PIN and from 3.8–300.0 in carcinoma cases. Integrin α_{11} IHC score ranged from 0–205 in benign cases and from 0–292 in carcinoma cases. The Gleason grading of the histological samples was done in adjacent HE stained sections.[38]

AR expression could be determined on 78 frozen sections corresponding to the samples used in integrin IHC. The AR IHC index was determined as percentage of positive nuclei in 3–5 individual visual fields within each particular slide. Number of total nuclei counted varied between 150–394 (mean = 253).

Statistical analysis

Statistical analyses were carried out using linear mixed models with histological group as fixed factor and subject as random to analyze mRNA levels. Multiple comparisons between histological groups were calculated using Tukey-Kramer method. Pearson correlation coefficients were calculated for integrin and AR mRNA levels. Residuals were checked for justification of analyses and log-transform was used when necessary. Non-parametric methods, Kruskal-Wallis and Mann-Whitney *U*-test, were used to analyze protein levels, except for AR where the data was distributed normally. The analyses were carried out using SAS system software (version 8.2, SAS Institute Inc., Cary, NC) and SPSS for Windows (version 11.0.1, SPSS Inc., Chicago, IL).

Results

Sarcoma cells express a larger variety of collagen receptor integrins than carcinoma cells

We used quantitative real-time PCR to analyze mRNA levels of collagen receptor integrins in various cancer cell lines. Because similar analysis has not been carried out previously we first compared the mRNA levels and corresponding protein levels in MG-63 osteosarcoma cells (Fig. 1). These cells expressed very low amounts of $\alpha_1\beta_1$ integrin and moderate amounts of $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ (Fig. 1*a*), which was in accordance with the previous results.[39] In real-time PCR the corresponding relative mRNA levels were 1.2% (α_1), 28.3% (α_2), and 3.2% (α_{11}) of β-actin mRNA level (Table I). The α_{10} C_T-values were considerably higher than those of the other α-subunits, indicating almost non-detectable absolute mRNA levels (Fig. 1*b*). No specific anti-α_{10} antibody was available to test its expression.

The integrin pattern of HeLa and KHOS-240 cells has also been tested at the protein level.[32,40] In this study, these results were compared to the corresponding relative mRNA levels. In conclusion, α_2 mRNA levels were in accordance with the expression levels of α_2 protein when different cell lines were compared to each other and this was also the case with $\alpha_1\beta_1$ integrin. Integrin protein could also be detected on cells with relatively low levels of the corresponding mRNA (~1.0%).

The mean mRNA expression of collagen receptor integrins in all the measured cancer cell lines are shown in Table I. Of the 24 cancer cell lines tested only SAOS osteosarcoma cells were negative for α_2, in accordance with our previous observations.[29] If 1% expression level was kept significant, 2/3 melanoma, 4/5 sarcoma and 7/16 carcinoma cell lines expressed α_1 mRNA. Fibrosarcoma cells expressed all 4 collagen receptors, whereas any of 3 osteosarcoma or one leiomyosarcoma cell lines did not have detectable α_{10} mRNA. None of the 3 melanoma, 14 carcinoma or 2 embryonic carcinoma cell lines had α_{10} mRNA. In addition to sarcomas only SK-MEL-5 melanoma cells, NCI-H1563 non-small cell lung cancer cells and Tera-2 embryonic carcinoma cells expressed noticeable amount of α_{11} mRNA.

Increased expression of α_{11} mRNA has been shown in clinically advanced non-small cell lung cancers[17] and it was surprising that most carcinomas, including 3 lung cancer cell lines, did not express significant levels of it. The data supported the idea that cell culture condition may select clones that express a different pattern of collagen receptors that is dominant in cells in the original tumors. To analyze this hypothesis further the studies were focused on prostate cancer, as we wanted to see whether $\alpha_2\beta_1$ integrin is as abundant in primary tumors as it is in various prostate carcinoma cells. We also wanted to test the expression of $\alpha_{11}\beta_1$ integrin in prostate cancer cells *in vivo*. Finally, we wanted to concomitantly analyze mRNA and protein levels in tissue samples and compare results to the data obtained with cell lines, because this could provide important information for further studies focused on integrin expression levels in cancer.

Integrin $\alpha_2\beta_1$ is expressed abundantly on benign and malignant prostate epithelial cells and its expression decreases during cancer progression

The strongest α_2 staining was seen in cell membranes of luminal epithelial cells and the intensity was strongest in benign glandular structures, where the lateral plasma membranes were clearly stained. There was no clear staining in the basal cells or in the epithelial-stromal borders, although occasional basal staining was detected (Fig. 2*a*). This epithelial staining decreased in PIN lesions (Fig. 2*b*) as well as in carcinomas (Fig. 2*c*,*d*).

The α_2 protein expression in epithelial cells decreased significantly in conjunction with the poorer histological glandular differentiation ($p = 0.0001$, Kruskal-Wallis test, Fig. 3*a*). The mean IHC score was 228.8 in benign foci ($n = 36$) and 222.2 in PIN foci ($n = 14$). In carcinomas the mean IHC score was 194.5 ($n = 5$), 187.1 ($n = 28$), 132.7 ($n = 15$), 86.1 ($n = 18$) and 35.4 ($n = 3$) in Gleason Grades 1–5 respectively. α_2 protein expression significantly correlated inversely with Gleason grade (Spearman rank order correlation $r = -0.621$, $p = 0.0009$) and the mean IHC scores were significantly different between Gleason grades ($p = 0.0002$, Kruskal-Wallis test, Fig. 3*a*).

In full accordance with the immunohistochemical stainings the expression of α_2 mRNA was lowest in high grade carcinomas and

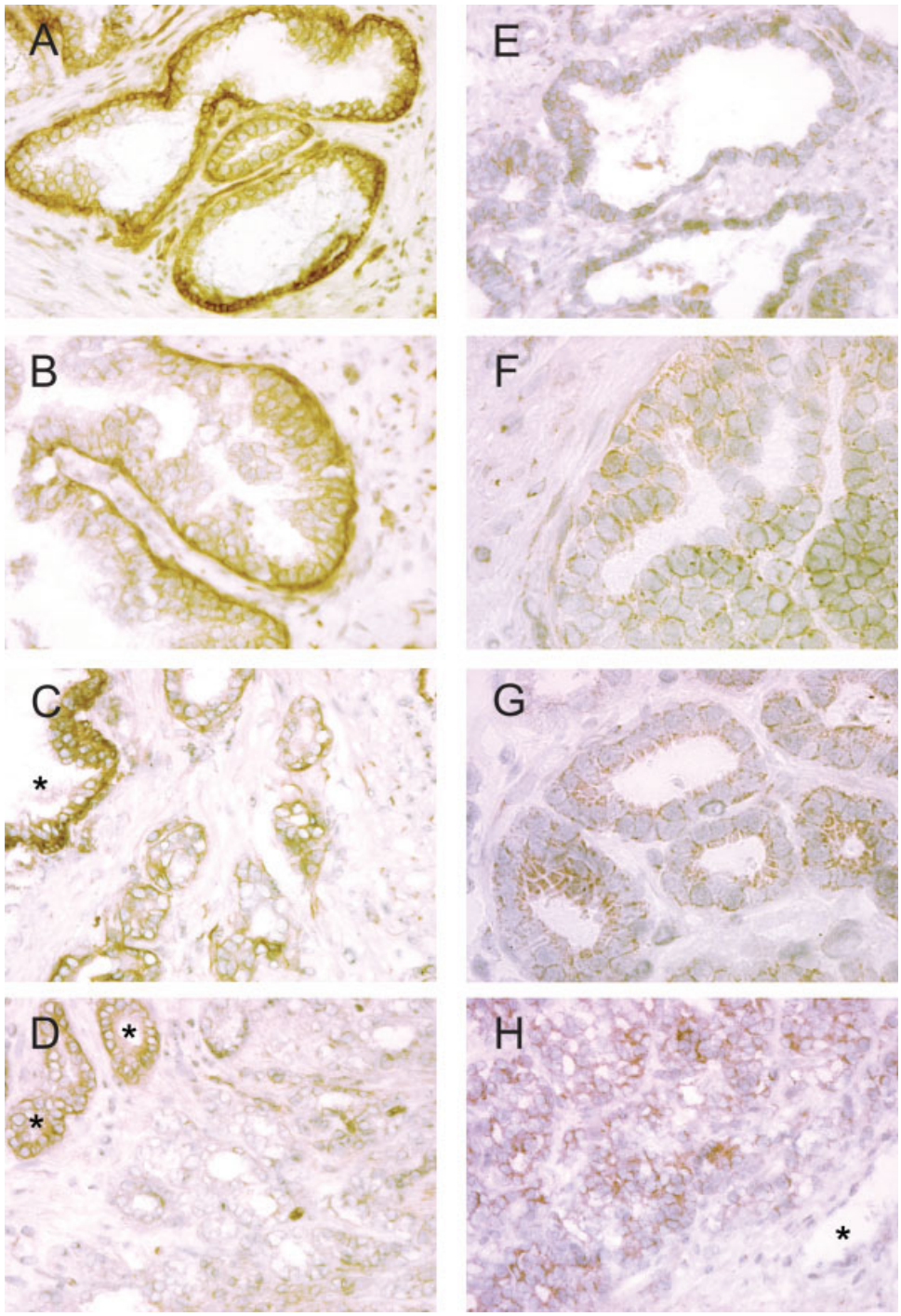

FIGURE 2 – Immunohistochemical staining of prostate tissue with α_2- and α_{11}-antibodies using ABC method. (*a*) α_2 staining in normal prostate showing continuous epithelial cell membrane staining and occasional basal staining in cell–matrix contacts (magnification = ×200). (*b*) PIN focus showing still continuous α_2 staining in luminal epithelial cells adjacent to basal lamina (magnification = ×200). (*c*) Gleason 2 carcinoma showing decreased α_2 staining (magnification = ×200). Note the adjacent benign gland staining more intensively (*). (*d*) A focus of Gleason Grade 4 carcinoma showing significantly less intensive α_2 staining than the adjacent benign glands (*) (magnification = ×200). (*e*) Benign glands showing luminal epithelial cell staining for α_{11} integrin (magnification = ×200). (*f*) α_{11} staining in PIN focus (magnification = ×400). (*g*) Gleason 2 carcinoma showing increased α_{11} staining in epithelium (magnification = ×400). (*h*) A focus of Gleason 5 carcinoma showing intense α_{11} staining (magnification = ×200). Note the difference to adjacent benign structures (*).

the levels were lower in carcinomas as compared to benign foci. The mean expression level was 21.8%, 15.6% and 12.0% of β-actin mRNA, for benign, Gleason 1–3 and Gleason 4–5 samples, respectively (Fig. 3*b*). The decrease was significant between the poorly differentiated carcinomas (Gleason 4 and 5) and benign prostate ($p = 0.0331$, Tukey-Kramer).

In prostate $\alpha_{11}\beta_1$ integrin is expressed on epithelial and stromal cells and the expression is enhanced during malignant transformation

The pattern of α_{11} protein staining was clearly different from α_2 pattern and the stromal elements were also variably stained (Fig. 2*e–h*). The intensity and the extent of stromal staining differed greatly from case to case and showed no clearly defined correlation to histological differentiation of lesion. The α_{11} antibody outlined larger blood vessels occasionally in a pattern compatible with α_{11} expression on smooth muscle cells, or associated stromal cells. For the α_{11} IHC score only the epithelial staining was evaluated. The luminal epithelial cell staining was located in the apical or lateral cell membranes. Occasional basal cell staining was seen, but it had no correlation with any histological feature or cell compartment.

There were no statistically significant differences in α_{11} IHC score in carcinomas of different Gleason grade, although the protein expression was highest in the most poorly differentiated group (Fig. 4*a*). Nevertheless, the α_{11} IHC score was sig-

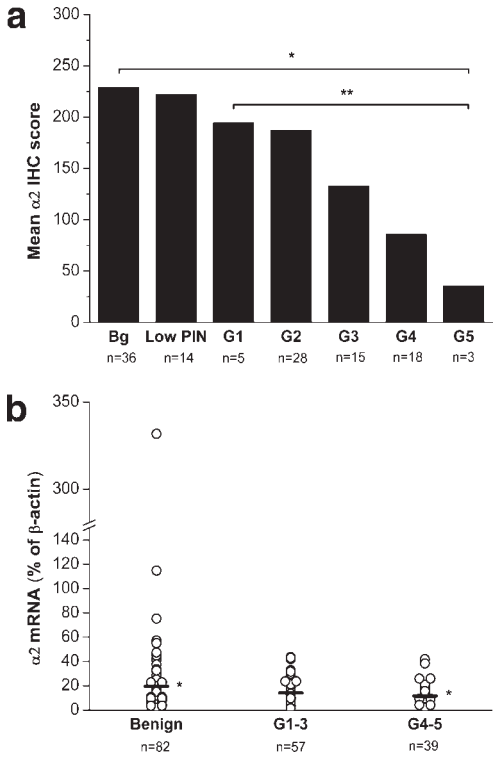

FIGURE 3 – α_2 subunit expression was measured in protein (immunohistochemical score) and mRNA (qRT-PCR) level. (*a*) Mean α_2 IHC scores in 119 independent foci of different histological grade. *IHC scores decreased significantly along the histological progression of the lesions ($p = 0.0001$, Kruskal-Wallis). **A significant difference was found when comparing different carcinoma grades to each other ($p = 0.0002$, Kruskal-Wallis). (*b*) mRNA was detected as a percentage of β-actin control mRNA in 178 samples. The samples were grouped in benign, Gleason 1–3 and Gleason 4–5 histological groups. Mean values for each group were: 21.8 (Benign), 15.6 (G1-3) and 12.0 (G4-5). *Difference in mean mRNA values was significant in $p = 0.0331$ level (Tukey-Kramer).

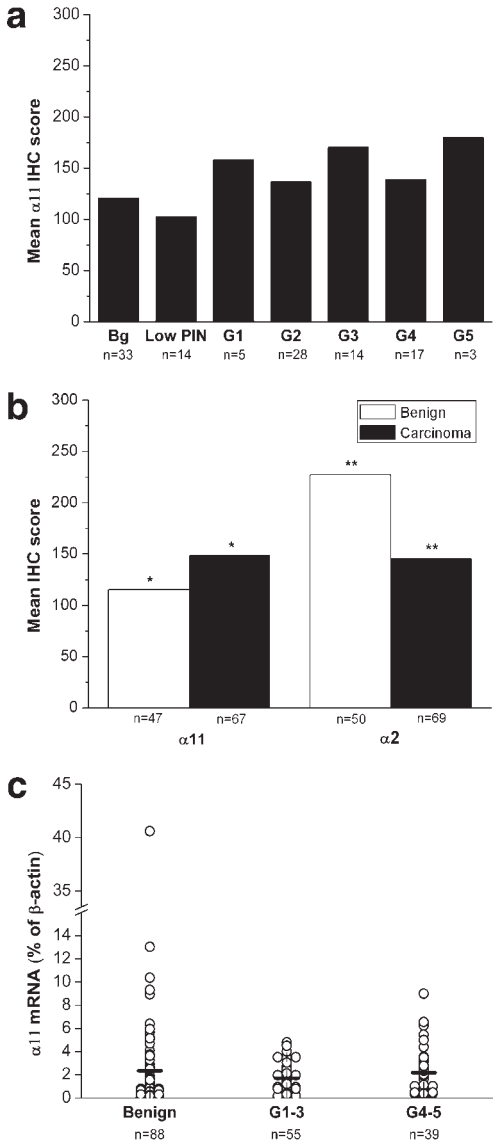

FIGURE 4 – α_{11} subunit expression was measured in protein (immunohistochemical score) and mRNA (qRT-PCR) level. (*a*) In a total of 114 independent foci, grouped in the same way as in α_2 analysis, there was a slight increase in α_{11} IHC scores in the high grade carcinomas. This increase, however, did not reach statistical significance. (*b*) *α_{11} IHC scores were significantly higher in pooled carcinomas with respect to benign lesions ($p = 0.029$, Mann-Whitney *U*-test). **In the same analysis the α_2 IHC scores were significantly lower in carcinoma lesions as compared to benign (including PIN) lesions ($p = 0.0005$, Mann-Whitney *U*-test). (*c*) α_{11} mRNA was detected as a percentage of β-actin control mRNA. Mean mRNA levels for α_{11} integrin were 2.4 (Benign), 1.7 (G1–3) and 2.2 (G4–5) in 182 samples.

nificantly higher in carcinomas than in benign lesions ($p = 0.029$, Mann-Whitney *U*-test, Fig. 4*b*). The benign foci had a mean IHC score of 115.3 and the carcinoma foci 148.3. In the same analysis α_2 IHC score in benign lesions (including PIN) was compared to carcinomas and there was a significant decrease in α_2 protein expression ($p = 0.0005$, Mann-Whitney *U*-test, Fig. 4*b*), suggesting that during prostate cancer progression $\alpha_{11}\beta_1$ may sometimes replace $\alpha_2\beta_1$ as the main collagen receptor.

In accordance with the fact that α_{11} protein could be stained in prostate samples also α_{11} mRNA was detected. As expected, based on the presence of α_{11} on epithelial and stromal cells in immunohistochemistry, mean α_{11} mRNA expression did not show any significant changes between histological groups. The expression was slightly higher in poorly differentiated carcinomas compared to well and moderately differentiated carcinomas: the mean α_{11} mRNA expression was 2.4%, 1.7% and 2.2% of β-actin mRNA, for benign, Gleason 1–3 and Gleason 4–5 samples, respectively (Fig. 4*c*).

Androgen receptor protein and mRNA levels correlate with the expression of $\alpha_2\beta_1$ integrin during cancer progression

Androgen receptor mRNA levels were measured similarly to integrin measurements in corresponding clinical samples. At protein level, nuclear AR expression was observed in all samples (range of AR IHC index in all samples = 1.3–77.0). The mean AR mRNA expression was 38.1%, 27.7% and 31.5% of β-actin mRNA, for benign, Gleason 1–3 and Gleason 4–5 samples, respectively (Fig. 5*a*), whereas AR IHC indices for the same groups were 43.9, 40.7 and 31.7 (Fig. 5*b*). Despite the fact that in Gleason 4–5 samples AR mRNA levels were slightly lower (17%) that in benign samples, the AR mRNA levels did not have significant correlation with histological differentiation or Gleason grade. A small (28%) decrease seen in AR IHC index between benign and Grade 4–5 samples was statistically significant ($p = 0.046$, *t*-test). Although there was no overall correlation to histological grade, the AR mRNA had a clear correlation with α_2 mRNA within each group of different samples (Fig. 5*c–e*). The correlation

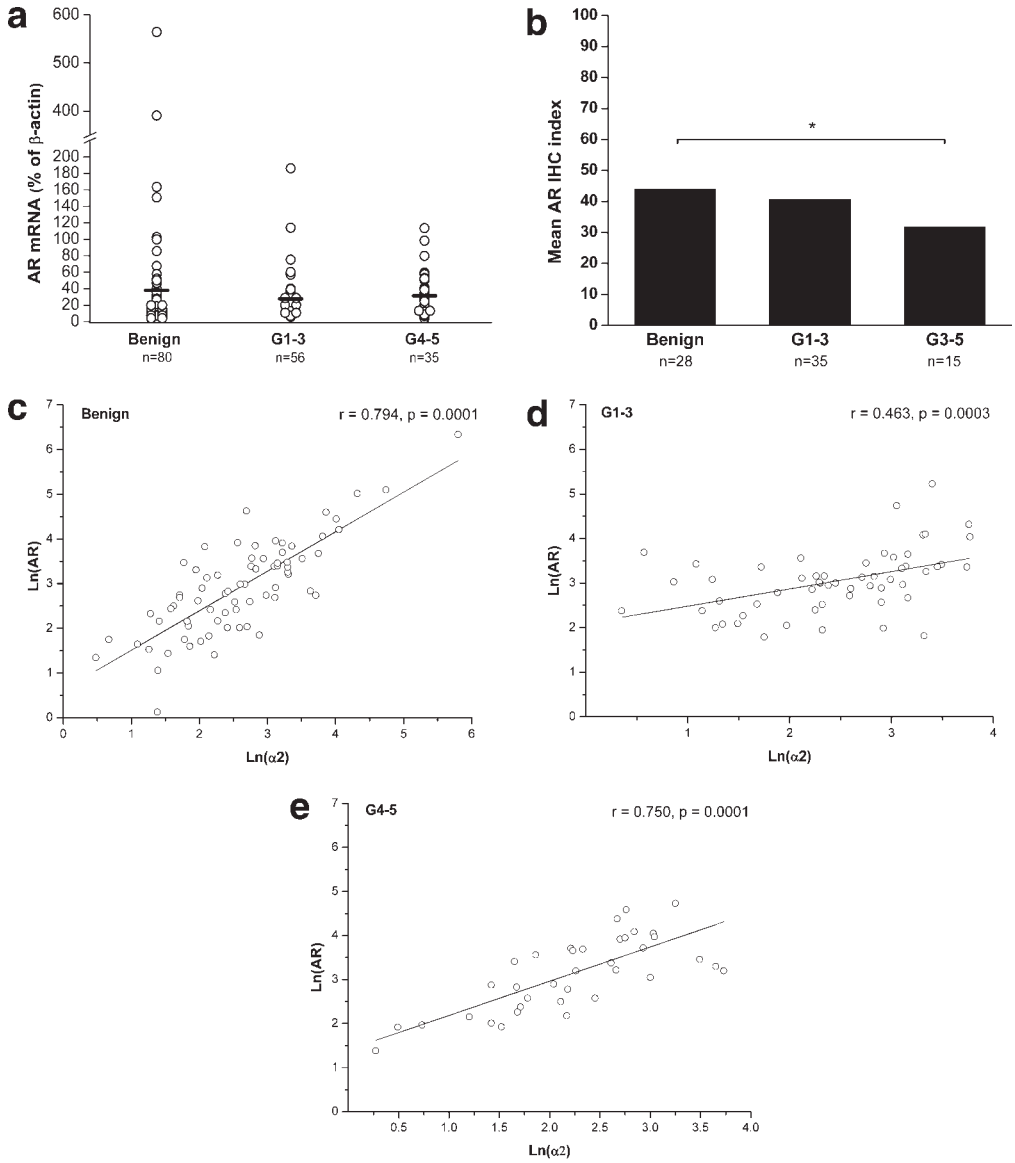

FIGURE 5 – Androgen receptor mRNA and protein levels in prostate samples were measured using quantitative RT-PCR analysis and immunohistochemistry. (*a*) Mean mRNA values were 38.1 in benign samples, 27.7 in Gleason 1–3 samples and 31.5 in Gleason 4–5 samples. The differences in AR mRNA levels were not significant between groups. (*b*) Mean AR IHC index was 43.9, 40.7 and 31.7 in benign, Gleason 1–3 and Gleason 4–5 samples, respectively. *There was a significant difference between benign and Gleason 4–5 samples ($p = 0.046$, *t*-test). Androgen receptor mRNA levels correlated significantly with α_2 integrin mRNA levels within benign (*c*), Gleason 1–3 (*d*) and Gleason 4–5 (*e*) samples. *r*, Pearson correlation coefficient; *p*, statistical significance.

was exceptionally good in benign samples ($r = 0.794$, $p = 0.0001$) and in the most poorly differentiated group of Gleason 4–5 carcinomas ($r = 0.750$, $p = 0.0001$). The correlation was not so clear in the Gleason 1–3 group ($r = 0.463$, $p = 0.0003$), possibly due to more heterogeneous collection of samples. Accordingly, there was a correlation between AR IHC index and α_2 protein levels ($r = 0.223$, $p = 0.049$, Spearman's rank order correlation).

α_2 integrin mRNA levels decreased during cancer progression, whereas significant decrease could not be seen in AR mRNA levels. At protein level, AR IHC index was not different when Gleason 1–3 samples were compared to benign samples, but α_2 levels degreased in the same samples. These results indicate that malignant transformation regulates α_2 expression with an AR independent mechanism. Inside the group of benign samples and the group of Grade 4–5 samples there was a clear correlation between AR and α_2 mRNA levels. Similarly there was a correlation between α_2 protein expression and AR IHC score. These observations strongly support the previous idea that AR can positively regulate α_2 expression.[30,41]

mRNA of $\alpha_1\beta_1$ and $\alpha_{10}\beta_1$ collagen receptors can be elevated in individual prostate tissue samples

In addition to α_2 and α_{11} also α_1 and α_{10} collagen receptor mRNAs were found in clinical prostate specimens. The mean α_1 mRNA expression was 25.0%, 13.2% and 15.9% of β-actin mRNA, for benign, Gleason 1–3 and Gleason 4–5 samples, respectively (Fig. 6*a*). α_1 mRNA levels were not significantly different between the different grade carcinomas or between benign and carcinoma samples, although the mean expression was lowest in Gleason 5 carcinomas.

The mean level of α_{10} mRNA was highest in the poorly differentiated group of Gleason 4 and 5 carcinomas. The mean α_{10}

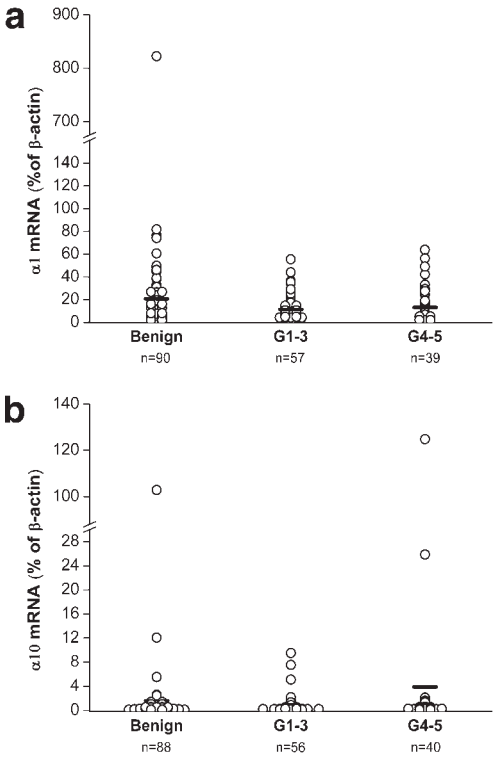

FIGURE 6 – α_1 and α_{10} collagen receptor integrin α-subunit mRNA levels in prostate samples were measured using quantitative RT-PCR analysis. (*a*) α_1 mRNA levels in 186 samples showed mean values of 25.0 (Benign), 13.2 (G1–3) and 15.9 (G4–5). (*b*) Mean mRNA levels for α_{10} integrin were 1.8 (Benign), 0.9 (G1–3) and 4.2 (G4–5) in 184 samples.

mRNA expression levels were 1.8%, 0.9% and 4.2% of β-actin mRNA, for benign, Gleason 1–3 and Gleason 4–5 samples, respectively (Fig. 6*b*). There were no statistically significant differences between groups. The high value of the mean α_{10} mRNA expression in Gleason 4–5 carcinomas is explained by exceptionally high expression in 2 individual samples. One benign sample had also very high levels of α_{10} mRNA and another benign sample had high levels of α_1 mRNA.

Discussion

The mRNA levels of all 4 collagen receptor integrin α subunits were measured by quantitative RT-PCR. We tested 24 cancer cell lines and confirmed some previous observations, but also unveiled new and unexpected facts. Integrin α_2 was strikingly the most abundant collagen receptor expressed in 23 of 24 cell lines. This collagen receptor may be essential for malignant process. It is also possible, however, that cell culture conditions favor α_2 positive cells. For example in breast cancer $\alpha_2\beta_1$ is usually downregulated[42] and $\alpha_2\beta_1$ has been shown to promote the orderly regulated glandular growth in breast[43,44] and still $\alpha_2\beta_1$ may promote the cell cycle progression and proliferation of breast cancer cells in cell culture.[45,46] Interestingly the only α_2 negative cell line (SAOS) had high α_{11} mRNA levels.

Relative mRNA levels of α_2 were constantly higher in both tissue- and cell-derived samples than the levels of other collagen receptor α subunits. When the syntheses of corresponding proteins were compared in MG-63 cells, metabolically labeled α_2 and α_{11} integrins were present in equal amounts. In the same cells the relative mRNA level of α_2 was 28.3% and α_{11} was 3.2%, suggesting that about 10-fold higher α_2 mRNA level is needed for the same protein synthesis rate. The reason for this phenomenon remains unknown.

The expression of α_1 was very variable, without a clear correlation to any special cancer type. Cervical adenocarcinoma-derived HeLa cells expressed $\alpha_1\beta_1$ integrin as their main collagen receptor that is in agreement with our previous analysis based on immunoprecipitations and function blocking antibodies.[40] PC-3 prostate carcinoma cells had high levels of α_1 mRNA. In normal human epithelial cells there is no regular expression pattern of $\alpha_1\beta_1$ and after malignant transformation, depending on the adenocarcinoma origin, the expression has been shown to be either up- or downregulated.[47–49] Previous studies have indicated that $\alpha_1\beta_1$ might promote cell proliferation and therefore it may support malignant phenotype.[50,51]

Expression of α_{10} in cancers has not been systematically studied previously and our results indicate that it may be limited to sarcomas, *e.g.*, fibrosarcoma. In normal tissues integrin α_{10} is expressed in bone and cartilage.[9]

Integrin α_{11} has been associated to non-small cell lung carcinoma progression *in vivo*.[17] We found low level expression of α_{11} in NCI-H1563 non-small cell lung cancer cells, but 3 other lung carcinoma cell lines as well as 10 other carcinomas were negative. The relatively low expression of α_{11} in lung and other carcinoma cell lines and the elevated expression in tissues might be connected to cell culture conditions and, for example, to the influence of stromal factors. Therefore, more detailed analyzes of integrin expression was carried out using prostate cancer tissue samples.

Quantitative RT-PCR analysis indicated that α_2 mRNA levels are significantly lower in tissue samples representing prostate carcinomas than in benign prostate tissue. This observation was confirmed by immunostainings and it is in accordance with one report published previously.[52] Prostate cancer progression seems to have similarities with breast cancer in terms of integrin expression.[42,53] Importantly, this observation does not exclude the possibility that $\alpha_2\beta_1$ integrin may later play an important role in invasion and metastasis formation.[13,52,54] It also indicated that quantitative RT-PCR is a useful method to analyze integrin expression, when background from stromal elements is taken into consideration. In the case of other collagen receptors no significant differences were detected in mRNA levels.

Because α_{11} mRNA was clearly present in prostate tissue samples and it has been connected previously to non-small cell lung cancer,[17] we also studied its expression at the protein level. Immunostaining indicated that α_{11} is expressed, in addition to epithelial cells, in stromal cells. This observation could explain why we could not see differences in mRNA levels, despite the fact that α_{11} expression was significantly enhanced in epithelial cells during cancer progression. α_{11} expression has not been described previously in prostate cancer and our results indicate that it may partially replace α_2 as the main collagen receptor of these cells. Based on our previous studies the ligand recognition pattern of both $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ is quite similar.[28] α_{11}I domains, however, seem to have lower affinity to fibrillar collagens than α_2I domains.[28] It is possible to speculate that this has an effect on cell migration. In addition, the signaling function of α_{11} may be different.

Single benign and tumor samples contained very high levels of α_{10} integrin. Similarly, single benign samples had very high levels of α_1 and α_2 mRNA. The reason for the occasional high expression remained unclear but for example inflammation may change integrin expression pattern.[55]

The mechanisms of collagen receptor regulation in prostate cancer are unknown. Some previous cell culture experiments propose interesting correlation between α_2 expression levels and AR. Nagakawa *et al.*[30] found recently that when AR-negative DU-145 prostate cancer cells are transfected with AR, there is a significant increase in α_2 integrin expression and concomitant increase in integrin-mediated cell adhesion to collagen I. We asked whether a similar correlation can also be found at tissue level. The previous results on AR expression in different grade tumors are controversial.[56,57] Our results on prostatectomy samples indicate that AR mRNA levels are slightly higher in benign tissues than in carcino-

mas but there are no significant changes after transformation and no significant correlation between AR mRNA levels and Gleason grade. Interestingly there was a clear correlation between AR and α_2 integrin mRNA expression inside both benign and transformed (Grades 4–5) sample groups, suggesting that AR expression levels may regulate integrin expression also *in vivo*. The analysis of AR positive nuclei in prostate sample supported these conclusions. It is probable, therefore, that malignant transformation regulates α_2 integrin expression with a mechanism that is independent of AR expression levels.

The downregulation of α_2 expression in locally advanced disease does not rule out the possibility that α_2 mediates metastasis formation in androgen-independent disease.[54] *In vivo* there can be a testosterone-regulated balance on AR mediated integrin expression and adhesion, which is then lost as the disease becomes androgen-independent. It has been shown that testosterone decreases α_2 expression in cell culture experiments.[30,41]

Previous studies have indicated that the expression pattern of integrin-type laminin receptors may change in prostate carcinoma.[58,59] Furthermore, prostate carcinoma cells may express a different splicing variant of β_1 integrin than their benign counterparts.[60–62] Our results indicate that there may be a switch from one collagen receptor to another. These changes in cell adhesion could play a significant role in cancer progression.

In more general terms, our results indicate that different combinations of collagen binding integrins contribute to the cell adhesion receptor repertoire of tumor cells. Most cancer cell lines seem to express α_2 integrin, but it actually might be downregulated in tumors. At the same time α_{11} is rare in cell lines, but it might be much more abundant in clinical carcinomas.

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