The influence of 1,25-dihydroxyvitamin D_3 and 1,24-dihydroxyvitamin D_3 on $\alpha_v\beta_3$ integrin expression in cancer cell lines

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Abstract. Integrins are cell-surface receptors engaged in important cancer invasion processes, such as adhesion, migration, proliferation and differentiation. The aim of this study was to evaluate the effect of 1,25-dihydroxyvitamin D_3 (calcitriol) and its metabolite 1,24-dihydroxyvitamin D_3 (PRI-2191) on $\alpha_v\beta_3$ integrin expression in various cancer cell lines. The expression levels of the β_3 and α_v integrins were reduced only in the WEHI-3 and LLC cell lines by the two compounds. Calcitriol or PRI-2191 treatment caused differentiation of WEHI-3 mouse leukemia cells, but apoptosis of LLC cells. WEHI-3 and LLC cells exposed to calcitriol or PRI-2191 lost their migratory and adhesive potentials. The inhibition of migratory potential was higher in the LLC cells than in the WEHI-3 cells and appeared to correlate with the increased down-regulation of $\alpha_v\beta_3$ integrin by calcitriol or PRI-2191. The observed *in vivo* effects (antitumor and antimetastatic) in mice bearing subcutaneously transplanted LLC cancer are possibly associated with inhibited migratory potential as a consequence of the lowered integrin expression caused by calcitriol or PRI-2191.

Introduction

Calcitriol, a hormonally active form of vitamin D_3 [1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$], regulates calcium and phosphorus homeostasis, but also exerts antitumor activity both *in vitro* and *in vivo*. In addition to its antiproliferative and differentiation-inducing effects, calcitriol induces apoptosis in a number of cancer cell lines *in vitro*. Studies performed in tumor-bearing animals treated with calcitriol have shown regression of tumors, inhibition of metastasis development, prolongation of survival time and an antiangiogenic effect (1-6).

Vitamin D primarily exerts its pleiotropic effects after binding to a specific receptor, which is a member of the steroid hormone receptor superfamily. The ligand-bound vitamin D receptor (VDR) then interacts with its cognate binding site, termed the vitamin D response element (VDRE), to affect the transcription of target genes (7,8). The transcriptional activity of VDR depends not only on the concentrations of receptor and hormone, but also on its heterodimer partner, retinoid X receptor (RXR), and on coactivator protein activities, which could influence the degree to which a particular target gene may be activated or repressed, depending on the cell line type (9,10).

It is known that calcitriol especially prompts hematopoietic precursor cells to develop along a monocyte/macrophage pathway (11-15). The human promyelocytic leukemia HL-60 cell line possesses adhesive properties and acquired $\alpha_v\beta_3$ integrin expression during monocytic differentiation after incubation with calcitriol (16). Furthermore, this integrin emerges during avian bone marrow differentiation into osteoclasts (17). It was also shown that calcitriol transcriptionally activates the α_v and β_3 genes in avian macrophages and the β_3 promoter contains a vitamin D_3 response element (VDRE) (18).

There are experimental data indicating that B16 mouse melanoma cells lose their adhesive and migratory properties *in vitro* and their metastatic potential *in vivo* after incubation with calcitriol (6). Similarly, inhibition of invasion and migration was observed in the human MDA-MB-231 breast cancer cell line after incubation with calcitriol (1). On the other hand, no inhibition of *in vitro* proliferation of these cells (B16 and MDA-MB-231) was observed. Thus an effect other than cytotoxic should be considered in explaining the effect on

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invasion and migration (1). Yudoh *et al* showed that calcitriol inhibits the expression of laminin on human fibrosarcoma cells (HT1080) and that this reduced expression leads to the inhibition of *in vitro* invasiveness through the extracellular matrix (5). In prostate cancer cell lines (human Du145 and PC-3), authors demonstrated that calcitriol decreases tumor cell adhesion and migration, due in part to decreased $\alpha_6ß_4$ integrin expression (19,20).

The integrins are a large family of cell-surface receptors that consist of α and ß subunits and are expressed on a wide variety of normal and cancer cells. Two members of the $ß_3$ integrin family,$\alpha_{IIb}ß_3$ and $\alpha_v ß_3$, mediate cell-cell and cell-extracellular matrix interactions. Dysregulation of $ß_3$ integrin expression is associated with the pathogenesis of certain diseases, including cancer (21-28). It was demonstrated that interruption of the adhesive activity of tumor cells may result in inhibition of tumor progression (25,29). It is still an open question whether interference with the integrin signaling pathway could suggest target(s) for the development of anticancer treatment by inhibition of cancer growth, progression, metastases and angiogenesis. Application of anti-integrin antibodies and RGD and related peptides have revealed promising effects in anticancer therapy (25,29-31).

In our previous studies, a series of vitamin D_2 analogs with a highly unsaturated side-chain and a series of vitamin D_3 analogs with one or two additional hydroxyl groups in the side-chain were examined for their antiproliferative activity *in vitro* against various human normal and cancer cell lines (32-34). The vitamin D_3 metabolite (24R)-1,24-dihydroxy-vitamin D_3 [tacalcitol, 1,24-$(OH)_2D_3$, PRI-2191] revealed higher antitumor and lower calcemic activity as well as lower toxicity than calcitriol (35). The antitumor effect could be attributed to the induction of cancer cell differentiation (14,33,34).

The aim of this study was to evaluate the effect of calcitriol and PRI-2191 on $\alpha_v ß_3$ integrin expression and the association of this effect with the anticancer activity of these agents.

Materials and methods

Compounds. Both calcitriol [1,25-$(OH)_2D_3$] and tacalcitol [1,24-$(OH)_2D_3$, PRI-2191] were certified synthetic materials obtained from the Pharmaceutical Research Institute, Warsaw, Poland. Samples of the compounds were stored in amber ampoules in an argon atmosphere at -20˚C. Prior to use, the compounds were dissolved in absolute ethanol to a concentration of 10^{-4} M and subsequently diluted in culture medium to achieve the required concentrations (ranging from 10^{-6} to 10^{-16} M). In the animal experiments, the compounds were dissolved in 99.8% ethanol, then diluted in 80% propylene glycol to achieve the required concentrations and administered to the mice in a volume 50 µl/10 g of body weight.

Cells. The cell lines used in these studies were obtained from the collections designated A-E: A, (ATCC) American Type Culture Collection (Rockville, MD, USA); B, (DKFZ) German Cell Line Collection (Tumorbank, Heidelberg, Germany); C, supplied to us from DKFZ by Professor J. Konopa (Technical University, Gdansk, Poland); D, received as a gift from Dr I. Wodinsky, National Cancer Institute, Bethesda, MD, USA; E, established at the Fibiger Institute, Copenhagen, Denmark and obtained from Dr J. Kieler.

The culture media used are designated i-iv: i) RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate, with 10% fetal bovine serum (all from Gibco, Scotland, UK); ii) RPMI-1640+Opti-MEM (1:1) medium supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, with 5% fetal bovine serum (all from Gibco). The culture of LoVo/DX cells was supplemented with 10 ng/100 ml doxorubicin (Institute of Biotechnology and Antibiotics, Warsaw, Poland). The culture of T47D cells was supplemented with 0.01 mg/ml bovine insulin and SCC-25 with 0.4 µg/ml hydrocortisone; iii) Minimum essential medium (Eagle's, from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, with 10% fetal bovine serum (all from Gibco). The culture of MCF-7 cells was supplemented with 0.01 mg/ml bovine insulin; iv) Dulbecco's modified Eagle's medium (Institute of Immunology and Experimental Therapy) with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose, with 10% fetal bovine serum (all from Gibco).

All the culture media were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were cultured at 37˚C in a 5% CO_2 humidified atmosphere.

In these studies, cells of human cancer cell lines used were prostate: LNCaP (A, i), Du145 (A, iii); breast: T47D and MDA-MB-231 (both A, ii), and MCF-7 (A, iii); head and neck: FaDu and Hep-2 (both A, iii), SCC-25 (A, ii); leukemia: CCRF/CEM, CEM/C2, MOLT-4, HL-60, HL-60/MX2, K562, U937, and CEMT-4 (all A, i); lung: A549 (A, ii); colon: Caco-2 (A, ii), CX-1/1 and HT-29 (both B, ii), SW-707, LoVo, and LoVo/DX (all C, ii); melanoma: Hs294T (A, iv); urinary bladder: HCV29T (E, ii); renal: A498 (A, ii); and pancreatic: AsPC-1 (A, i). The mouse cancer cell lines were lung: LLC (D, iv); mammary gland: 4T1 (A, i); melanoma: B16 (A, iv); and leukemia: P388 (C, i), L1210 (D, i), and WEHI-3 (A, i). In parentheses are listed the source of the cell line and the type of culture medium used.

Antibodies. For FACS analysis, the anti-mouse fluorescein isothiocyanate (FITC)-conjugated CD61 (integrin $ß_3$ chain, subclone of HM $ß_3$-1[1]) and phycoerythrin (PE)-conjugated CD51 (integrin α_v chain), anti-human FITC-conjugated CD51/CD61 (integrin $\alpha_v/ß_3$), and anti-human and anti-mouse (FITC)-conjugated CD41 (integrin α_{IIb} chain) antibodies were purchased from BD Pharmingen (San Diego, CA, USA). The antibodies were used at the concentrations recommended by the suppliers.

For Western blot analysis, the anti-human and anti-mouse primary antibodies purified mouse anti-integrin $ß_3$, 250 µl/ml (BD Pharmingen) and polyclonal rabbit anti-integrin α_{IIb}, 200 µg/ml (Santa Cruz Biotechnology, Heidelberg, Germany) and the secondary biotinylated antibodies anti-mouse

immunoglobulins (Dako, Denmark) and anti-rabbit IgG (RD Systems), both 250 μg/ml, were used.

In vitro antiproliferative assay. One to two hours before the addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of $1x10^4$ cells per well for the adherent cell lines. An assay was performed after 96 h of exposure to verify the concentrations of the tested agents. The results were calculated as the IC_{50} (50% inhibitory concentration), i.e. the dose of tested agent, which inhibits 50% of the proliferation of the cancer cell population. IC_{50} values were calculated for each experiment separately and mean values ± SD are presented in the tables. Each compound at each concentration was tested in triplicate in a single experiment, which was repeated 3-7 times. Ethanol, which was used as a solvent (in a dilution corresponding to its highest concentration applied to the tested compounds), did not exert any inhibitory effect on cell proliferation ($p<0.05$). In the antiproliferative assays to evaluate cytostatic effect, the SRB or MTT methods were applied, as described previously (36).

Cell cycle and cell-surface antigens. The cultured cells were seeded at a density of $1x10^5$ cells/ml of culture medium on 24-well plates (Sarstedt) to a final volume of 2 ml. The cells were exposed to the study compound at concentrations ranging from 10-1000 nM (depending on the cell line type) for 48, 72, or 96 h. Ethanol alone (a solvent for the compounds tested) at a concentration corresponding to that used for the compounds was used in each experiment as a control. After incubation, the cells were collected by centrifugation, washed in phosphate-buffered saline (PBS), and counted in a hemacytometer.

Cell-cycle analysis. Cells ($1x10^6$) were washed twice in cold PBS and fixed for 24 h in 70% ethanol at -20˚C. Then the cells were washed twice in PBS and incubated with RNase (50 μg/ml) at 37˚C for 1 h. The cells were stained for 30 min with propidium iodide (50 μg/ml) at 4˚C and the cellular DNA content was determined using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

Expression of cell-surface integrins. To determine $ß_3$, α_v and α_{IIb} integrin expression by flow cytometry, $2.0x10^5$ cancer cells in 200 μl of PBS [supplemented with 0.1% fetal bovine serum (FBS)] were mixed with an appropriate volume of moAb solution (prechilled to 4˚C). The cells were incubated in darkness for 30 min on ice and subsequently washed twice with 500 μl of PBS (supplemented as above). FITC-conjugated IgG1 was used as the negative control. Cell-surface fluorescence was measured using a FACS Calibur flow cytometer (Becton Dickinson). Damaged cells were labeled by adding 10 μl of propidium iodide solution (20 μl/ml) to each test tube just before data acquisition. Data analysis was performed using WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA, USA).

To determine $ß_3$, α_v, or α_{IIb} integrin expression by Western blotting, the collected cells were rinsed once in PBS and lysed on ice for 45 min in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 1% Triton X-100, supplemented with the complete-TM mixture of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). The lysates were sonicated and cleared by microcentrifugation at 11,000 x g for 10 min. Protein concentrations were determined using the protein assay described by Boratynski (37). Equal amounts of protein (50 μg) were separated in an 8% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (0.45 Micron; Osmonics, GE Water & Technologies, Trevose, PA, USA). Protein loading and efficiency of transfer were monitored by 0.1% Ponceau S-Red staining. The membranes were blocked for 1 h at room temperature in 1% blocking reagent (5% non-fat dry milk in 0.1% PBS/Tween-20), then washed three times (3x10 min) with 0.05% PBST and incubated for 1 h with the primary (first layer) antibody. After incubation, the blot was washed three times with 0.1% PBST and incubated for 1 h with the secondary antibody. Secondary antibodies were detected with horseradish peroxidase-conjugated IgG (Avidin HRP, Dako, Denmark).

Apoptosis determination by Annexin V staining. The cells ($1x10^6$) were washed twice with PBS. FITC-Annexin V (Alexis Biochemicals, San Diego, CA, USA) was diluted to a concentration of 1 mg/ml in binding buffer (Hepes buffer: 10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and the cells were suspended in 1 ml of this solution (freshly prepared each time). Then, after 15 min of incubation in the dark at room temperature, the propidium iodide (PI) solution (10 mg/ml) was added prior to analysis to give a final concentration of 1 mg/ml. Data analysis was performed by flow cytometry using the CellQuest program for data acquisition. The data were displayed as a two-color dot plot with FITC-Annexin V (FL1-H, Y axis) vs. PI (FL3-H, X axis). Double-negative cells were live cells, PI^+/Annexin V^+ were late apoptotic or necrotic cells and PI^-/Annexin V^+ early apoptotic cells.

Reverse transcriptase-polymerase chain reaction. Total RNA was isolated using Trizol reagent (Invitrogen) from cultures of cells. Genomic DNA was removed from preparations of RNA with the use of DNase I. First-strand cDNA was prepared using reverse transcriptase SuperScript III (Invitrogen) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed on the amounts of cDNA corresponding to 1/25, 1/250 and 1/2500 of the serially diluted volume after reaction with reverse transcriptase. PCR was performed in a total volume of 20 μl containing cDNA, 0.4 μM each of 5' and 3' primers, and 0.5 unit Taq polymerase (Fermentas). Expression of mRNA for $ß_3$ integrin was tested using the primers: forward 5'-TCA GATGCGCAAGCTTACTA and reverse 5'-TCAGCACGT GTTTGTAGCCAA. PCRs with GAPDH primers were performed to test the amount and quality of the cDNA using the primers: forward 5'-ATGACATCAAGAAGGTGGTG and reverse 5'-CATACCAGGAAATGAGCTTG. The samples were subjected to 35 cycles (for $ß_3$ integrin) or 30 cycles (for GAPDH) of PCR according to the following scheme: 94˚C for 30 sec, 55˚C (for $ß_3$ integrin), or 45˚C (for GAPDH) for 30 sec and 72˚C for 1 min. The absence of contaminating genomic DNA in the RT-PCR analysis was demonstrated by

parallel RT reactions from the same RNA in which the reverse transcriptase was omitted. The amplified products were resolved on 2% agarose gels and visualized by ethidium bromide staining under UV illumination.

Migration assay. FALCON cell culture inserts for 24-well plates containing PET membranes with 8-μm pores and FALCON 24-well Companion Plates (Becton Dickinson Biosciences, USA) were used as invasion chambers. The inserts were coated with Matrigel Basement Membrane Matrix (Becton Dickinson Biosciences), 22 μg per insert, i.e. 73.3 μg/cm^2 of the membrane, using cooled pipettes and accessories. Matrigel was gently gelled and dried in 37˚C. The matrigel was rehydrated for 1 h with DMEM [0.7 ml to the top chamber (insert), 0.7 ml to the bottom chamber] and then the DMEM was carefully removed.

The LLC and WEHI-3 cells were harvested after 72 h of incubation with 100 nM (LLC) or 10 nM (WEHI-3) of calcitriol or PRI-2191, washed and suspended with DMEM (from the Institute of Immunology and Experimental Therapy). Cells (1x10^5) in 0.5 ml of DMEM per insert were added. The bottom chamber was filled with 5-10% fetal bovine serum medium as a chemoattractant. Migration was conducted for 5 h (37˚C, CO_2). The cells were fixed and stained with a DiffQuick set (Medion Diagnostics, Dudungen, Switzerland) and counted by light microscopy. The average number of cells per view for one insert was calculated and the mean ± SD of these values in groups was presented.

Adhesion assay. After 48 h of incubation with 100 nM calcitriol or PRI-2191, the LLC cells were harvested, washed, and resuspended in TSM buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) containing 0.5% BSA at a concentration of 5x10^5 cells/ml. Ninety-six-well NUNC Maxisorp plates were coated overnight at 4˚C with 50 μl of fibrinogen (Sigma-Aldrich, Saint Louis, MO, USA) (10 μg/ml in 7.5% $NaHCO_3$), washed twice with 300 μl TSM and blocked for 30 min at 37˚C with 1% BSA/TSM (50 μl). The 50-μl cell suspension was allowed to adhere for 60 min at 37˚C and non-adherent cells were removed with warm TSM (300 μl, twice). Adherent cells were lysed and subjected to crystal violet assay. Fifty microliters of 0.2% crystal violet in 20% MetOH was added to each well and incubated for 30 min at 4˚C. After the incubation time was complete, the wells were washed twice with 300 μl PBS (-Ca^{++}, Mg^{++}), then 80 μl of the 100 μl of MetOH was added to each well. The optical densities of the samples were read after 30 min on a Multiskan RC photometer (Labsystems, Helsinki, Finland) at 570 nm.

Antitumor effect in vivo

Mice. Eight- to ten-week-old female C57Bl/6 mice weighing 18-20 g supplied by the Animal Breeding Center of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland, were maintained under standard laboratory conditions. All the experiments described were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

Schedule of treatment protocol. C57BL/6 female mice were inoculated subcutaneously (s.c.) in the right flank of the abdomen with a 20% (w/v) suspension of LLC tumor cells in 0.2 ml of saline. Calcitriol or PRI-2191 was administered orally (p.o.) at 5 μg per kg body weight on days 3, 6, 8, 10, 13 and 15 (i.e. six times) after cancer cell inoculation.

Evaluation of therapeutic effect. Tumor volume was calculated using the formula (a^2 x b)/2, where a is the smaller tumor diameter in mm and b the larger tumor diameter in mm. Inhibition of tumor growth was calculated using the formula: TGI (%) (tumor growth inhibition) = (W_T/W_C) x 100 - 100%, where W_T is the median tumor volume of the treated mice and W_C that of the untreated control animals. The animals were sacrificed 20 days after inoculation of the LLC cells and the lungs were examined macroscopically for metastatic foci. For histological studies, the subcutaneous tumors were removed, fixed with 4% buffered formalin and processed routinely. The preparations were subsequently stained with hematoxylin and eosin. The vessel diameter was measured at a magnification of x400.

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test or a Mann-Whitney U Test was applied. P-values <0.05 were considered significant.

Results

The influence of calcitriol and PRI-2191 on $\alpha_v\beta_3$ integrin expression. The effect of the tested compounds on integrin expression was investigated in selected human and mice cell lines which express α_vß$_3$ integrin confirmed by Western blotting and FACS analysis (Figs. 1-3). FACS analysis showed from among the mouse cancer cell lines the expression of the ß$_3$ integrin subunit in 4 (LLC, WEHI-3, B16 and P388) and of the subunit α_v in 5 mouse cancer cell lines (LLC, WEHI-3, B16, P388 and 4T1) (Fig. 1). Only four human cancer cell lines, namely A498, Hs294T, Du145, HCV29T (Fig. 2) and LoVo/DX (only 7% of cells, data not shown), expressed α_vß$_3$ integrin detected by this method. The α_{IIb} integrin subunit on the cell lines tested was not detectable by flow cytometry. However, the results obtained by Western blotting confirmed the results obtained only for positive cells detected by FACS analysis. Western blot analysis additionally showed expression of the ß$_3$ integrin subunit in 12 human cell lines, i.e. LNCaP, MDA-MB-231, MCF-7, T47D, SW-707, CX-1, A549, SCC-25, Hep-2, ASPC-1, CEMT-4 and HT-29 (an example of blots in Fig. 3A). By using this method, expression of the α_{IIb} integrin subunit on the LNCaP, Hs294T and FaDu cell lines could be shown as well (Fig. 3B).

The human cancer cell lines Hs294T, Du145, A498 and HCV29T incubated for 96 h with 1000 nM of calcitriol or PRI-2191 showed a tendency to decrease α_vß$_3$ integrin expression (data not shown). Two mouse cancer cell lines,

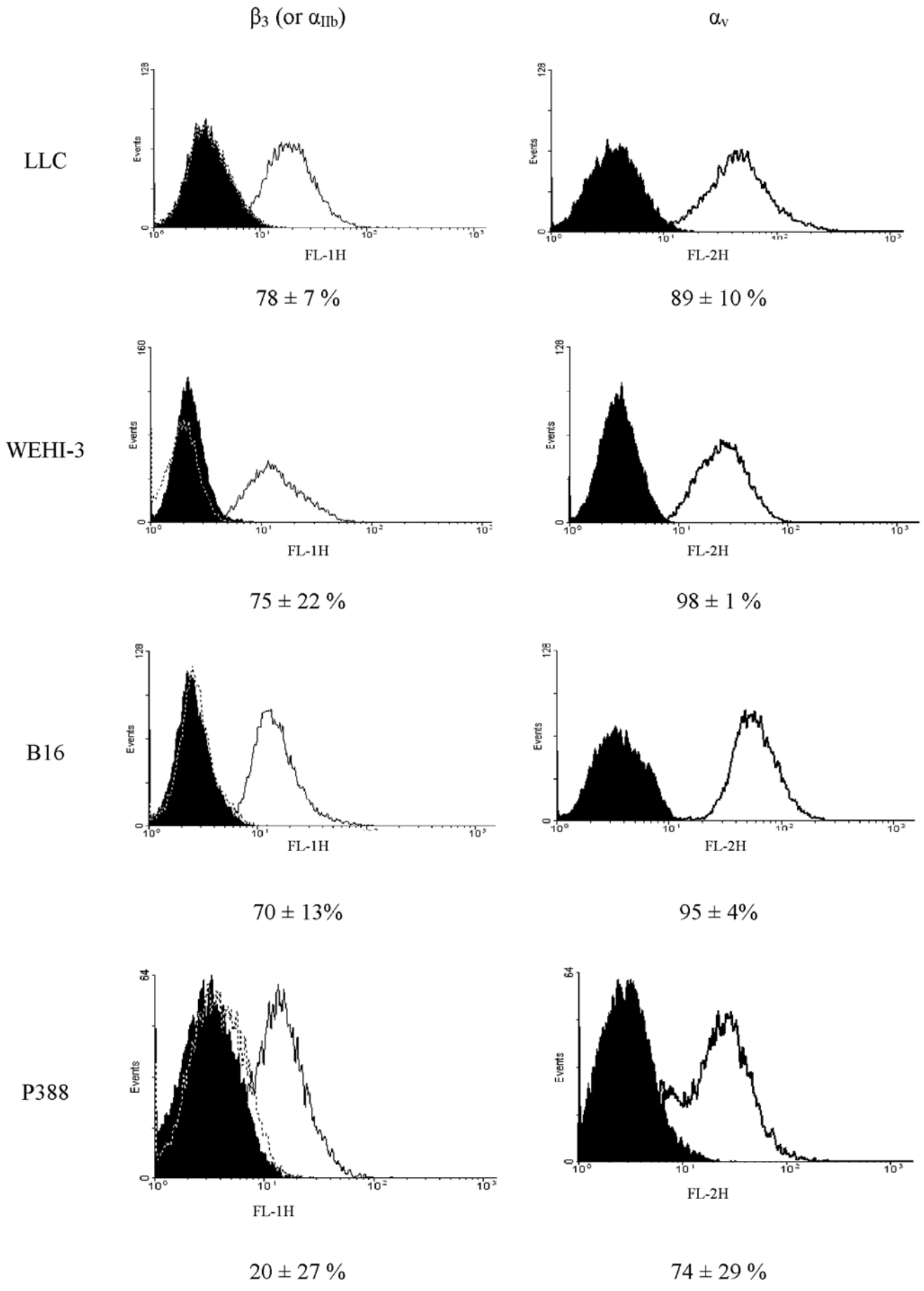

Figure 1. FACS analysis of the expression of α_v, α_{IIb} and $ß_3$ integrins in mouse cancer cell lines. Black area, isotype control; black line, anti-$ß_3$ or -α_v Ab; dotted line, anti-α_{IIb} Ab. The percentage of positive cells (± SD) was calculated by subtracting the common area under the graphs (the graph representing the negative control and the graph representing the expression of antigen) from the area under the graph representing the expression of antigen.

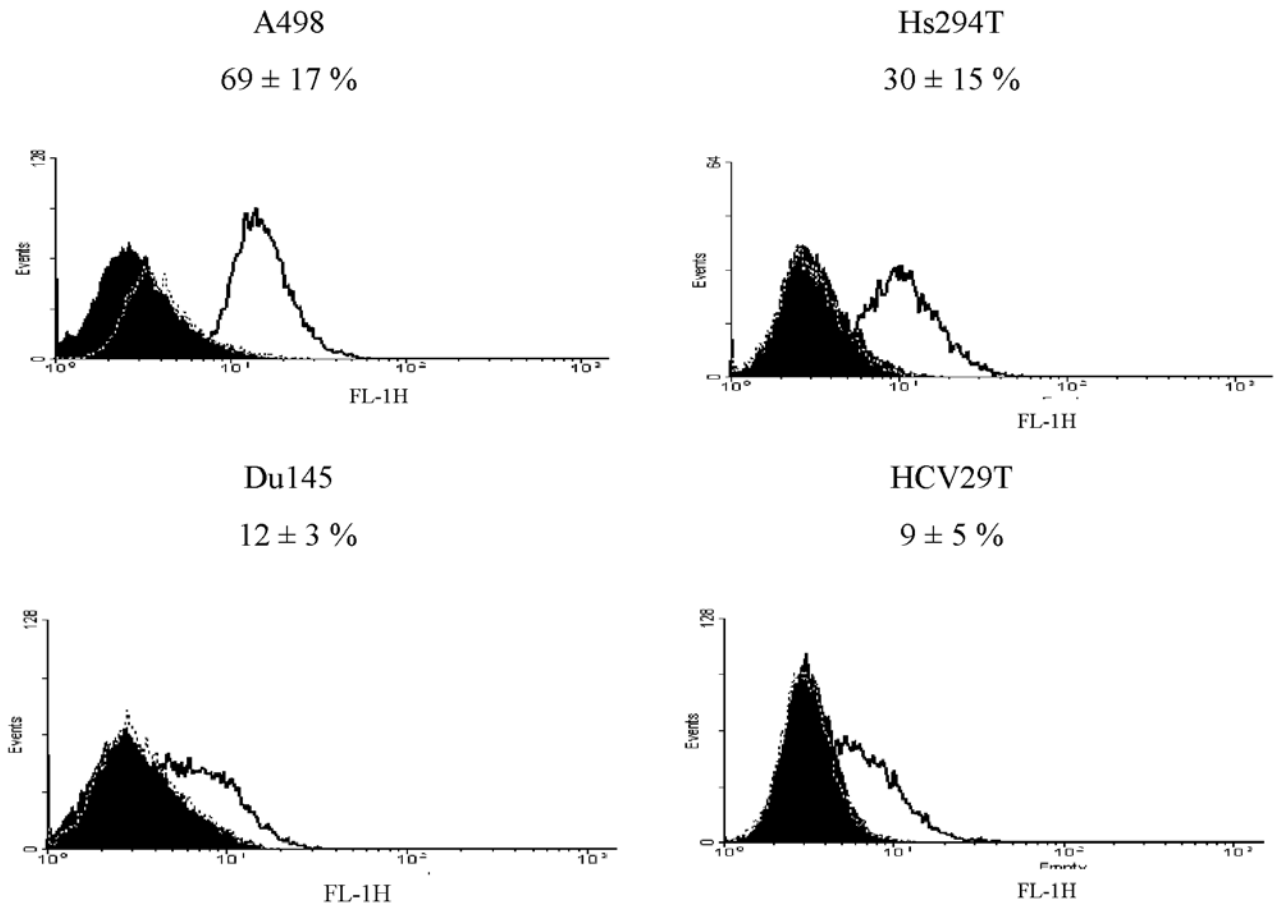

Figure 2. FACS analysis of the expression of $\alpha_v ß_3$ and α_{IIb} integrins in human cancer cell lines. Black area, isotype control; black line, anti-$\alpha_v ß_3$ antibody; dotted line, anti-α_{IIb} antibody. The percentage of positive cells (±SD) was calculated by subtracting the common area under the graphs (the graph representing the negative control and the graph representing the expression of antigen) from the area under the graph representing the expression of antigen.

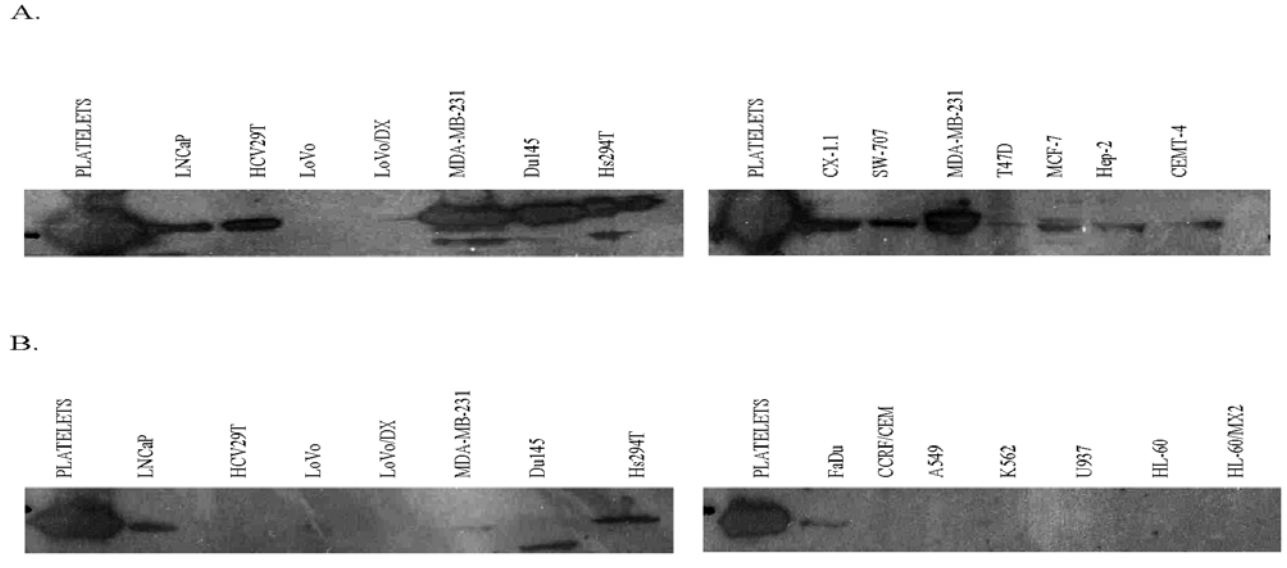

Figure 3. Western blot analysis of the ß3 and α_{IIb} integrin subunits. (A) The expression of $ß_3$ integrin in human cancer cell lines (104 kDa). (B) The expression of α_{IIb} integrin in human cancer cell lines (120 kDa). Lysates from human platelets were used as a positive control.

LLC and WEHI-3, expressing high levels of $\alpha_v ß_3$ integrin, were exposed to calcitriol or PRI-2191 for 48, 72 or 96 h. It appears that 48 h of incubation with 100 nM calcitriol or PRI-2191 was sufficient to significantly reduce (by 25%) the percentage of $ß_3$-positive LLC cells. These compounds decreased integrin expression by 50% during the 96 h of

Table I. The influence of calcitriol and PRI-2191 (100 nM) on $\alpha_v\beta_3$ integrin expression in the mouse Lewis lung carcinoma cell line (LLC).

Integrin subunit	Incubation time (h)	Control		Calcitriol		PRI-2191	
		%	MFC	%	MFC	%	MFC
β_3	48	82.77±14.26	31.59±5.90	57.54±15.05[a]	26.52±5.69	62.38±8.12	26.51±5.93
	72	82.71±6.35	23.38±2.42	30.67±6.98[a]	20.29±0.16	26.86±6.33[a]	19.73±0.68[a]
	96	80.29±12.12	23.3±4.68	25.94±12.54[a]	21.35±3.28	29.65±10.68[a]	20.14±3.41
α_v	48	89.87±3.78	58.52±9.93	59.02±13.15	44.94±0.53	57.53±17.25	44.62±0.13
	72	88.44±3.57	54.94±11.12	36.97±11.96[a]	42.48±2.62	34.78±9.72[a]	41.64±2.73
	96	88.46±12.32	53.29±19.68	33.67±13.09[a]	38.54±0.71	33.41±15.44[a]	37.66±1.37

[a]P<0.05 (Kruskal-Wallis, Mann-Whitney U test). Results of 4-6 experiments are presented as the percentage of positive cells (%) and the mean fluorescence channel (MFC) of the positive cell population (± SD). The percentage of positive cells was calculated by subtracting the common area under the graphs (the graph representing the negative control and the graph representing the expression of antigen) from the area under the graph representing the expression of antigen.

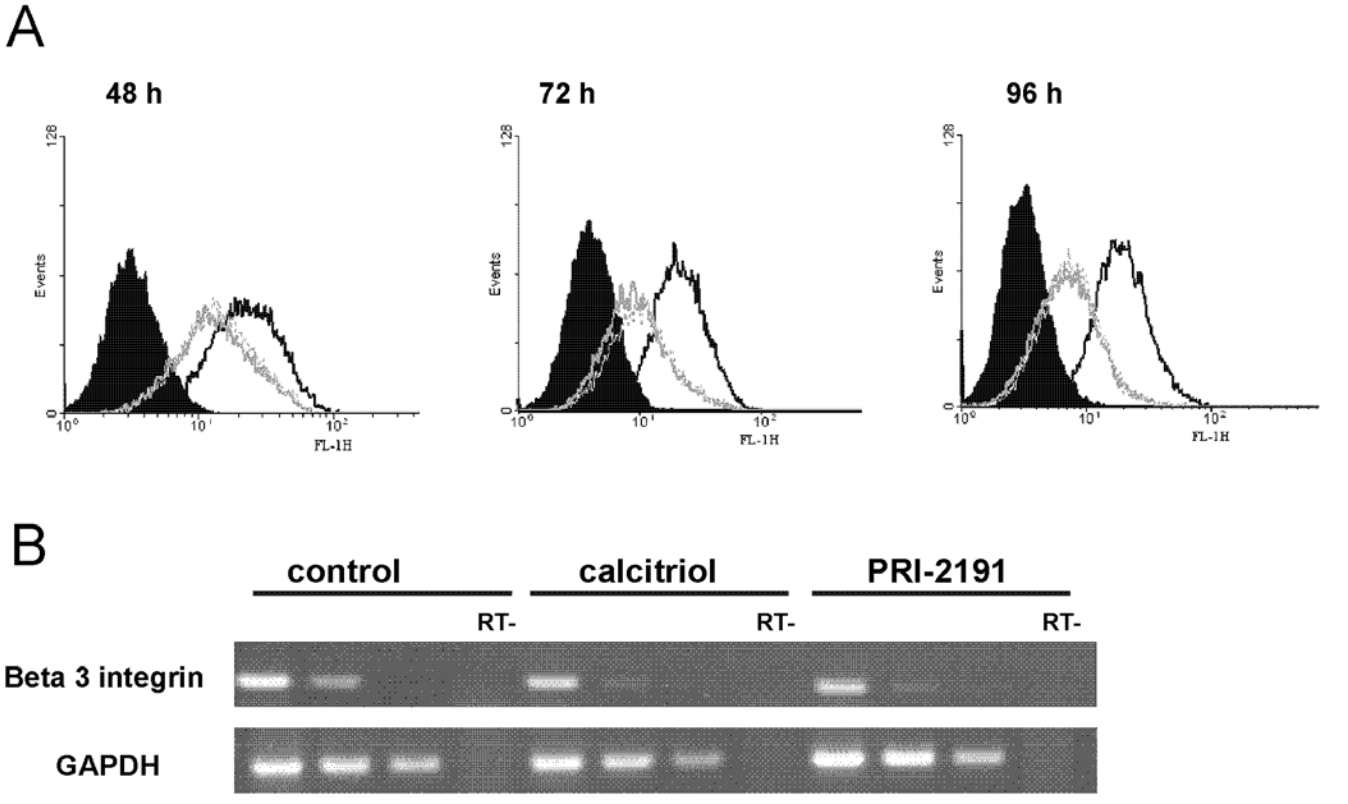

Figure 4. The expression of β_3 integrin on LLC cells. (A) FACS analysis after various incubation times with calcitriol or PRI-2191 (representative histograms). Black area, isotype control; black line, ethanol control with anti-β_3 antibody; grey line, calcitriol with anti-β_3 antibody; grey dotted line, PRI-2191 with anti-β_3 antibody. (B) RT-PCR analysis for mRNA encoding β_3 integrin in LLC cells after 96 h of incubation with calcitriol or PRI-2191. The PCRs were performed on cDNAs serially diluted (1/25, 1/250 and 1/2500). GAPDH primers were used to test the amount and quality of cDNAs. Results are representative of several experiments using cells from three different refrozen samples.

incubation (Table I, Fig. 4A). Similarly, a reduction in the α_v integrin subunit was observed, though this decrease was statistically significant only after 72 and 96 h (not after 48 h) of incubation (Table I). A decrease in β_3 integrin mRNA was also shown by RT-PCR analysis of LLC cells incubated for 96 h with calcitriol or PRI-2191 (Fig. 4B).

Calcitriol or PRI-2191 caused similar, although lower, changes in the profile of integrin expression in mouse WEHI-3 leukemia (~26% decrease, not statistically significant) (Table II). The diminishing effect on integrin expression on LLC or WEHI-3 cells was similar or slightly higher for calcitriol than for its analog, PRI-2191 (Fig. 4, Tables I and II).

Table II. The influence of calcitriol and PRI-2191 (10 nM) on $\alpha_v\beta_3$ integrin expression in the mouse WEHI-3 leukemia cell line.

Integrin subunit	Incubation time (h)	Control		Calcitriol		PRI-2191	
		%	MFC	%	MFC	%	MFC
β_3	48	73.07±13.07	16.45±3.23	58.25±8.4	11.46±1.58	57.57±12.99	11.86±1.66
	72	85.54±13.4	20.45±6.65	62.49±21.05	11.52±2.71[a]	69.58±18.35	13.05±2.40
	96	87.18±8.58	20.85±4.19	63.32±13.63	12.23±1.39	62.04±12.20	12.11±1.30
α_v	48	87.6±13.4	23.03±7.23	78.18±9.87	14.79±1.38	80.44±10.9	15.45±1.52
	72	83.66±7.9	18.47±4.48	63.1±6.99	14.05±2.88	64.80±6.51	13.28±2.35
	96	85.31±10.22	23.31±6.85	59.61±12.07	12.45±5.49	71.04±7.94	15.81±2.70

[a]P<0.05 (Kruskal-Wallis, Mann-Whitney U test). Results of 4-6 experiments are presented as the percentage of positive cells (%) and the mean fluorescence channel (MFC) of the positive cell population (± SD). The percentage of positive cells was calculated by subtracting the common area under the graphs (the graph representing the negative control and the graph representing the expression of antigen) from the area under the graph representing the expression of antigen.

Table III. Inhibition of proliferation of mouse cell lines expressing $\alpha_v\beta_3$ integrin by calcitriol or PRI-2191 after 96 h of incubation.

Cell line	Inhibition of proliferation (%) ± SD					
	Calcitriol		PRI-2191		Ethanol (control)	
	100 nM	10 nM	100 nM	10 nM	0.1%	0.01%
LLC	51.4±11.7	25.0±1.4	58.7±6.0	47.0±2.8	3.2±13.0	0
WEHI-3	62.6±8.9	44.5±11.3	67.2±7.3	53.0±17.4	7.4±4.1	9.8±6.3

One to two hours before the addition of the tested compounds, the cells were plated in 96-well plates at densities of $1x10^4$ cells per well. The assay was performed after 96 h of exposure to varying concentrations of the tested agents. Ethanol at corresponding concentrations was used as a control.

Table IV. Tumor and lung weight and tumor vasculature (day 20) in mice bearing LLC tumors transplanted subcutaneously.

	Propylene glycol 80%	Calcitriol	PRI-2191
No. of mice	8	7	7
Tumor weight (g)			
Median	4.65	2.3[a]	2.7[a]
Mean ± SD	4.39±0.72	2.24±1.44	2.93±1.61
Lung weight (g)			
Median	0.20	0.10[a]	0.10[a]
Mean ± SD	0.24±0.12	0.13±0.05	0.11±0.04
Vessel diameter (μm^2)			
Median	554	445	433
Mean ± SD	550±48	461±205	425±185

[a]P<0.05 Mann-Whitney U Test. Mice bearing LLC cancer transplanted subcutaneously were administered calcitriol or PRI-2191 or propylene glycol (as a control) orally (p.o.) according to the following schedule: 5 μg/kg/day on days 3, 6, 8, 10, 13 and 15 (six times).

The 96-h incubation of the other mouse cell lines, i.e. B16 melanoma and P388 leukemia, expressing $\alpha_v\beta_3$ integrin revealed no effect on their expression even at a high dose (1000 nM) of calcitriol or PRI-2191 (data not shown).

The influence of calcitriol and PRI-2191 on the proliferation, apoptosis and cell-cycle division of selected cell lines expressing $\alpha_v\beta_3$ integrin. Proliferation of the mouse cell lines LLC and WEHI-3 was inhibited after incubation with the two

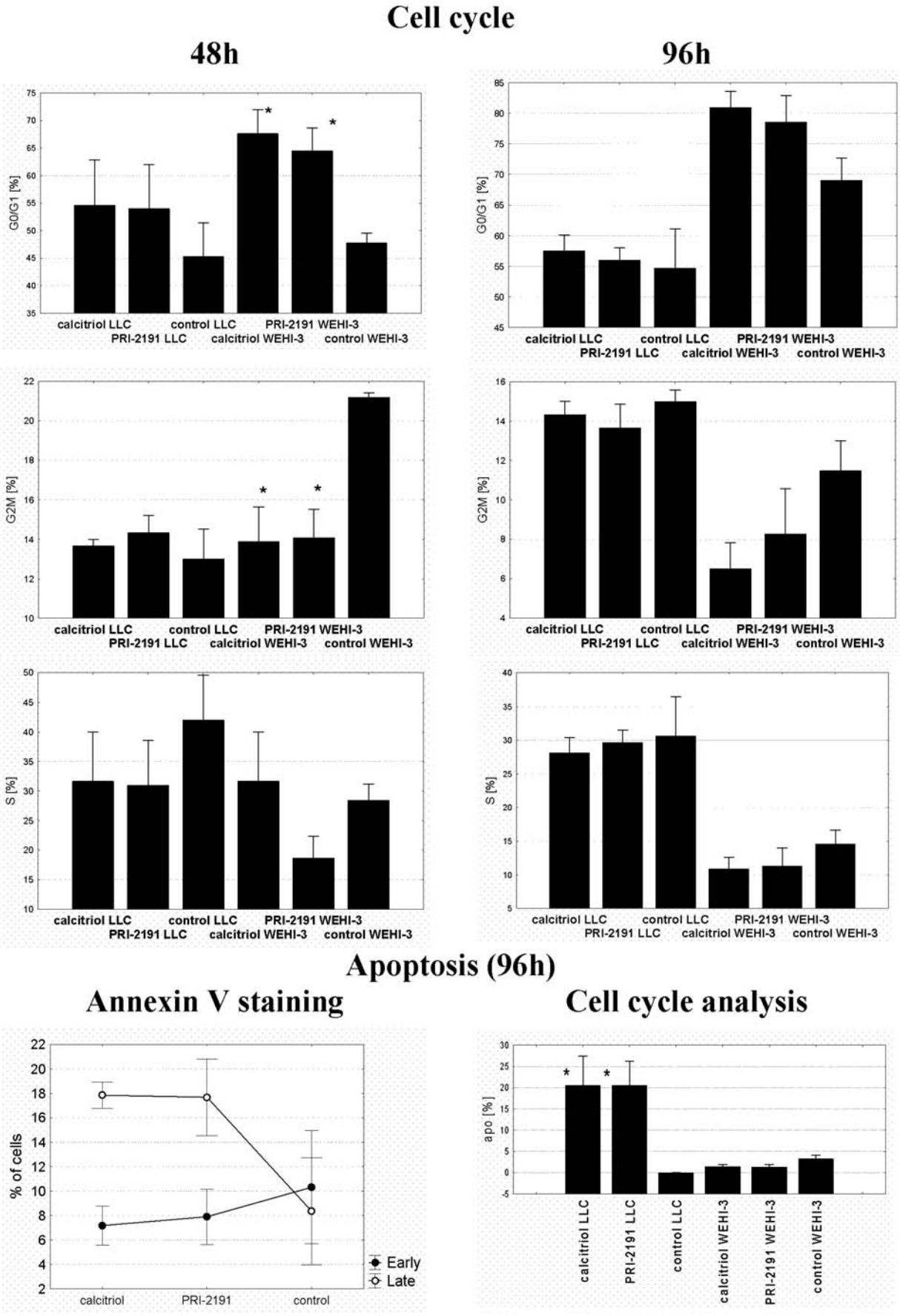

Figure 5. The effect of calcitriol and PRI-2191 on cell-cycle distribution and apoptosis of the LLC and WEHI-3 cell lines. Cell-cycle: The results are presented as the mean percentage of the cell population qualified to one of the four groups: M1, cells in phase G0/G1; M2, cells in phase S; M3, cells in phase G2/M and M4, apoptotic cells (Apo). The cells were incubated with 100 (LLC) or 10 (WEHI-3) nM of calcitriol or PRI-2191 for 48 or 96 h. In the control, ethanol was used in the appropriate concentration. Apoptosis by annexin V staining: The data are displayed as a two-color dot plot with FITC-Annexin V (FL1-H, Y axis) vs. PI (FL3-H, X axis). Double-negative cells were live cells, PI^+/Annexin V^+ late apoptotic or necrotic cells and PI^-/Annexin V^+ early apoptotic cells. The data presented are the mean ± SE.

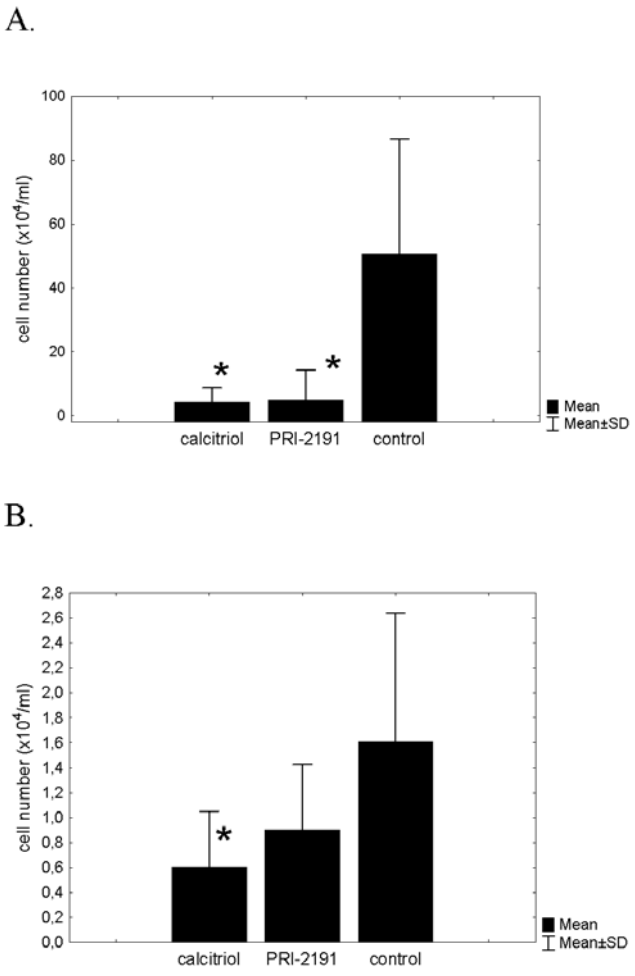

Figure 6. The migration of LLC (A) and WEHI-3 (B) cells through matrigel. The cells were incubated for 72 h with 10 nM (WEHI-3) or 100 nM (LLC) calcitriol or PRI-2191, then allowed to migrate in matrigel. N (WEHI-3), control-12, calcitriol-11, PRI-2191-10. N (LLC), control-8, calcitriol-8, PRI-2191-8. *P<0.05 Multiple-Comparison Kruskal-Wallis test.

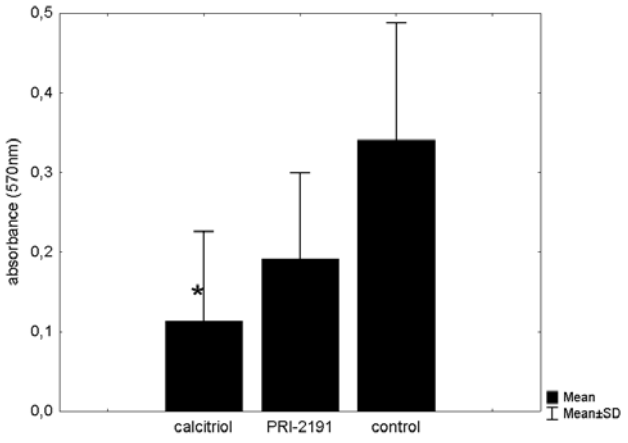

Figure 7. Adhesion of LLC cells to fibrinogen. The LLC cells were incubated for 48 h with 100 nM calcitriol or PRI-2191, then allowed to adhere to fibrinogen. N, control-6, calcitriol-5 and PRI-2191-5. *P<0.05 Multiple-Comparison Kruskal-Wallis test.

tested compounds. WEHI-3 cells were the most sensitive among the cell lines tested. This antiproliferative activity was higher after exposing the cells to PRI-2191 than to calcitriol (Table III). Moreover, Du145 human prostate cancer cells were sensitive to calcitriol and PRI-2191 (~20% inhibition at the maximal dose applied, i.e. 1000 nM, data not shown). Other cell lines with integrin expression, namely B16, P388, Hs294T, HCV29T and A498, appeared to be insensitive to the antiproliferative activity of calcitriol or its metabolite after 96 h of incubation (data not shown).

The effect of calcitriol or PRI-2191 on the cell cycle was examined in the LLC and WEHI-3 cancer cell lines. These cells are most sensitive to the antiproliferative activity and diminishing effect on integrin $\alpha_v ß_3$ of vitamin D compounds (Tables I-III). After 48 h of incubation, WEHI-3 cells accumulated in the G_0/G_1 cell-cycle phase, with a parallel decrease in cells in the G_2M and S phases. In the case of LLC cells, an increase in the percentage of cells in the G_0/G_1 phase and a decrease in S was observed (Fig. 5). After 96 h of incubation, the cell-cycle of the WEHI-3 cells was similar to that after 48 h. The LLC cells underwent apoptosis after 96 h of incubation with calcitriol or PRI-2191 (Fig. 5). We showed by Annexin V staining of LLC cells that the percentage of cells incubated for 96 h with calcitriol or PRI-2191 in early apoptosis decreased, though the percentage of cells in late apoptosis or necrotic cells increased compared with the control cells (Fig. 5).

The influence of calcitriol and PRI-2191 on adhesion and invasion in vitro and tumor growth and metastasis in vivo. The growth of subcutaneously inoculated LLC tumor cells was reduced after treatment of mice with calcitriol or PRI-2191. The TGI (tumor growth inhibition) values were 51 and 42%, respectively. Both agents also led to an ~50% reduction in lung metastasis in the mice (Table IV). Moreover, a tendency to decrease blood vessel diameter was shown (Table IV). The number of blood vessels remained unchanged (data not shown). Under our experimental conditions, WEHI-3 leukemia inoculated i.p. or s.c. into BALB/c mice appeared to be insensitive to such treatment (unpublished data).

The number of LLC cells which migrated through matrigel was significantly reduced after incubation with calcitriol or PRI-2191 (92 and 91%, respectively). A similar effect was observed in WEHI-3 cells, although it was lower and statistically significant only after exposure of the cells to calcitriol (67% reduction of cell migration after incubation with calcitriol and 51% after incubation with PRI-2191) (Fig. 6). Moreover, LLC cells incubated with 100 nM calcitriol or PRI-2191 lost adhesive capacity to fibrinogen by 67% ($p<0.05$) and 44%, respectively (Fig. 7).

Discussion

Calcitriol appeared to inhibit the invasiveness of MDA-MB-231 breast cancer, B16 melanoma, Lewis lung carcinoma (LLC), Du145 and PC-3 prostate cancer, and HT1080 fibrosarcoma cells (1,5,6,19,20,38). The reduced invasiveness of LLC cells treated with calcitriol is thought to be a result of decreased protein kinase A (PKA) activity and altered actin polymerization (39,40). In our previous studies, we observed LLC tumor growth delay after treating mice with calcitriol, calcipotriol or its two analogs (PRI-2202 or PRI-2205) (36).

Young *et al* showed the results of studies in which mice inoculated with LLC cells were treated with calcitriol, which resulted not only in reduced tumor growth, but also in fewer lung metastases (38). In the present study, we showed that calcitriol and its analog PRI-2191 inhibit growth and metastasis of LLC cells transplanted subcutaneously. Moreover, a tendency to decrease blood vessel diameter, without influencing their number (data not shown), was observed. This observation may suggest the influence of calcitriol and its analog on tumor growth not only directly, but also through normalization of the tumor vasculature. Since previous studies with other cells correlated similar responses with changes in cellular integrin profiles (6,20), we hypothesize that calcitriol or its analog might act through specific integrins to inhibit metastasis.

It is known that $ß_3$ integrins may contribute to the metastatic progression of cancer by mediating the adhesion of cancer cells to endothelium and migration through the extracellular matrix (41). High expression levels of $\alpha_v ß_3$ integrin have been shown on cancer cells and on endothelial cells from peritumoral blood vessels of various cancers, including prostate, breast, colon, lung, ovary, and cervical cancer and melanoma (6,21-23,25-28,42-44).

Among the cell lines expressing $\alpha_v ß_3$ integrin (measured by flow cytometry and Western blotting), only the LLC and WEHI-3 cells were sensitive to the diminishing effect of calcitriol or PRI-2191 on this integrin's expression. Moreover, the number of LLC cells which migrated through matrigel was significantly reduced after incubation with calcitriol or PRI-2191. A similar effect was observed in WEHI-3 cells, but it was lower and statistically significant only after exposure of the cells to calcitriol. These observations correlated with the degree of the diminishing effect on integrin expression: more profound on the LLC than on the WEHI-3 cell line. However, it is interesting that a higher inhibition of proliferation of the WEHI-3 than of the LLC cell line was observed. Moreover, LLC cells incubated with calcitriol or PRI-2191 lost their adhesive capacity to fibrinogen.

Yudoh *et al* observed that calcitriol inhibited *in vitro* invasiveness (through the extracellular matrix) and *in vivo* pulmonary metastasis of B16 mouse melanoma, but it did not reveal an antiproliferative effect *in vitro* or an antitumor effect *in vivo* (primary tumor growth) (6). The role of $ß_3$ integrin was postulated as a possible mechanism of the antimetastatic activity of calcitriol in this mouse tumor model (6). Our data confirm these observations concerning the inhibition of B16 cell proliferation (45) and an antimetastatic effect (unpublished data) caused by calcitriol or its analogs, but the mechanism of these effects remain unclear, especially in light of our observation that integrin $ß_3$ expression in B16 cells was not influenced by calcitriol or PRI-2191.

The explanation for the observed diminishing effect on integrin expression caused by calcitriol may be related to the VDRE, which was reported in the avian $ß_3$ gene (18). Although calcitriol transcriptionally activates the α_v and $ß_3$ genes (18), there is no information on the presence of a $ß_3$ integrin VDRE in cancer cells. Moreover, some authors observed increased $\alpha_v ß_3$ integrin expression on the HL-60 cell line (16). However, it is known that VDR requires an accessory nuclear factor for high-affinity DNA-binding interactions and members of the retinoid X receptor (RXR) family have been identified that can act as heterodimer partners with the VDR to promote such interactions (46,47). Changes in the expression of either receptor or their mutually exclusive availability to interact with each other within a target cell could influence the degree to which certain gene targets may be activated or repressed (9,48).

All the presented results suggest that the *in vivo* inhibition of LLC tumor growth and metastasis after treatment with calcitriol or PRI-2191 could be a consequence of the diminished integrin $\alpha_v ß_3$ expression caused by these agents. Tumor cell invasion and metastasis are complex processes involving many cellular changes and regulatory molecules. Thus, the actions of calcitriol and PRI-2191 on $\alpha_v ß_3$ integrin expression is probably one of several mechanisms by which these compounds may alter invasion and metastasis.

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