

α 5-integrin is crucial for L1CAM-mediated chemoresistance in pancreatic adenocarcinoma

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Abstract. We recently showed that the adhesion molecule L1CAM (CD171) is overexpressed in pancreatic adenocarcinoma (PDAC) essentially contributing to chemoresistance of PDAC cells. In search of the mechanisms of this effect we now identified α 5-integrin as the L1CAM ligand being essential for L1CAM-mediated chemoresistance of these highly malignant tumor cells. Thus, blockade or knock-down of α 5-integrin in the L1CAM expressing PDAC cell lines PT45-P1res, Colo357 and Panc1 increased anti-cancer drug sensitivity. In line with the previously reported NO-dependent caspase inhibition resulting from L1CAM induced iNOS expression, the loss of chemoresistance upon α 5-integrin inhibition was preceded by decreased iNOS expression and enhanced caspase-3/-7 activation. Accordingly, the loss of anti-cancer drug protection by α 5-integrin inhibition could be overcome by administration of the NO-donor SNAP. Moreover, the gain of chemoresistance of parental PT45-P1 cells when transfected with L1CAM was abrogated by α 5-integrin inhibition, whereas transfection of PT45-P1 cells with an integrin binding-deficient L1CAM mutant (L1mutRGE) did neither induce chemoresistance or iNOS expression nor conferred sensitivity to α 5-integrin inhibition as seen upon transfection with wild-type L1CAM. Thus, mutational loss of the integrin binding site in the L1CAM molecule or the

blockade of α 5-integrin abolished the induction of iNOS expression and chemoresistance by L1CAM, indicating that both a functional L1CAM and α 5-integrin are indispensable of L1CAM-induced drug resistance in PDAC cells.

Introduction

In recent years, the adhesion molecule L1CAM (CD171) has attracted much attention because it is overexpressed in various tumors, e.g., in ovarian carcinoma, melanoma and colon carcinoma, being associated with short survival and poor prognosis (1-5). Moreover, L1CAM has also been detected in pancreatic adenocarcinoma (PDAC) (6).

L1CAM is a 200-220-kDa transmembrane glycoprotein of the immunoglobulin (Ig) superfamily composed of six Ig-like domains and five fibronectin type III repeats followed by a transmembrane region and a highly conserved cytoplasmic tail (7). Previous studies demonstrated that L1CAM can interact either with itself (homophilic) or with a variety of heterophilic ligands such as integrins α 5 β 1, α v β 3 or α v β 3, neurocan or neuropilin-1 (8-13). In many incidences, the binding sites in the L1CAM molecule have been mapped, e.g., the RGD site(s) in the 6th Ig domain (1 in the human, 2 in the mouse) supporting α 5 β 1, α v β 3 or α v β 3 integrin-mediated cell binding (10-12). The 1st Ig domain can bind to the proteoglycan neurocan (14) and the VEGF-R2-coreceptor neuropilin-1 (8,13).

The mechanisms by which L1CAM contributes to tumor progression are now beginning to emerge. Several studies have demonstrated that L1CAM augments tumor growth in NOD/SCID mice, enhances cell motility on extracellular matrix proteins and increases Matrigel invasion (2,15-18), thus indicating a role in tumor cell migration and metastasis. In ovarian and pancreatic carcinoma cells, L1CAM was found to mediate also resistance to apoptotic stimuli (6,19), thereby accounting for the profound chemoresistance of these highly malignant tumors. Using the well characterized model system of long-term drug treated, chemoresistant PT45-P1res cells and its parental chemosensitive counterpart PT45-P1 (20), we recently demonstrated that the chemoresistant phenotype of PT45-P1res cells highly depends on L1CAM expression, itself induced by IL1 β . Moreover, we identified a mechanism by which L1CAM mediates chemoresistance in

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Abbreviations: iNOS, inducible nitric oxide synthetase; NO, nitric oxide; PDAC, pancreatic ductal adenocarcinoma; RT, reverse transcription; siRNA, small interfering RNA; SNAP, S-Nitroso-N-acetyl-D,L-penicillamine

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PDAC cells: IL1 β mediated L1CAM expression leads to the induction of the inducible nitric oxide synthetase (iNOS), resulting in enhanced levels of nitric oxide (NO) and thereby in the inactivation of caspases (6,20). Meanwhile, antibodies to L1CAM or knock-down of L1CAM were shown to abolish chemoresistance (6,19) and to reduce cell proliferation *in vitro* as well as *in vivo* using a xenograft mouse model (21-23). However, so far it is not known whether these L1CAM-mediated processes require ligand binding.

Addressing this issue, the present study investigated the involvement of potential ligands in L1CAM-mediated chemoresistance of PDAC cells, identifying $\alpha 5$ -integrin to be essential in this process.

Materials and methods

Cell lines and culture. The human PDAC cell lines PT45-P1, Colo357 and Panc1 have been described previously (24). PT45-P1 and PT45-P1res cells were kept in culture (37°C, 5% CO₂, 85% humidity) using RPMI-1640 medium (PAA Laboratories, Cölbe, Germany) supplemented with 1% glutamine (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). The generation of chemoresistant PT45-P1res cells deriving from PT45-P1 cells has been described elsewhere (20). Colo357 and Panc1 cells were cultured in RPMI-1640 medium (PAA Laboratories) supplemented with 1% glutamine (Invitrogen), 10% FCS (Biochrom KG) and 1% sodium pyruvate (PAA).

Reagents. Etoposide was purchased from Bristol Myers Squibb (München, Germany). For functional blocking of integrins, a mouse IgG1 anti-human $\alpha 5$ -integrin antibody, a mouse IgG1 anti-human $\beta 1$ -integrin antibody or an isotype matched control antibody (all from Chemicon via Millipore, Schwalbach/Ts., Germany) were used, each at a concentration of 10 μ g/ml.

Site directed mutagenesis of L1CAM. Mutagenesis of L1CAM in position D556E (resulting in RGD \rightarrow RGE) was performed with the QuikChange™ Site-Directed Mutagenesis Kit according to the instructions of the manufacturer (Stratagene, Heidelberg, Germany) and as described recently (25). All constructs were verified by sequencing.

Surface staining of L1CAM, $\alpha 5$ - and $\beta 1$ -integrin and flow cytometry. After washing with PBS, cells grown in 6-well culture plates were detached with 5 mmol/l EDTA/PBS and then washed with PBS. Blocking was conducted in 1% BSA/PBS for 60 min at room temperature. Incubation with either a monoclonal mouse IgG1 anti-L1CAM antibody [L1-11A, clone UJ127 (16)], a mouse IgG1 anti- $\alpha 5$ -integrin (clone PID6 from Santa Cruz Biotechnology, Heidelberg, Germany), a mouse IgG1 anti- $\beta 1$ -integrin antibody (clone 6S6, Chemicon) or an isotype matched control antibody (R&D Systems, Wiesbaden, Germany), each diluted 1:1000 in 1% BSA/PBS, was performed at 4°C overnight. Then, cells were washed three times with PBS followed by incubation (60 min, 37°C) with an Alexa flour 488 conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany), diluted 1:500 in 1% BSA/PBS. After washing with PBS, cells were resuspended in 500 μ l PBS and analysed by fluorescence flow cytometry.

Measurement of caspase-3/-7 activity. Cells (1x10⁵ cells/well) were grown in a 12-well culture plate for 24 h. Then, medium was replaced and cells were left untreated or treated as indicated. Apoptosis was determined by detection of the caspase-3/7 activity, using a homogeneous luminiscent assay following the manufacturer's instructions (Promega, Mannheim, Germany). All samples were measured in duplicates.

Cell transfection. For plasmid transfection, PT45-P1 cells were seeded into 6-well plates (2x10⁵ cells/well) and grown overnight, followed by transfection with 5 μ l/well DIMRIE reagent (Invitrogen) and 0.4 μ g/well of the following plasmids: pcDNA3.1 (mock), pcDNA3.1-L1CAM wild-type (L1wt) or pcDNA3.1-L1CAMmutRGE (L1mutRGE). Upon transfection for 18 h, 1 ml medium containing 20% FCS was added and cells were left untreated or were treated as indicated for further 24 h. For siRNA treatment, cells were seeded into 12-well plates (1x10⁵ cells/well) and grown overnight followed by transfection with 12 μ l/well RNAiFect reagent (Qiagen, Hilden, Germany) and 0.4 μ l/well of either Stealth negative control siRNA, Stealth $\alpha 5$ -integrin siRNA or Stealth neuropilin-1 siRNA (each 20 μ M, Invitrogen). After overnight transfection, cells were either left untreated or treated as indicated for further 24 h.

Western blotting. Preparation of whole cell lysates as well as gel electrophoresis and immunoblotting have been described previously (6,26). For detection of L1CAM, a monoclonal antibody (clone UJ127 from Acris Antibodies) was diluted 1:1000 in 5% non-fat milk powder and 0.05% Tween in TBS (blotto-TBST) and incubated overnight at 4°C. For the detection of $\alpha 5$ - and $\beta 1$ -integrin, polyclonal goat antibodies (both from Santa Cruz Biotechnology) were each diluted 1:200 in blotto-TBST. Soluble neuropilin-1 was detected by a polyclonal rabbit antibody (Zymed via Invitrogen) diluted 1:250 in blotto-TBST. For detection of caspases, the following antibodies (all from Cell Signaling via New England Biolabs, Frankfurt a.M., Germany) were used at the indicated dilutions: anti-caspase-3 (proenzyme), 1:1000 in blotto-TBST; anti-cleaved caspase-3, 1:250 in blotto-TBST; anti-caspase-7 (pro- and cleaved enzyme), 1:1000 in blotto-TBST; anti-caspase-8 (pro- and cleaved enzyme), 1:1000 in 5% BSA (Serva) in TBST; anti-caspase-9 (proenzyme), 1:1000 in blotto-TBST; anti-caspase-9 (cleaved), 1:250 in blotto-TBST. iNOS was detected by a polyclonal rabbit anti-human iNOS antibody (BD Biosciences, Heidelberg, Germany), diluted 1:1000 in blotto-TBST. As control of equal protein load, a polyclonal rabbit antibody for HSP90 (Santa Cruz Biotechnology) was diluted 1:2000 in blotto-TBST. For detection of the primary antibodies, anti-rabbit, anti-goat and anti-mouse HRP-linked antibodies (Cell Signaling), respectively, were used at a dilution of 1:2000 in blotto-TBST.

Real-time PCR. Total RNA (2 μ g) was reverse-transcribed into single-stranded cDNA, as described previously (27). 1.3 μ l of cDNA and 0.2 μ mol/l gene-specific primers were adjusted with RNase-free water to a volume of 10 μ l. To this mixture, 10 μ l of iQ SYBR Green Supermix (Bio-Rad) was

added. The primer sequences and the PCR conditions for the detection of iNOS were: forward, CATAGAGATGGCCTGT CCTTGGG; reverse, TCCAGCTTGACCAGAGATTCTGG; 95°C/3 in; 95°C/1 min, 62°C/1 min, 72°C/20 sec for 3 cycles; 95°C/1 min, 61°C/1 min, 72°C/20 sec for 3 cycles; 95°C/1 min, 60°C/1 min, 72°C/20 sec for 3 cycles; 95°C/1 min, 58°C/1 min, 72°C/20 sec for 36 cycles and 72°C/10 min. For normalization of the mRNA content, β -actin was amplified in parallel using primers from Takara (via Lonza, Potsdam, Germany). The real-time PCR was performed using a MyiQ Single Color Real-time PCR Detection System (Bio-Rad). Data were collected during annealing steps and were further analysed by using the i-Cycler iQ Optical system software (Bio-Rad). All samples were analysed in duplicates.

Statistical analysis. Data are presented as mean \pm standard deviations and were analyzed by Student's t-test using the simple interactive statistical analysis (SISA) program (<http://home.clara.net/sisa/>). A $p < 0.05$ was considered as statistically significant.

Results

Chemosensitive PT45-P1 and chemoresistant PT45-P1res cells differentially express L1CAM, but exhibit a similar expression of potential L1CAM ligands. Recently, we have shown that the PDAC cell line PT45-P1 becomes chemoresistant (PT45-P1res) upon long-term drug treatment (20). Moreover, PT45-P1res cells are characterized by an elevated expression of L1CAM accounting for acquired drug resistance as similarly shown by diminished Annexin V binding and caspase-3/7 activity (6). To investigate whether L1CAM-mediated chemoresistance depends on heterophilic ligands, PT45-P1 and PT45-P1res cells were screened by Western blotting for the expression of neuropilin-1 as well as of $\alpha 5$ -, αv -, $\beta 1$ -, $\beta 3$ - and $\beta 5$ -integrins, all being potential ligands of L1CAM. Whereas expression of αv -, $\beta 3$ - and $\beta 5$ -integrin could not be found in either cell line (data not shown), PT45-P1res and PT45-P1 cells showed similar expression levels of $\alpha 5$ -, $\beta 1$ -integrin and of neuropilin-1 (Fig. 1A). Interestingly, in both cell lines only expression of the soluble form of neuropilin-1 (90 kDa) but not of the 130-kDa membrane-bound form (data not shown) could be detected. According to our previous findings, chemoresistant PT45-P1res cells exhibited an elevated expression of L1CAM compared to its chemosensitive counterpart PT45-P1 (Fig. 1A). Fluorescence flow cytometry confirmed cell surface expression of L1CAM only in PT45-P1res cells and $\alpha 5$ - as well as $\beta 1$ -integrin expression in both cell lines (Fig. 1B).

Blocking or knock-down of $\alpha 5$ -integrin increases chemosensitivity in PT45-P1res cells. Since neuropilin-1 has been described to promote chemoresistance in certain PDAC cell lines (28), we analysed whether this potential L1CAM ligand is involved in L1CAM-mediated chemoresistance of PT45-P1res cells. Transfection of these cells with neuropilin-1 specific siRNA clearly reduced neuropilin-1 expression and did not affect that of L1CAM (Fig. 2A), confirming specificity of the neuropilin-1 siRNA. Under these conditions, the apoptotic response towards etoposide treatment (20 μ g/ml for

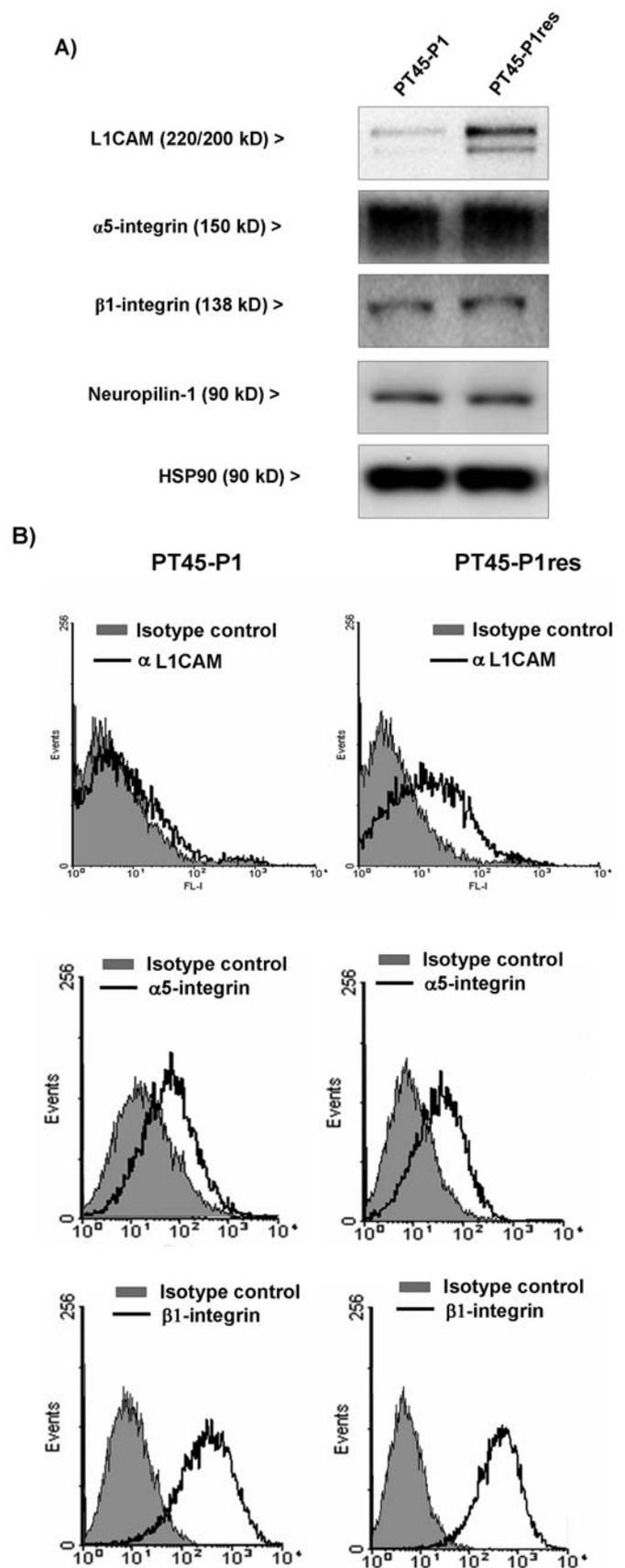


Figure 1. Expression of L1CAM and L1CAM ligands in PT45-P1 and PT45-P1res cells. (A) Cellular lysates were subjected to Western blotting detecting L1CAM, soluble neuropilin-1, $\alpha 5$ - and $\beta 1$ -integrin. HSP90 was detected as control for equal protein load. (B) Representative histograms from fluorescence flow cytometry of surface stainings of L1CAM, $\alpha 5$ - and $\beta 1$ -integrin, each compared with an isotype matched control antibody.

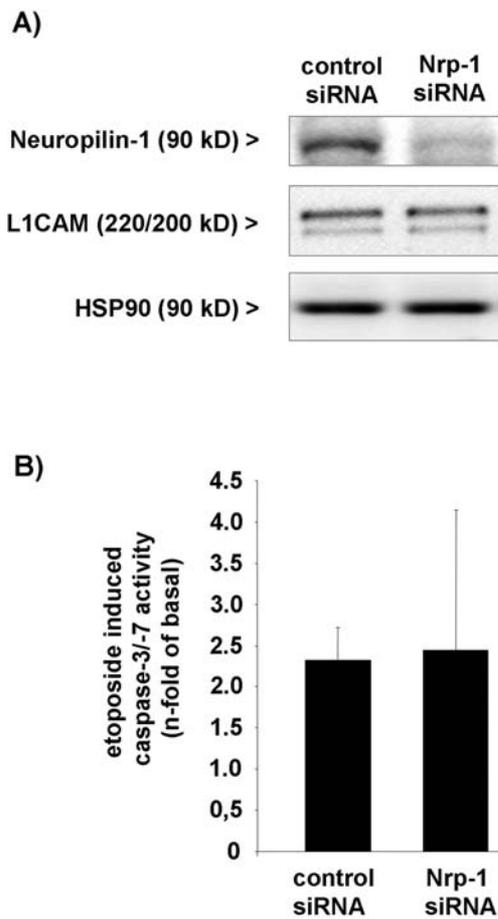


Figure 2. Neuropilin-1 is not involved in chemoresistance in PT45-P1res cells. (A) PT45-P1res cells were transfected with control siRNA or with a neuropilin-1 specific siRNA. After 48 h, Western blotting for the detection of neuropilin-1, L1CAM and of HSP90, used as a control for equal protein load, was performed. (B) In parallel, siRNA transfected PT45-P1res cells were treated with 20 $\mu\text{g/ml}$ etoposide or not for 24 h and caspase-3/-7 activity was determined. Data are expressed as etoposide induced caspase-3/-7 activity (n-fold of basal). Means \pm SD from 6 independent experiments are shown.

24 h) remained unchanged as indicated by a similar activation of caspase-3/-7 compared to control transfected cells (Fig. 2B), thus excluding neuropilin-1 as potential ligand of L1CAM-mediated chemoresistance.

To analyse whether $\alpha 5$ - and $\beta 1$ -integrin are involved in the mediation of chemoresistance, drug-induced apoptosis was measured in PT45-P1res cells subject to treatment with blocking antibodies against the respective integrin. Although $\beta 1$ -integrin blocking antibodies abolished migration of PT45-P1res cells (data not shown), no chemosensitizing effect was observed in these cells (Fig. 3A). In contrast, blocking of $\alpha 5$ -integrin significantly increased chemosensitivity, as indicated by the elevated caspase-3/-7 activity in response to etoposide which rose by >50% compared to PT45-P1res cells treated with an isotype matched control antibody (Fig. 3A). To confirm the involvement of $\alpha 5$ -integrin in the chemoresistant phenotype of PT45-P1res cells, expression of $\alpha 5$ -integrin was reduced by transfection of $\alpha 5$ -integrin siRNA, not affecting expression of L1CAM (Fig. 3B). Under these conditions, etoposide induced

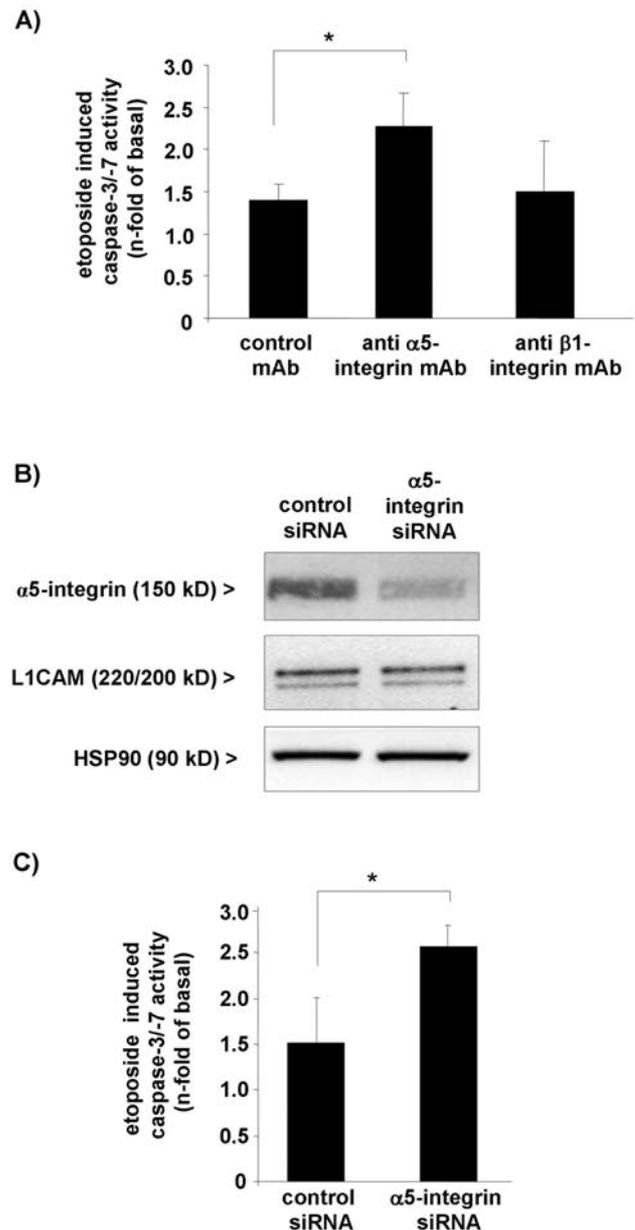


Figure 3. Blocking or knock-down of $\alpha 5$ -integrin increases chemosensitivity in PDAC cell lines. (A) PT45-P1res cells were either left untreated or were treated with 20 $\mu\text{g/ml}$ etoposide in the presence of either an anti- $\alpha 5$ -integrin antibody, an anti- $\beta 1$ -integrin antibody or an isotype matched control antibody (each at 10 $\mu\text{g/ml}$). After 24 h, caspase-3/-7 activity was determined (n=3). (B) PT45-P1res cells were transfected with control siRNA or with $\alpha 5$ -integrin specific siRNA. After 48 h, Western blotting for the detection of $\alpha 5$ -integrin, L1CAM and of HSP90, used as a control for equal protein load, was performed. (C) In parallel, siRNA transfected PT45-P1res cells were treated with 20 $\mu\text{g/ml}$ etoposide or not for 24 h and caspase-3/-7 activity was determined (n=4).

caspase-3/-7 activity was increased in PT45-P1res cells (Fig. 3C) to a similar extent as seen upon treatment with blocking antibodies (Fig. 3A), indicating that the chemoresistant phenotype of these cells is dependent on $\alpha 5$ -integrin. Furthermore, knock-down (Fig. 3D) and blocking of $\alpha 5$ -integrin both similarly increased drug induced caspase-3/-7 activity in the two L1CAM expressing PDAC cell lines Colo357 and Panc1 (Fig. 3E), supporting a general role of $\alpha 5$ -integrin in chemoresistance of PDAC cells.

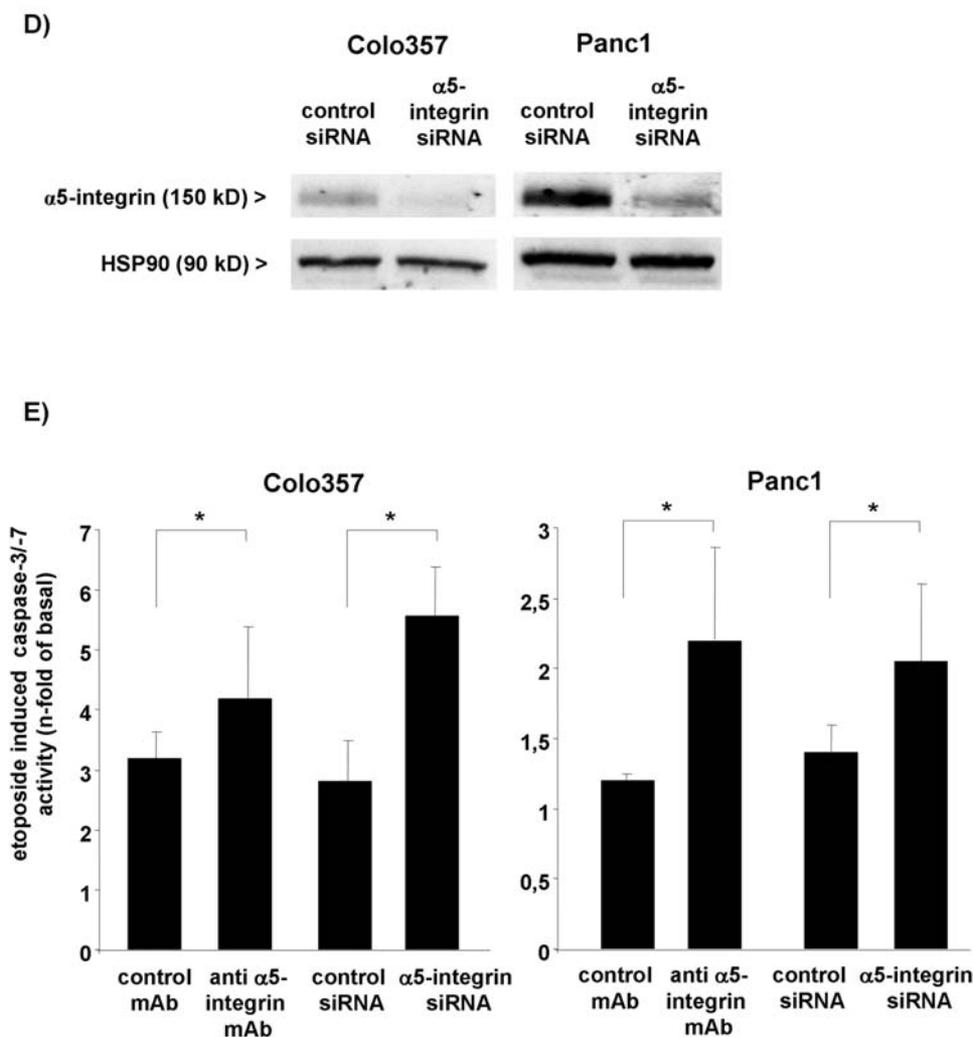


Figure 3 (continued). (D) Colo357 and Panc1 cells were transfected with control siRNA or with $\alpha 5$ -integrin specific siRNA. After 48 h, Western blotting for the detection of $\alpha 5$ -integrin and of HSP90, the latter used as a control for equal protein load, was performed. (E) Colo357 and Panc1 cells were either transfected as described in D or were left untransfected, the latter either treated with an isotype matched control antibody or with an anti- $\alpha 5$ -integrin antibody (each at 10 $\mu\text{g/ml}$). Transfected and antibody treated cells were either left untreated or stimulated with 20 $\mu\text{g/ml}$ etoposide. After 24 h, caspase-3/-7 activity was determined (n=3). Data are expressed as etoposide induced caspase-3/-7 activity (n-fold of basal). Means \pm SD from 3 (A and E) and 4 (C) independent experiments are shown. *p<0.05.

$\alpha 5$ -integrin is involved in the induction of iNOS in LICAM expressing PT45-P1res cells. We previously showed that acquired chemoresistance results from an increased expression of the inducible nitric oxide synthetase (iNOS) and an elevated secretion of NO leading to the inhibition of caspases (20). Since LICAM is essential for iNOS induction, thereby accounting for caspase inhibition, we looked whether $\alpha 5$ -integrin is involved in this mechanism of LICAM mediated chemoresistance. Blocking of $\alpha 5$ -integrin (data not shown) or knock-down of $\alpha 5$ -integrin expression by siRNA transfection clearly diminished the expression of iNOS in PT45-P1res cells as demonstrated by real-time PCR analysis (Fig. 4A) and by Western blot analysis (Fig. 4B). In accordance with these findings, the enhanced drug induced caspase-3/-7 activity that was seen upon knock-down of $\alpha 5$ -integrin could be reversed in these cells by addition of the NO donor SNAP. As shown in Fig. 4C, administration of SNAP almost completely diminished the etoposide-induced caspase-3/-7 activity in $\alpha 5$ -integrin siRNA treated PT45-P1res cells.

$\alpha 5$ -integrin is involved in the inhibition of caspases in LICAM expressing PT45-P1res cells. Given a role of $\alpha 5$ -integrin in the induction of iNOS expression, as a consequence one would expect that this LICAM ligand is also involved in the inhibition of caspases. Thus, we next analysed the effect of $\alpha 5$ -integrin knock-down on the activation of caspases in chemoresistant PT45-P1res cells. Whereas knock-down of $\alpha 5$ -integrin did not affect the expression level of the pro-caspases-3, -7, -8 and -9 upon etoposide treatment (Fig. 5), the cleavage of these caspases was clearly enhanced, thus indicating their increased activation under these conditions (Fig. 5).

LICAM mediated chemoresistance requires an intact integrin binding site. To further support that LICAM-mediated chemoresistance requires $\alpha 5$ -integrin, we used a LICAM variant bearing a mutation within the RGD site in the 6th Ig domain (L1mutRGE) that affects integrin binding. LICAM negative PT45-P1 cells were transfected with either a control

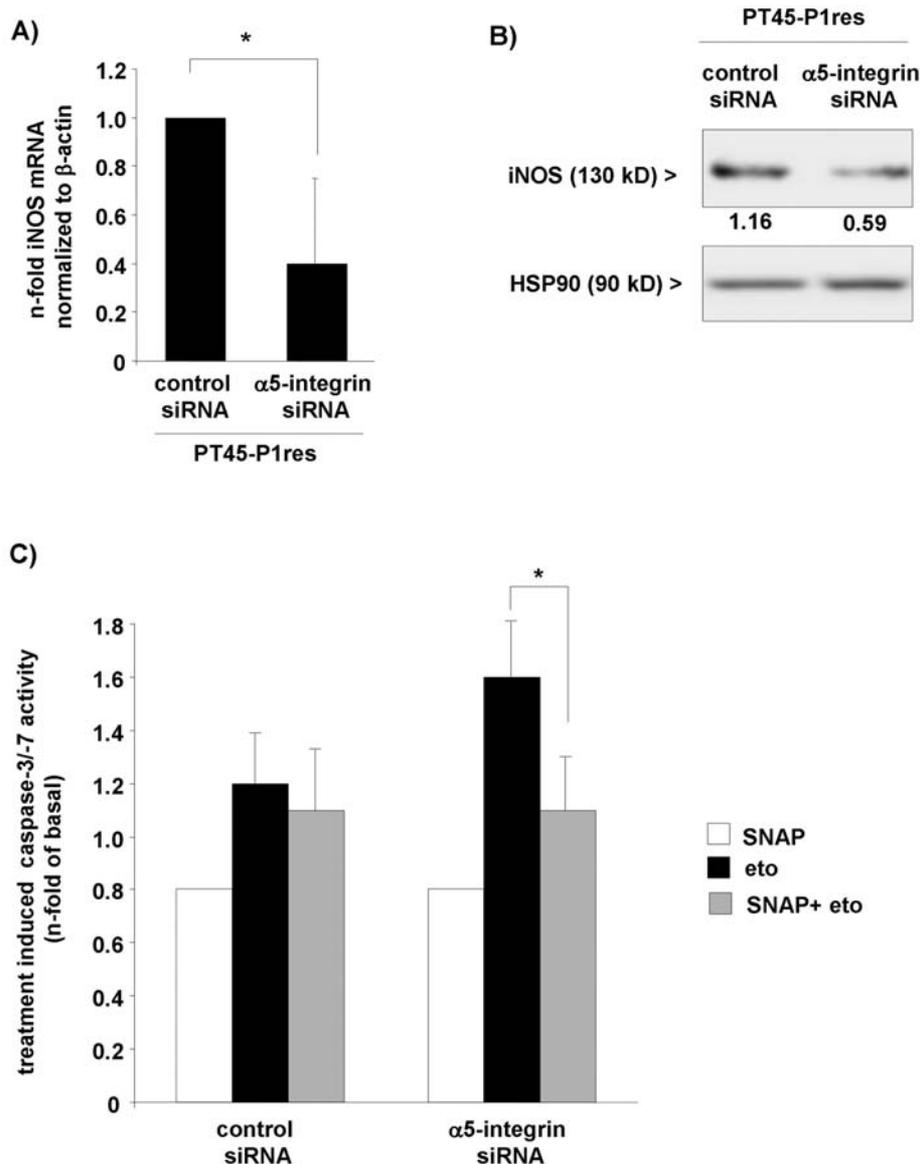


Figure 4. $\alpha 5$ -integrin is involved in the induction of iNOS in L1CAM expressing PT45-P1res cells. PT45-P1res cells were transfected with control or $\alpha 5$ -integrin specific siRNA. (A) RNA from transfected PT45-P1res cells (24 h) was subjected to RT and subsequent real-time PCR using primers specific for iNOS and for β -actin, used for normalization of mRNA content. Normalized mRNA levels of iNOS in PT45-P1 cells, either upon $\alpha 5$ -integrin blocking or upon $\alpha 5$ -integrin knock-down, were related to normalized mRNA levels in respective control treated cells. (B) Cellular lysates of siRNA transfected PT45-P1res cells were subjected to Western blotting detecting iNOS and HSP90, used as control for equal protein load. Numbers displayed below the images represent the band intensity level obtained from densitometric analysis and subsequent normalization to HSP90. (C) siRNA transfected PT45-P1res cells were left untreated or were treated with 200 μ mol/l SNAP, 20 μ g/ml etoposide or with a combination of both. After 24 h, caspase-3/-7 activity was determined. Data are expressed as treatment induced caspase-3/-7 activity (n-fold of basal). Means \pm SD from three independent experiments are shown. * $p < 0.05$.

plasmid (pcDNA3.1), wild-type (L1wt) or mutant L1CAM (L1mutRGE). After transfection, comparable amounts of both L1CAM proteins could be detected in these cells by Western blot analysis (Fig. 6A) as well as by L1CAM staining and subsequent flow cytometry analysis (Fig. 6B). Transfection of chemosensitive PT45-P1 cells with the wild-type form of L1CAM significantly decreased caspase-3/-7 activation upon etoposide treatment by 50%, whereas expression of mutant L1CAM hardly affected the drug induced caspase-3/-7 activation in these cells (Fig. 6C).

In line with these findings, antibody blockage of $\alpha 5$ -integrin only reversed the chemoresistant phenotype of PT45-P1 cells

gained after transfection with wild-type L1CAM, whereas chemosensitivity of L1mutRGE expressing cells was not altered. As shown in Fig. 6D, etoposide induced caspase-3/-7 activity was completely abolished in L1wt expressing PT45-P1 cells in the presence of $\alpha 5$ -integrin blocking antibodies, supporting the hypothesis that $\alpha 5$ -integrin is crucial for L1CAM-mediated chemoresistance in PDAC cells.

Expression of iNOS is elevated in PT45-P1 cells expressing the wild-type but not the RGE mutant form of L1CAM. Next, PT45-P1 cells were transfected with either L1CAM construct in the absence or presence of $\alpha 5$ -integrin blocking antibodies and

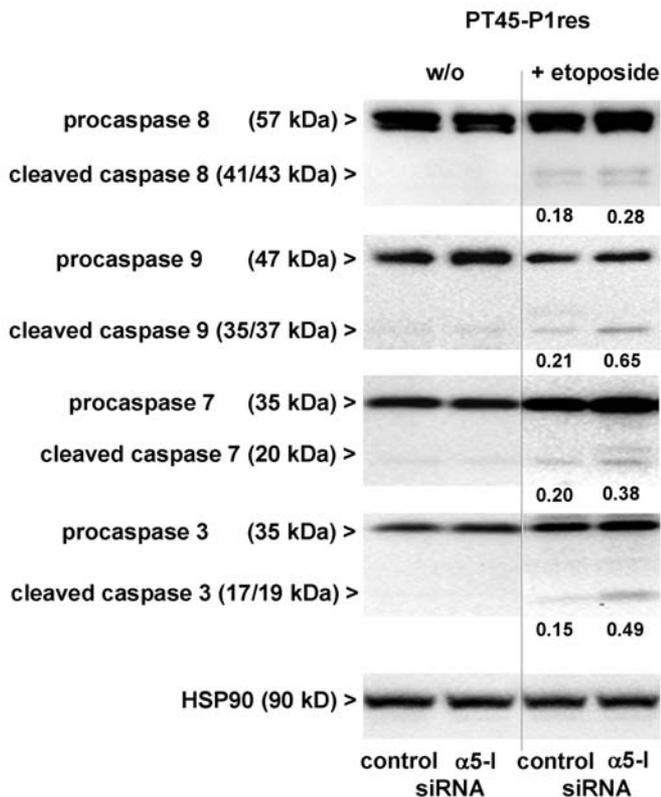


Figure 5. $\alpha 5$ -integrin is involved in the inhibition of caspases in L1CAM expressing PT45-P1res cells. PT45-P1res cells were either transfected with control siRNA or with $\alpha 5$ -integrin specific siRNA ($\alpha 5$ -I siRNA) followed by treatment with 20 $\mu\text{g}/\text{ml}$ etoposide for 48 h or not. Cellular lysates were submitted to Western blotting using antibodies for the detection of full length (procaspase) and cleaved caspase-3, -7, -8 and -9. A HSP90 antibody was used as a control for equal protein load. Numbers displayed below the images represent the band intensity level obtained from densitometric analysis and subsequent normalization to HSP90.

were analysed for iNOS induction. In line with the findings in PT45-P1res cells, transfection of PT45-P1 cells with L1wt significantly increased expression of iNOS as demonstrated by real-time PCR (Fig. 7A) and Western blot analysis (Fig. 7C). In contrast, this effect was not observed after transfection with L1mutRGE (Fig. 7A and C) indicating that L1CAM requires a functional integrin binding site to induce iNOS expression. Furthermore, blocking of $\alpha 5$ -integrin clearly abrogated the increased iNOS expression level in PT45-P1 cells transfected with L1wt (Fig. 7B and D), whereas iNOS expression in L1mutRGE expressing PT45-P1 cells remained unaffected. These data clearly point to a combined role of L1CAM and $\alpha 5$ -integrin in the induction of iNOS in PT45-P1 cells.

Discussion

Enhanced L1CAM expression could be detected in PDAC accounting for acquired (6) as well as for innate (unpublished data) chemoresistance in PDAC cells. Regarding the role of L1CAM in carcinogenesis, so far only the impact of L1CAM on tumor cell migration and metastasis has been described in some detail (2,15,16,29). Besides L1CAM itself, both in its membrane-bound and soluble form, other adhesion

molecules known to be involved in tumor cell migration have been identified as L1CAM ligands, e.g., the integrins $\alpha 5\beta 1$, $\alpha v\beta 1$ and $\alpha v\beta 3$ (30,31). Thus, binding to and signaling through integrins might be one mechanism by which L1CAM promotes tumor cell migration (16), the upregulation of integrin expression, as demonstrated for $\beta 3$ -integrin, might be another (18). However, little is known about the exact mechanisms how L1CAM mediates cell migration, in particular which ligands and cellular signals are involved. This even more applies for the mechanisms underlying L1CAM-mediated chemoresistance. Several studies have already shown that integrin dependent adhesion to extracellular matrix proteins or to adjacent stroma cells confers apoptosis protection towards cytostatic drugs, death ligands or ionizing radiation in various tumor cells (32-34). Since integrins as well as neuropilin-1 are expressed in PDAC (28,35) and also represent potential ligands of L1CAM, it is tempting to speculate that one of these ligands is involved in L1CAM-mediated chemoresistance of PDAC cells. We have already shown that PT45-P1res cells acquiring a chemoresistant phenotype upon long-term drug treatment are characterized by an elevated expression of L1CAM compared to the chemosensitive parental cell line PT45-P1 (6). Moreover, L1CAM expression in PT45-P1res cells accounts for increased expression of iNOS and elevated NO levels resulting in caspase inhibition (20). Since it has been shown that proteolytic cleavage of L1CAM is dispensable for chemoresistance in PDAC as well as in ovarian carcinoma cells (6,19), soluble L1CAM can be excluded as potential ligand in this context. Instead, homophilic interaction of membrane-bound L1CAM with other membrane-bound L1CAM molecules, either on the same cell or on adjacent cells, might be crucial for mediation of chemoresistance. This also applies for heterophilic L1CAM ligands, including $\alpha 5$ - and $\beta 1$ -integrin as well as the soluble form of neuropilin-1 that have been detected in PT45-P1res and PT45-P1 cells. It can be assumed that L1CAM expressing tumor cells interact with these ligands on adjacent tumor or stromal cells (e.g., myofibroblasts). Contact to neighbouring cells could favour a bidirectional signaling via L1CAM and its binding to the respective ligand because ligand and receptor are often expressed by the same cell. So far, it is not known whether the anti-apoptotic signaling originates from L1CAM or from its ligand.

Wey *et al* demonstrated that transfection of PDAC cells with neuropilin-1 conferred drug resistance and, in turn, knock-down of neuropilin-1 increased chemosensitivity of PDAC cells (28), pointing to a role for neuropilin-1 in chemoresistance of this tumor entity. However, knock-down of neuropilin-1 in PT45-P1res cells did not affect drug induced apoptosis, indicating that neuropilin-1 is apparently not involved in the mediation of chemoresistance in this cell line. One explanation might be that soluble neuropilin-1, as detected in PT45-P1res cells, does not confer chemoresistance, at all. In contrast, blocking and knock-down of $\alpha 5$ -integrin clearly increased the chemosensitivity not only of PT45-P1res cells but also of the PDAC cell lines Colo357 and Panc1 pointing to an essential role of this integrin in the mediation of chemoresistance in L1CAM expressing PDAC cells. Moreover, the abrogation of $\alpha 5$ -integrin expression was associated with a decreased expression of iNOS and

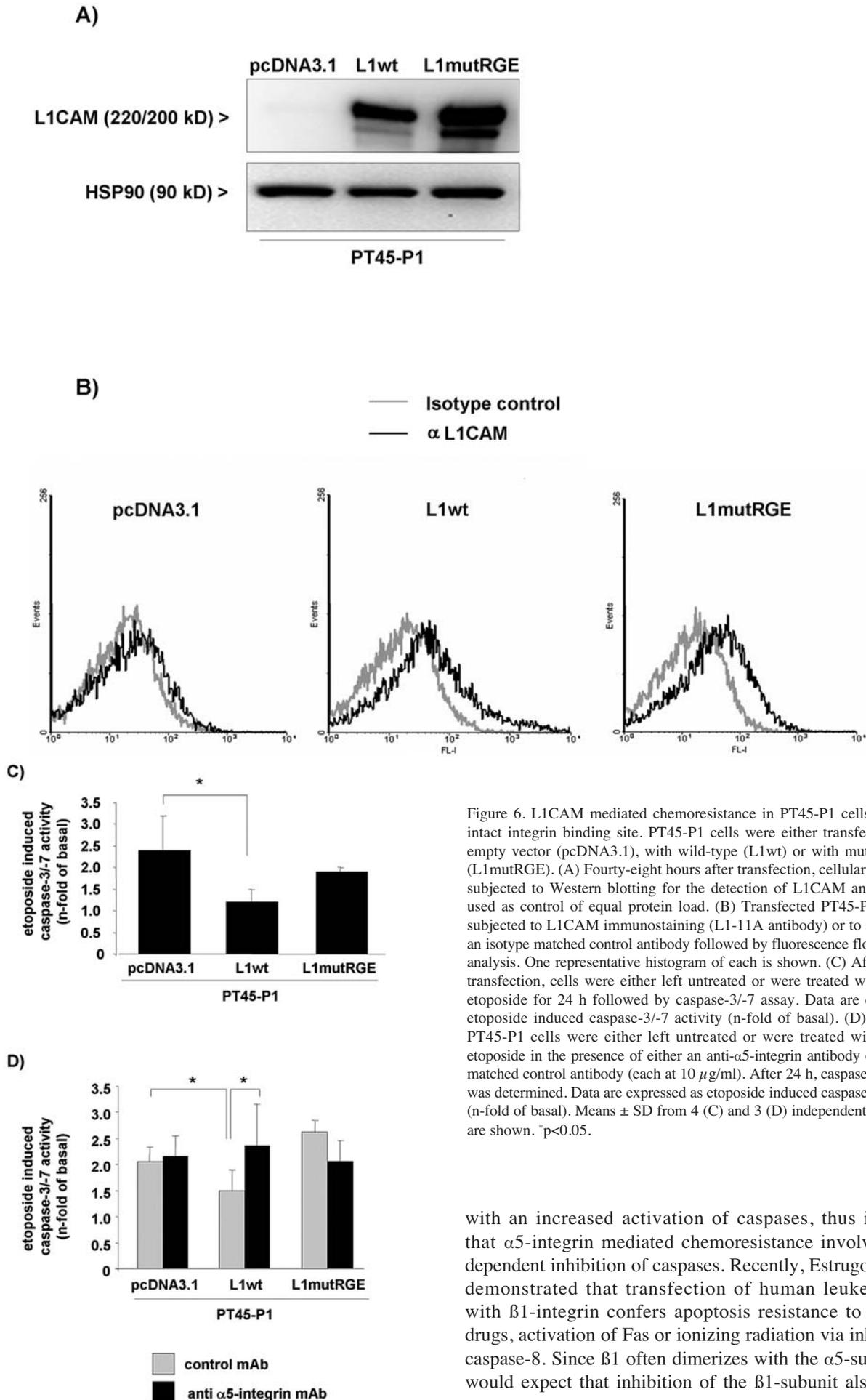


Figure 6. L1CAM mediated chemoresistance in PT45-P1 cells requires an intact integrin binding site. PT45-P1 cells were either transfected with an empty vector (pcDNA3.1), with wild-type (L1wt) or with mutant L1CAM (L1mutRGE). (A) Forty-eight hours after transfection, cellular lysates were subjected to Western blotting for the detection of L1CAM and of HSP90, used as control of equal protein load. (B) Transfected PT45-P1 cells were subjected to L1CAM immunostaining (L1-11A antibody) or to staining with an isotype matched control antibody followed by fluorescence flow cytometry analysis. One representative histogram of each is shown. (C) After overnight transfection, cells were either left untreated or were treated with 20 μ g/ml etoposide for 24 h followed by caspase-3/-7 assay. Data are expressed as etoposide induced caspase-3/-7 activity (n-fold of basal). (D) Transfected PT45-P1 cells were either left untreated or were treated with 20 μ g/ml etoposide in the presence of either an anti- α 5-integrin antibody or an isotype matched control antibody (each at 10 μ g/ml). After 24 h, caspase-3/-7 activity was determined. Data are expressed as etoposide induced caspase-3/-7 activity (n-fold of basal). Means \pm SD from 4 (C) and 3 (D) independent experiments are shown. * p <0.05.

with an increased activation of caspases, thus indicating that α 5-integrin mediated chemoresistance involves a NO-dependent inhibition of caspases. Recently, Estrugo *et al* (32) demonstrated that transfection of human leukemia cells with β 1-integrin confers apoptosis resistance to cytostatic drugs, activation of Fas or ionizing radiation via inhibition of caspase-8. Since β 1 often dimerizes with the α 5-subunit, one would expect that inhibition of the β 1-subunit also leads to

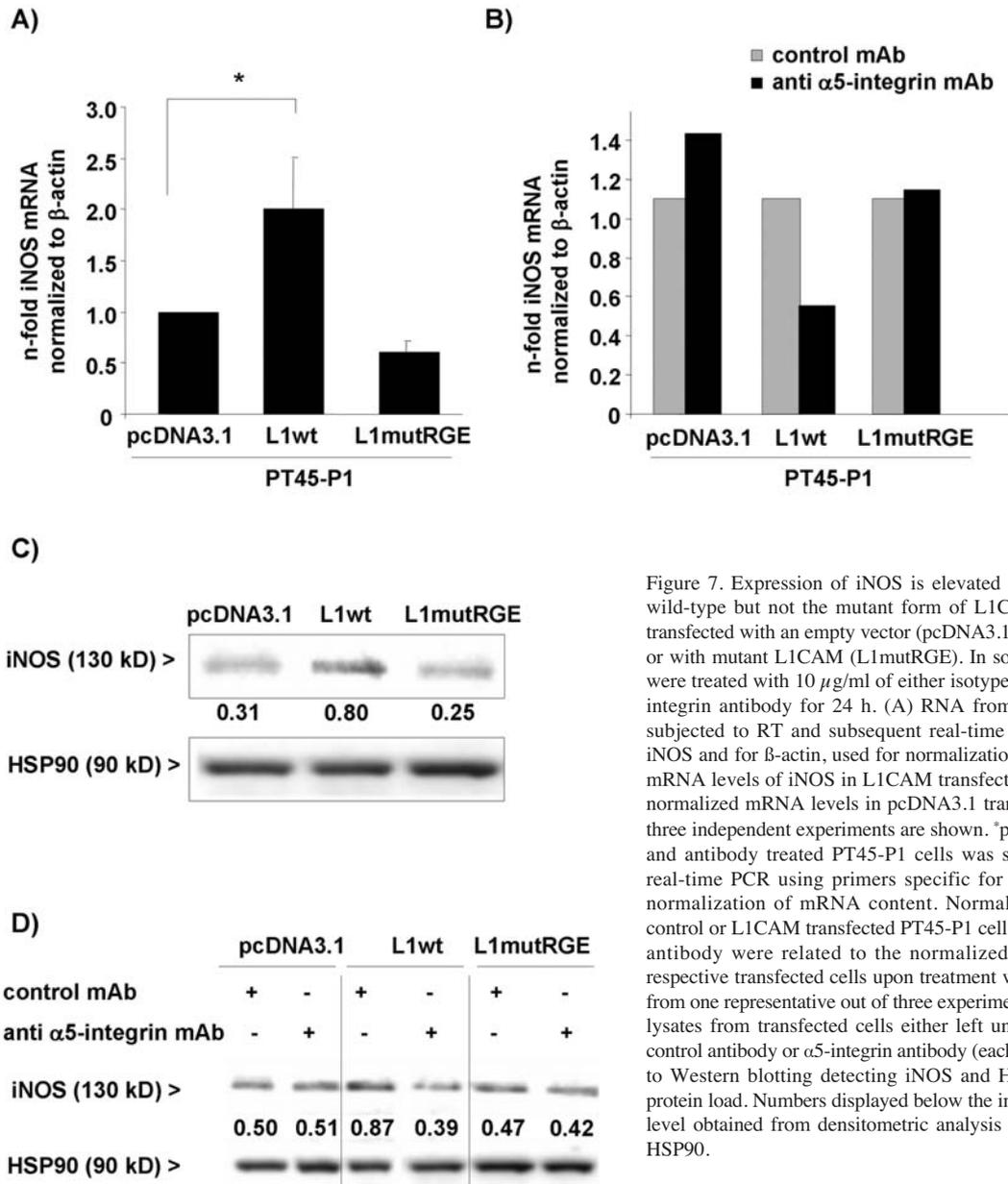


Figure 7. Expression of iNOS is elevated in PT45-P1 cells expressing the wild-type but not the mutant form of L1CAM. PT45-P1 cells were either transfected with an empty vector (pcDNA3.1), with wild-type L1CAM (L1wt) or with mutant L1CAM (L1mutRGE). In some experiments (B and D), cells were treated with 10 μ g/ml of either isotype matched control antibody or α 5-integrin antibody for 24 h. (A) RNA from transfected PT45-P1 cells was subjected to RT and subsequent real-time PCR using primers specific for iNOS and for β -actin, used for normalization of mRNA content. Normalized mRNA levels of iNOS in L1CAM transfected PT45-P1 cells were related to normalized mRNA levels in pcDNA3.1 transfected cells. Means \pm SD from three independent experiments are shown. * p <0.05. (B) RNA from transfected and antibody treated PT45-P1 cells was subjected to RT and subsequent real-time PCR using primers specific for iNOS and for β -actin, used for normalization of mRNA content. Normalized mRNA levels of iNOS in control or L1CAM transfected PT45-P1 cells upon treatment with α 5-integrin antibody were related to the normalized mRNA levels of iNOS in the respective transfected cells upon treatment with the control antibody. Results from one representative out of three experiments are shown. (C and D) Cellular lysates from transfected cells either left untreated (C) or exposed to either control antibody or α 5-integrin antibody (each at 10 μ g/ml) (D), were subjected to Western blotting detecting iNOS and HSP90 used as control for equal protein load. Numbers displayed below the images represent the band intensity level obtained from densitometric analysis and subsequent normalization to HSP90.

an abrogation of the chemoresistant phenotype of PT45-P1res cells. Interestingly, we observed an increasing effect by inhibition of α 5-integrin but not of β 1-integrin on caspase activation and chemosensitivity in PT45-P1res cells. This might be explained by the fact that β 1 can dimerize with most of the other α -subunits as well (36,37), predominating over α 5 β 1 integrin formation as described e.g., for α 6 β 1 expression in PDAC (37-39). To support the involvement of α 5-integrin particularly in L1CAM-mediated chemoresistance, we made use of a L1CAM variant (L1mutRGE) bearing a mutated integrin binding site in the 6th Ig domain. Transfection of chemosensitive PT45-P1 cells with wild-type L1CAM (L1wt) increased expression of iNOS along with a reduced caspase activation upon etoposide treatment. In contrast, chemosensitivity as well as iNOS expression remained unchanged in L1mutRGE expressing PT45-P1 cells. These

data already point to the necessity of a functional integrin binding site in the L1CAM molecule. Moreover, it is important to note that blocking of α 5-integrin reduced expression of iNOS and abolished the chemoresistant phenotype only in L1wt expressing PT45-P1 cells but not in PT45-P1 cells expressing either no L1CAM at all (parental or mock) or L1CAM with a non-functional integrin binding site (L1mutRGE). Thus, L1CAM fails to induce iNOS mediated chemoresistance in PT45-P1 cells if lacking this integrin binding site as well as if α 5-integrin is blocked or α 5-integrin expression is silenced. In conclusion, this study deepens the understanding of L1CAM-mediated chemoresistance in PDAC and provides evidence that α 5-integrin is of pivotal importance in this process. Whether the activated signaling cascade leading to iNOS induction and drug resistance originates from L1CAM or from α 5-integrin is a matter of ongoing studies.

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