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The novel synthetic ether lipid inositol-C2-PAF inhibits phosphorylation of the tyrosine kinases Src and FAK independent of integrin activation in transformed skin cells

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ABSTRACT

New alkyl-phospholipids that are structurally derived from platelet-activating factor are promising candidates for anticancer treatment. The mechanism of action of derivatives of the platelet-activating factor is distinctly different from that of known DNA- or tubulin-targeting anticancer agents because they are incorporated into cell membranes, where they accumulate and interfere with a wide variety of key enzymes. We recently presented evidence of a novel group of alkyl-phospholipids, glycosidated phospholipids that efficiently inhibit cell proliferation. One member of this group, inositol-C2-PAF (Ino-C2-PAF), displays high efficacy and low cytotoxicity in HaCaT-cells, an immortalized non-tumorigenic skin keratinocyte cell line.

Here, we show that Ino-C2-PAF also inhibits the motility of the skin-derived transformed cell lines HaCaT and squamous cell carcinoma (SCC)-25. This decrease in motility is accompanied by an altered F-actin cytoskeleton, increased clustering of integrins, and increased cell–matrix adhesion. Despite enhanced integrin clustering and matrix adhesion, we observed less phosphorylation of the cytoplasmic tyrosine kinases focal adhesion kinase (FAK) and Src, key regulators of cellular motility, at focal adhesion sites. Transient transfection of constitutively active variants of FAK and Src could at least in part bybass this inhibitory effect of Ino-C2-PAF. This fact indicates that Ino-C2-PAF interferes with the fine-tuned balance between adhesion and migration. Ino-C2-PAF at least partially uncouples integrin-mediated attachment from subsequent integrin-dependent signaling steps, which inhibits migration in transformed keratinocyte cell lines.

1. Introduction

1.1. Biological effects of alkyl-phospholipids

Migration of malignant cells or metastasis is one of the hallmarks of tumor progression. Therefore, efforts have been made to develop anticancer drugs that inhibit both the expansion of tumor cells as well as metastasis.

Alkyl-phospholipids (APLs), compounds with aliphatic side chains that are ether-linked to a glycerol backbone, are structurally derived from platelet-activating factor (PAF) and are a new class of drugs with anti-proliferative properties in tumor cells [1,2]. In contrast to the commonly used cisplatin or vincristine, APLs do not interfere with DNA or the mitotic spindle apparatus of the cell; instead, they are incorporated into cell membranes and exhibit less unwanted toxicity [3]. Recent work has shown that these compounds induce selective apoptosis in malignant cells, but do not affect normal cells [4–6]. At higher concentrations, APLs induce cell lysis due to their detergent-like structure [7].

The prototype of anti-proliferative APLs is *rac*-1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (Et-18-OCH3; edelfosine). This compound affects various processes and signaling pathways, leading to cell cycle arrest and apoptosis, which may explain the anti-proliferative properties of APLs [5,8,9].

Hexadecylphosphocholine (HePC, Miltefosine®), an APL without the glycerol backbone, was the first to be clinically tested for the topical treatment of skin metastases stemming from mammary carcinomas [10,11].

1.2. Glycosidated phospholipids

Recently, we presented evidence of glycosidated phospholipids, a novel group of APLs that efficiently inhibit the proliferation of

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premalignant keratinocyte HaCaT cells [12–14]. Among the glycosidated phospholipids, 1–*O*-octadecyl-2-*O*-(2-(*myo*-inositolyl)-ethyl)-*sn*-glycero-3-(r/s)-phosphatidylcholine (Ino-C2-PAF) shows very high efficacy and low cytotoxicity. This compound has previously been shown to increase the expression and activity of terminal differentiation markers, such as involucrin and transglutaminase, in HaCaT cells [15].

1.3. The role of the non-receptor tyrosine kinases FAK and Src in integrin-dependent signaling

Integrins are transmembrane receptors that mediate cell attachment to the extracellular matrix (ECM) as well as cell migration [16]. They are heterodimeric proteins comprised two subunits, α and β, which bind to ECM components. Each subunit contains a large extracellular domain, a single transmembrane segment, and a short cytoplasmic tail of 15–70 amino acids. On the cell surface, integrins exist in several activation states. Activation induces integrin clustering, leading to the recruitment of multiple signaling molecules, the formation of actin filaments [17], and thus the regulation of various signal transduction cascades. Because integrins lack intrinsic enzymatic activity, their signaling function is dependent on non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and Src. FAK is activated upon integrin engagement and physically interacts with the β_1 cytoplasmic tail as well as various signaling molecules at focal adhesions [18–20]. Once activated, FAK forms a dual kinase complex with Src, which can subsequently phosphorylate various adaptor proteins such as p130Cas and paxillin [21].

In the present study, we show that Ino-C2-PAF increases attachment to ECM components and reduces the motility of skin-derived transformed cells, such as HaCaT and SCC-25 cells. Furthermore, the organization of the F-actin cytoskeleton is shifted toward more pronounced cortical actin rather than actin stress fibers. While β_1 integrin-containing focal complexes are enlarged in Ino-C2-PAF-treated cells, subsequent integrin-mediated signaling events such as phosphorylation of FAK and Src, which supports migration, are inhibited. Transient transfection of constitutively active variants of FAK and Src could at least in part bybass this inhibitory effect of Ino-C2-PAF.

2. Materials and methods

2.1. Cell culture

SCC (squamous cell carcinoma)-25 cells were grown in Ham's F12/DMEM medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), L-glutamine (440 mg/l), hydrocortisone 0.4 μg/ml), and heat-inactivated fetal bovine serum (10%). HaCaT cells were grown in RPMI medium supplemented with heat-inactivated fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (440 mg/l). Cells were disaggregated with trypsin and 0.02 mM EDTA. Two days prior to experimentation, cells were adapted to defined keratinocyte serum-free medium with growth supplements (pituitary extract including insulin, epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Gibco/BRL). Experiments were performed in defined keratinocyte serum-free medium.

2.2. Antibodies and reagents

The monoclonal FITC-conjugated anti-β_1 integrin antibody was obtained from Immunotech (Hamburg, Germany). Antibodies raised against the integrin subunits α_3 and β_4 were purchased from Chemicon (Canada). The anti-α_6 integrin antibody as well as the secondary FITC-conjugated goat anti-mouse antibody was obtained from BD Bioscience (Heidelberg, Germany). The secondary peroxidase-conjugated rat anti-mouse immunoglobulin G (IgG) was purchased from Dianova (Hamburg, Germany). Anti-pFAK (Y397) and anti-pSrc (Y418) polyclonal phosphorylation site-specific antibodies were obtained from Invitrogen (Karlsruhe, Germany). The monoclonal anti-α-tubulin antibody was purchased from Abcam (Cambridge, UK).

Collagen IV (human placenta), laminin-111 (from Engelbreth-Holm-Swarm mouse sarcoma), fibronectin (from human plasma), and phalloidin (TRITC- and CPITC-conjugated) were purchased from Sigma–Aldrich (Munich, Germany). cDNA constructs used in this study were kindly provided by other groups: GFP-FAK by Jun-Lin Guan (University of Michigan Medical School, Ann Arbor, USA), CD2-FAK by Shuang Huang (Medical College of Georgia, Augusta, USA) and Src-Y527F-GFP by Margaret Frame, (Edinburgh Cancer Research Centre, Edinburgh, Scotland).

2.3. Cell attachment assay

For attachment assays, HaCaT cells were incubated for 48 h with or without Ino-C2-PAF. At the same time, 96-well plates were coated with the extracellular matrix components as indicated (20 μg/ml PBS; Sigma, Munich, Germany) and incubated for 16 h at 4 °C. Non-specific binding was blocked with 1% BSA for 4 h at 4 °C. A total of 5×10^4 HaCaT cells in 100 μl medium per well were plated onto the indicated matrix proteins. After 2 h of incubation at 37 °C, non-attached cells were removed by washing with PBS. Attached cells were fixed with 1% glutardialdehyde in PBS and stained with 0.1% crystal violet. Plates were photometrically measured at 570 nm after Triton X-100 dye solubilization.

2.4. Immunofluorescence studies

HaCaT and SCC-25 cells were incubated with or without Ino-C2-PAF for the indicated times. A total of 5.5×10^4 cells were seeded onto 8-well Permanox® slides (Nunc, Wiesbaden, Germany) coated with collagen IV. After a 2-h attachment period, cells were washed with PBS, fixed, and permeabilised with 3.7% paraformaldehyde/0.025% saponin in PBS for 10 min at room temperature. After blocking for 10 min with 1% BSA in PBS, cells were incubated with either anti-β_1 integrin FITC-conjugated antibody (40 μg/ml; Immunotech, Hamburg, Germany) or with TRITC-conjugated phalloidin (1:500) at 4 °C over night. To detect phosphorylated FAK and Src, cells were blocked with a solution of 1% BSA in TBS, incubated first with the respective primary antibody diluted in TBS at 4 °C overnight and then with FITC-conjugated goat anti-rabbit IgGs for 2 h at room temperature. Slides were analyzed on a Zeiss Axiovert 200 microscope with an Axiocam and Axiovision software (AxioVs40 V; Zeiss, Jena, Germany). Images were further processed using Adobe Photoshop (version 8.0.1). To compare images image acquisition was carried out on identical conditions. Digital images of the cells were taken at the times indicated using a 63× objective.

2.5. Flow cytometry

HaCaT and SCC-25 cells were incubated with Ino-C2-PAF for 48 h or left untreated. Cells were detached with accutase. For flow cytometry, 5×10^5 cells were stained with integrin antibodies as indicated for 45 min on ice. Cells were then washed twice with PBS and incubated for another 45 min with the respective secondary antibody. Cells were rinsed twice, and surface integrin expression was measured using a FACScan (Beckton Dickinson, Heidelberg, Germany). For the controls, cells were incubated with the respective secondary antibody only. To assess the level of active of β1 integrins on the cell surface the 12G10 antibody was used (AbD Serotec, Düsseldorf, Germany).

2.6. Western blotting

For Western blotting, cells were lysed in Triton X-100 buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.8, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF, aprotinin, leupeptin, pepstatin A, each 10 μg/ml) and were centrifuged for 10 min at 4 °C at 6000 × g. Supernatants were denatured by boiling with Laemmli's sample buffer. Samples were separated by 7.5% SDS–PAGE under reducing conditions. Western blotting and subsequent protein detection on nitrocellulose by the HRP/ECL method (Amersham, Freiburg, Germany) was performed as previously described [19]. Signals were visualized using a Digital Imaging System (LAS-100) by Fuji (Raytest, Straubenhardt, Germany).

2.7. Cell migration assay

Haptotactic transwell migration assays were performed using transwell plates (Costar, Corning, NY, USA) with a pore size of 8 μm, as described previously [22]. The underside of the filter was coated with collagen IV (20 μg/ml) for 30 min at room temperature followed by blocking of non-specific binding sites with 1% BSA for 30 min at room temperature. A total of 600 μl medium was added per well. The upper compartment was filled with 100 μl of a cell suspension containing 5.5×10^4 cells. Cells were then allowed to migrate for 6 h at 37 °C. For migration experiments with transfected cells migration time was elongated to 16 h. Afterwards, cells were removed from the upper surface with cotton swabs, and the filters were washed in PBS. Migrated cells were fixed in 4% paraformaldehyde in PBS containing 0.025% saponin for 30 min, stained with 0.1% crystal violet, and counted using a 20× objective and a 10 × 10 grid. Multiple fields were counted and averaged for each condition.

2.8. Transfection of cells

GFP-FAK, Src-WT-GFP, CD2-FAK, EGFP were transfected into cells using the Magnetofection™ transfection reagent PolyMag (OZ Bioscience, Marseille, France). 24 h before transfection, 6×10^5 cells were plated on a 35 mm dish in complete culture medium. 2 μg DNA were diluted in 200 μl culture medium free of antibiotics, and subsequently mixed with 2 μl of PolyMag. After 20 min of incubation, the complex was added to the cells and the dish was placed upon the magnetic plate for 20 min. The medium was changed 2 h after transfection and cells were incubated for further 24 h at 37 °C. Subsequently, cells were used for migration assays. Transfection efficiency was between 50% and 80% depending on the respective cDNA construct.

2.9. Wound healing assay

A total of 2×10^5 cells were seeded onto a collagen IV-coated 8-well chamber and incubated with defined keratinocyte medium supplemented with bovine pituitary extract (Gibco/BRL) over night at 37 °C. After washing, a scratch was induced using a pipette tip, and the wounded monolayer was carefully washed again. Cells were then incubated in serum-free medium with Ino-C2-PAF or left untreated for the indicated times. Due to the different velocities of the two cell lines, digital images of the wounds were taken after 1 and 6 h for SCC-25 cells and after 3 and 24 h for HaCaT cells. The F-actin cytoskeletons of cells at the wound edge were analyzed using TRITC-phalloidin. For detailed phosphorylation analysis of cells at the wound edge, cells were incubated with phosphorylation site-specific antibodies and the respective secondary antibodies. Digital images of the cells were taken after 6 h using a 63× objective.

2.10. Proliferation assay

A colorimetric immunoassay (cell proliferation ELISA with bromodeoxyuridine (BrdU); Roche) was used to detect replicating cells. Cells were seeded in a 96-well microtiter plate at 1.5×10^4 cells per well and incubated with Ino-C2-PAF. BrdU labeling solution (100 mM) was then added to give a final concentration of BrdU of 10 mM per well. Further steps were performed according to the manufacturer's instructions. The colorimetric reaction was determined photometrically at 405 nm and subsequently quantified. The untreated cells were set at 100%.

2.11. Determination of substance-induced cytotoxicity

The cytotoxic effect of Ino-C2-PAF was determined using the cytotoxicity detection kit (LDH) from Roche. In brief, HaCaT cells (1.5×10^4 cells per well) were incubated with Ino-C2-PAF or left untreated. All steps were conducted according to the manufacturer's instructions. The colorimetric reaction was measured photometrically at 492 nm and subsequently quantified. The untreated cells served as a negative control (0% cytotoxicity), whereas cells lyzed with Triton X-100 (0.5%) served as a positive control and were set at 100% cytotoxicity.

2.12. Sample analysis and statistical analysis

In each case three independent experiments were performed and analyzed (unless stated otherwise). For migration, attachment and proliferation assays sample count was at least $n = 4$. In wound healing assays at least four different sections of each wound were analyzed. In flow cytometry analysis each sample was performed in duplicate. The arithmetic mean and standard deviation of the mean were calculated.

The Shapiro–Wilk test was used to check for Gaussian distribution, followed by the Student's *t*-test. Significance was set at $p = 0.05$. Significant differences are marked with an asterisk.

3. Results

3.1. Ino-C2-PAF reduced migration of SCC-25 and HaCaT cells

Migration of epithelial cells is an important step in metastasis. *In vitro* cell motility can be studied using haptotactic migration assays and a collagen IV gradient. To assess whether the motility of SCC-25 and HaCaT cells is influenced by non-toxic concentrations of Ino-C2-PAF, cells were treated with 5 μM or without Ino-C2-PAF for 48 h and detached from the plate. 5.5×10^4 cells were allowed to migrate toward a collagen gradient for 6 h at 37 °C (Fig. 1A). Ino-C2-PAF significantly reduced migration by 50% in both cell lines. To be sure that the reduction of migrated cells was not due to an effect on proliferation we performed a proliferation assay in parallel with SCC-25 cells (Fig. 1B). We observed no significant effect on proliferation after 6 h. This has also been shown for HaCaT cells in an earlier study [15]. Also viability is unaffected under these conditions (Fig. 1C).

3.2. Ino-C2-PAF induced the redistribution of the F-actin cytoskeleton

Cell movement is driven by reorganization of the F-actin cytoskeleton. Therefore, we induced motility in confluent monolayers of HaCaT and SCC-25 cells by mechanical scratch wounding and analyzed the cytoskeletons of motile cells at the wound edge with TRITC-conjugated phalloidin in the presence or absence of Ino-C2-PAF (Fig. 2). Due to different migration velocities, SCC-25 cells were analyzed after 1 and 6 h, and HaCaT cells were analyzed after 3 and 24 h. Black and white images were imported into Adobe

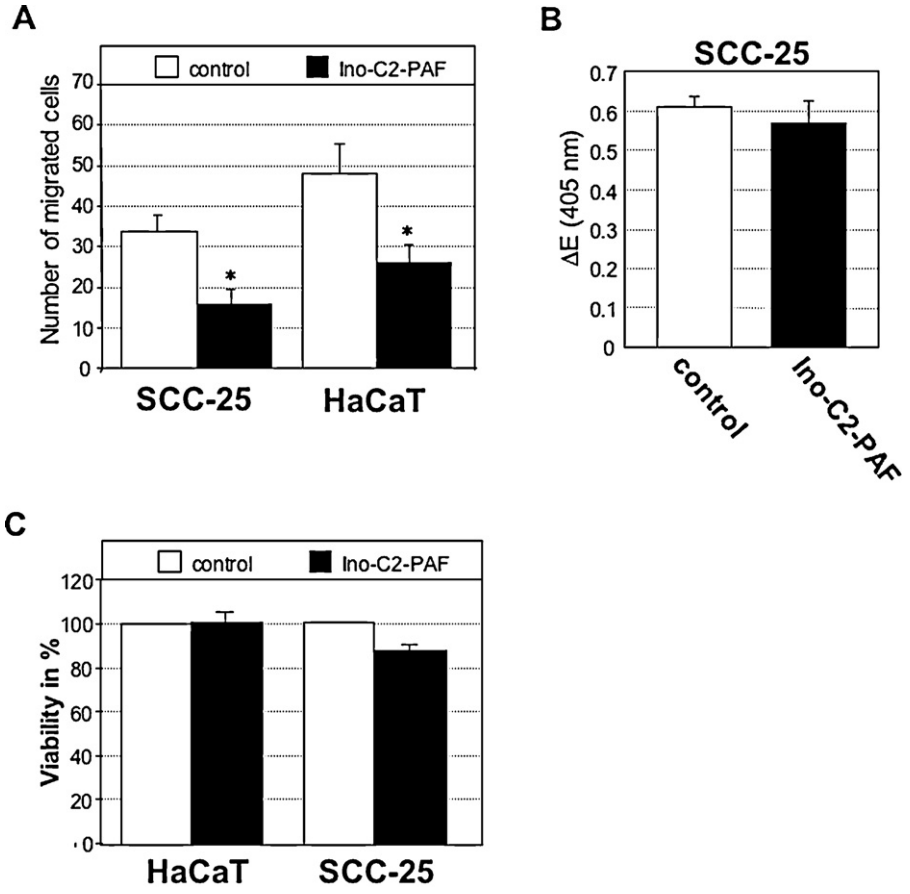

Fig. 1. Ino-C2-PAF inhibits migration of SCC-25 and HaCaT cells. (A) SCC-25 or HaCaT cells (5.5×10^4) were incubated in serum-free medium in the presence or absence of 5 μM Ino-C2-PAF for 48 h. Cells were allowed to migrate toward a collagen IV gradient for 6 h in haptotactic transwell chamber assays. Three independent experiments have been performed. $^*p < 0.05$. (B) To determine the influence of Ino-C2-PAF on proliferation of SCC-25 cells, 1.5×10^4 cells per well were treated as described in (A) and were further incubated for 6 h in the presence of bromodeoxyuridine (BrdU). The colorimetric reaction was detected photometrically at 405 nm. Three independent experiments were performed. The arithmetic mean and standard deviation (SD) are shown. (C) To determine the influence of Ino-C2-PAF on viability of SCC-25 and HaCaT cells 1.5×10^4 cells per well were treated as described in (A) and were further incubated for 6 h. Cytotoxicity was determined by monitoring the activity of the enzyme lactate dehydrogenase.

Photoshop and inverted. Once inverted, F-actin appeared in black. This helped to visualize very thin F-actin-containing cell protrusions. Analysis of the F-actin cytoskeletons of cells at the wound edge revealed a decrease in protrusion outgrowth in the presence of Ino-C2-PAF compared to untreated cells. In untreated SCC-25 cells, the F-actin at the wound edge was orientated toward the wound bed (arrow), whereas F-actin was predominantly localized at cell-cell contacts in Ino-C2-PAF-treated SCC-25 cells (Fig. 2; white arrowheads). In HaCaT cells treated with Ino-C2-PAF for 3 h, F-actin was predominantly found at cell–cell junctions (arrowheads). In contrast, F-actin was orientated toward the wound bed in untreated cells. After 24 h, cell-cell contacts via thin actin-rich cell protrusions could still be detected in Ino-C2-PAF-treated cells. Here, F-actin bundles appeared to be orientated randomly and were concentrated in the cell periphery (arrow). However, after the same period of time, untreated cells had already started to migrate into the wound bed and displayed a polarized cytoskeleton with actin bundles oriented perpendicularly to the leading edge of the outgrowing cells (Fig. 2).

3.3. Ino-C2-PAF increases attachment to extracellular matrix components and causes redistribution of β_1 integrins

Integrin-mediated cell attachment to the ECM is fundamental to migration. To assess whether the inhibition of migration and the redistribution of the F-actin cytoskeleton altered integrin function, we quantified attachment of both cell lines to collagen IV, fibronectin, and laminin-111 in the presence and absence of Ino-C2-PAF (Fig. 3A). Ino-C2-PAF increased attachment to all ECM components tested in a concentration-dependent manner. This effect was most pronounced on collagen IV, on which adhesion was enhanced by 43% in SCC-25 cells and 70% in HaCaT cells relative to untreated cells. In SCC-25 cells, attachment to fibronectin and laminin-111 was increased by 23% and 15%, respectively, after incubation with 5 μM Ino-C2-PAF. In HaCaT cells, attachment to these two matrix components was increased by 40%. This increase in adhesion was clearly integrin-dependent, as background attachment to poly-L-lysine was unaffected in both cell lines.

To ascertain whether expression of integrin subunits accounts for the differences observed we performed microarray experiments with RNA of Ino-C2-PAF-treated and untreated HaCaT cells (Fig. 3B). It turned out that expression of none of the integrin subunits present in HaCaT cells was altered significantly by Ino-C2-PAF. Furthermore, we quantified surface expression of the integrin subunits, α_3, α_6, β_1 and β_4 in Ino-C2-PAF-treated and untreated cells using flow cytometry. Surface expression of the α_3 and β_1 integrin subunit remained unaltered after incubation with Ino-C2-PAF in both cell lines (Fig. 3C). However, in HaCaT cells surface expression of the α_6 and β_4 subunits, which assemble into integrin $\alpha_6\beta_4$, was elevated significantly (Fig. 3C). In SCC-25 cells both subunits are only slightly but non-significantly elevated.

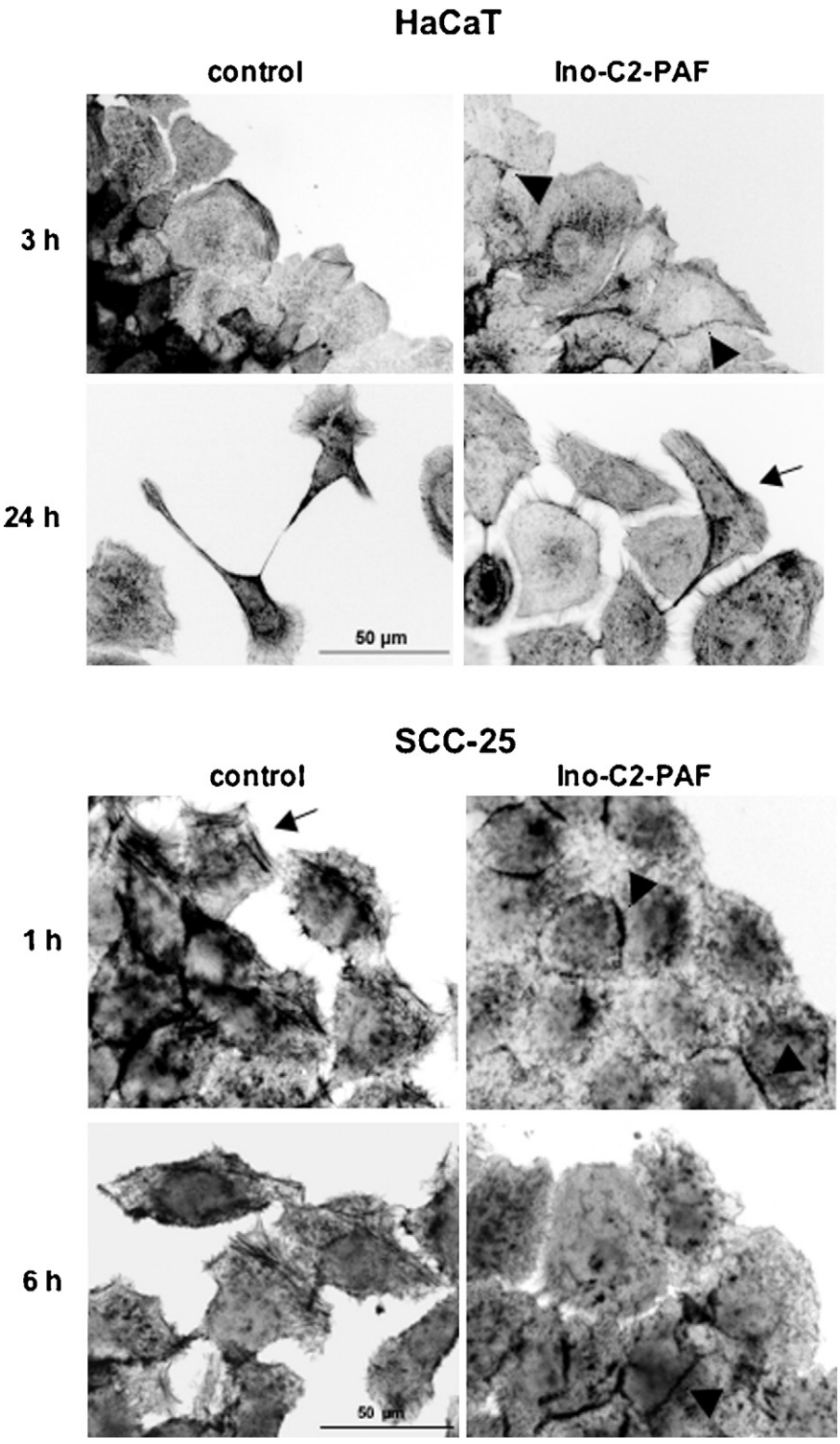

Fig. 2. Ino-C2-PAF causes redistribution of the F-actin cytoskeleton. To analyze the F-actin cytoskeletons of cells at the wound edge, cells were treated with or without 5 μM Ino-C2-PAF. Cells were allowed to migrate into the wound bed for the indicated times. To visualize very thin F-actin-containing protrusions, black and white images taken on a Zeiss Axiovert 200 microscope were imported into Adobe Photoshop and inverted. F-actin appears in black. Arrowheads indicate F-actin in cell-cell contacts arrows point to lamellipodia of cells at the wound edge. The wound bed is on the right side.

Thus, the observed rise in matrix attachment determined in both SCC-25 and HaCaT cells could not directly be due to the overexpression of the $\alpha_6\beta_4$ integrin since the surface expression of the $\alpha_6\beta_4$ integrin is only elevated in one of both cell lines and mainly binding to β_1 integrin ligands was affected.

Therefore, we checked whether the activation state of the β_1 integrin was influenced by Ino-C2-PAF by the use of the antibody 12G10, which recognizes the high-affinity state of β_1 integrins. As shown by flow cytometry, neither 0.6 μM nor 5 μM Ino-C2-PAF was capable of changing the activation state of β_1 integrins in both

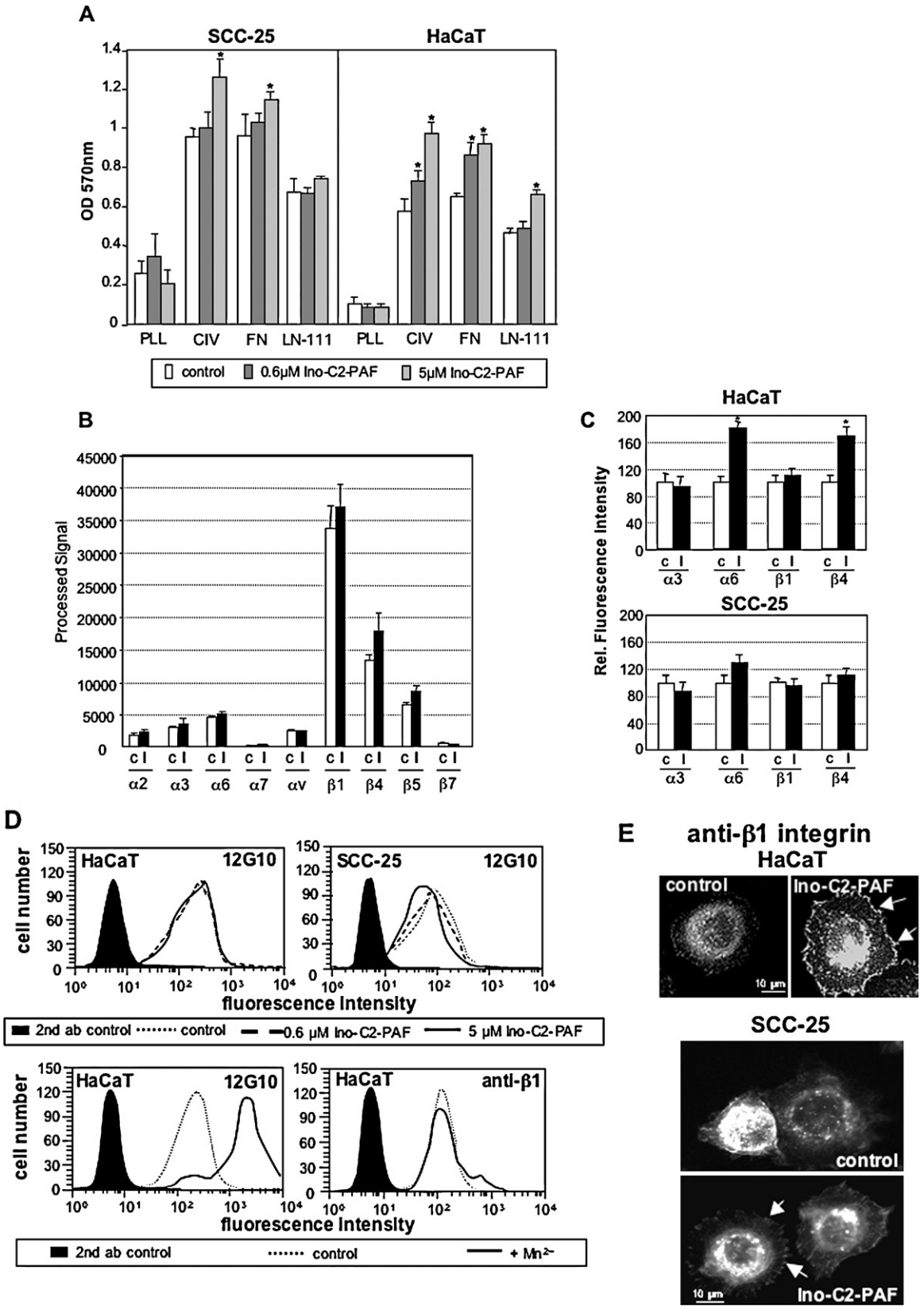

Fig. 3. Ino-C2-PAF increases β_1 integrin-dependent attachment and induces clustering of β1 integrins. (A) For monitoring cell-matrix attachment, cells were incubated with 0.6 or 5 μM Ino-C2-PAF or were left untreated for 48 h. Cells were then plated onto matrix proteins as indicated for 2 h, washed, fixed, and stained with crystal violet. Plates were measured photometrically at 570 nm after TritonX-100 dye solubilization. $^*p < 0.05$. PLL: poly-L-lysine, CIV: collagen IV, FN: fibronectin, LN-111: laminin111. (B) Expression level of integrin subunits was analyzed by microarray technology. HaCaT cells were incubated in serum-free medium in the presence or absence of 5 μM Ino-C2-PAF. Isolated total RNA was then used for microarray analysis with Agilent whole human genome microarray kit 4 × 44 K arrays. Three independent experiments were conducted. Data are presented as a mean value of processed signals. (C) To determine the surface expression of the integrin subunits α_3, β_1, α_6 and β_4, HaCaT and SCC-25 cells incubated in the presence or absence of Ino-C2-PAF were analyzed by flow cytometry. After Ino-C2-PAF treatment, cells were incubated with the specific anti-α or anti-β integrin antibody and the appropriate secondary antibody and subsequently analyzed. Results were presented in a bar diagram, where control values were set at 100%.

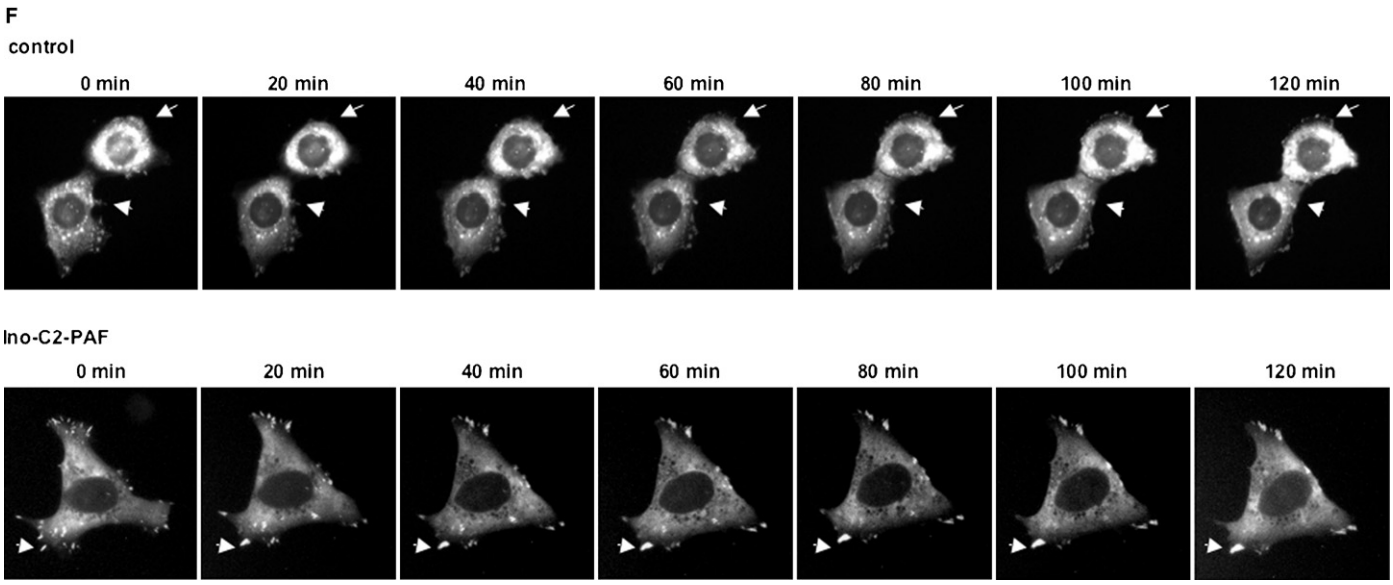

Fig. 3. (*Continued*).

cell lines (Fig. 3D, upper panel). As a positive control $MnCl_2$ was used as a known positive regulator of integrin affinity (Fig. 3D, lower panel). While the amount of β_1 integrin remained unaltered Mn^{2+} increased the amount of 12G10 binding.

Even though the surface expression and the activation state of β_1 integrins was unchanged, immunofluorescence analysis with an anti-β_1 integrin antibody showed that Ino-C2-PAF increased the size and number of β_1 integrin-containing focal contacts upon attachment to collagen IV relative to untreated control cells (Fig. 3E). This implied that rather the avidity of integrins is increased than the affinity. The increase in size of focal complexes could also be visualized in HaCaT cells transiently transfected with GFP-FAK (Fig. 3F). Control cells transfected with GFP-FAK reveal a normal turnover of focal complexes with detachment of focal complexes (arrowhead) and the subsequent formation of new focal adhesions in other regions of the cell (upper panel; arrow). In cells treated with Ino-C2-PAF some of the focal complexes increase in size (arrowhead) but the formation of new focal complexes could not be observed.

3.4. Ino-C2-PAF modulates phosphorylation of the tyrosine kinases FAK and Src

The cytoplasmic tyrosine kinases FAK and Src are key regulators of focal adhesions, which can be regarded as integrin-dependent dynamic protein complexes through which the cytoskeleton of a cell connects to the extracellular matrix. Upon adhesion, FAK is activated by autophosphorylation on tyrosine residue 397 (Y397), which creates a binding site for Src, which in turn is also phosphorylated at tyrosine residue 418 (Y418). The FAK/Src complex regulates a variety of signaling cascades that modulate focal contact dynamics in motile cells [23]. Therefore, we studied the phosphorylation of Src at position 418 and FAK at position 397 by Western blotting with phosphorylation site-specific antibodies in cell lysates of HaCaT and SCC-25 cells after treatment with Ino-C2-PAF. Phosphorylation of Src(Y418) was clearly reduced after treatment for 1, 3, and 6 h relative to untreated control cells. Phosphorylation of FAK(Y397) was also attenuated under these conditions in both cell lines (Fig. 4A and B, upper panel). The microarray analysis revealed that expression of FAK and Src is not affected by Ino-C2-PAF-treatment (Fig. 4C).

Indirect immunofluorescence revealed that untreated motile HaCaT and SCC-25 cells at the "front row" of the wound edge contained phosphorylated Src(Y418) in focal adhesions at the leading edge of the cells. In Ino-C2-PAF-treated cells, only small Src(Y418) puncta could be detected in cells facing the wound bed. Furthermore, the residual phosphorylated Src was primarily localized in the cell–cell junctions (Fig. 4D). The total amount of phosphorylated Src(Y418) and FAK(Y397) was noticeably higher in SCC-25 cells than in HaCaT cells.

FAK(Y397), which is normally localized in the focal complexes of the lamellipodia of migrating cells, could not be detected in focal complexes after Ino-C2-PAF-treatment (Fig. 4E). This exclusion of pSrc(Y418) and pFAK(Y397) from focal complexes was also observed in cells kept under subconfluent conditions (data not shown).

3.5. Constitutively active variants of Src and FAK can in part rescue the inhibition of migration caused by Ino-C2-PAF

To evaluate whether the inhibition of migration caused by Ino-C2-PAF could be rescued we performed migration assays in the absence or presence of Ino-C2-PAF with cells that were transiently transfected with constitutively active Src (Src-Y527F) or FAK (CD2-FAK). It turned out that in HaCaT cells transfection of Src-Y527F

$^*p < 0.05$ c: control; I: 5 μM Ino-C2-PAF. (D) To assess the activity of β_1 integrins, HaCaT and SCC-25 cells treated with Ino-C2-PAF or left untreated were analyzed by flow cytometry using the 12G10 antibody. Cells incubated with the respective secondary antibody only (2nd ab control) served as negative controls. As a positive control cells were stimulated with 5 mM $MnCl_2$ in TBS. (E) To visualize the distribution of β_1 integrins on the cell surface, cells were incubated in serum-free medium with or without 5 μM Ino-C2-PAF for 48 h. Cells were then plated onto collagen IV for 2 h, washed, fixed, and blocked with 1% BSA. β_1 integrin was visualized using an anti-β_1 integrin FITC-conjugated antibody. Immunostaining was then analyzed on a Zeiss Axiovert 200 microscope, using a 100× magnification. Representative cells are shown. Arrows mark some of the enlarged focal adhesions at the cell periphery. (F) To monitor focal adhesion dynamics cells were transiently transfected with GFP-FAK, replated on collagen IV-coated glass coverslips and subsequently incubated in the presence or absence of 5 μM InoPAF in serum-free medium. Typical time courses of GFP-FAK are shown for control cells (upper panel) and Ino-C2-PAF-treated cells (lower panel). Arrows and arrowhaeads indicate individual focal adhesions that appear or disappear in control cells or that persist and/or enlarge in Ino-C2-PAF-treated cells.

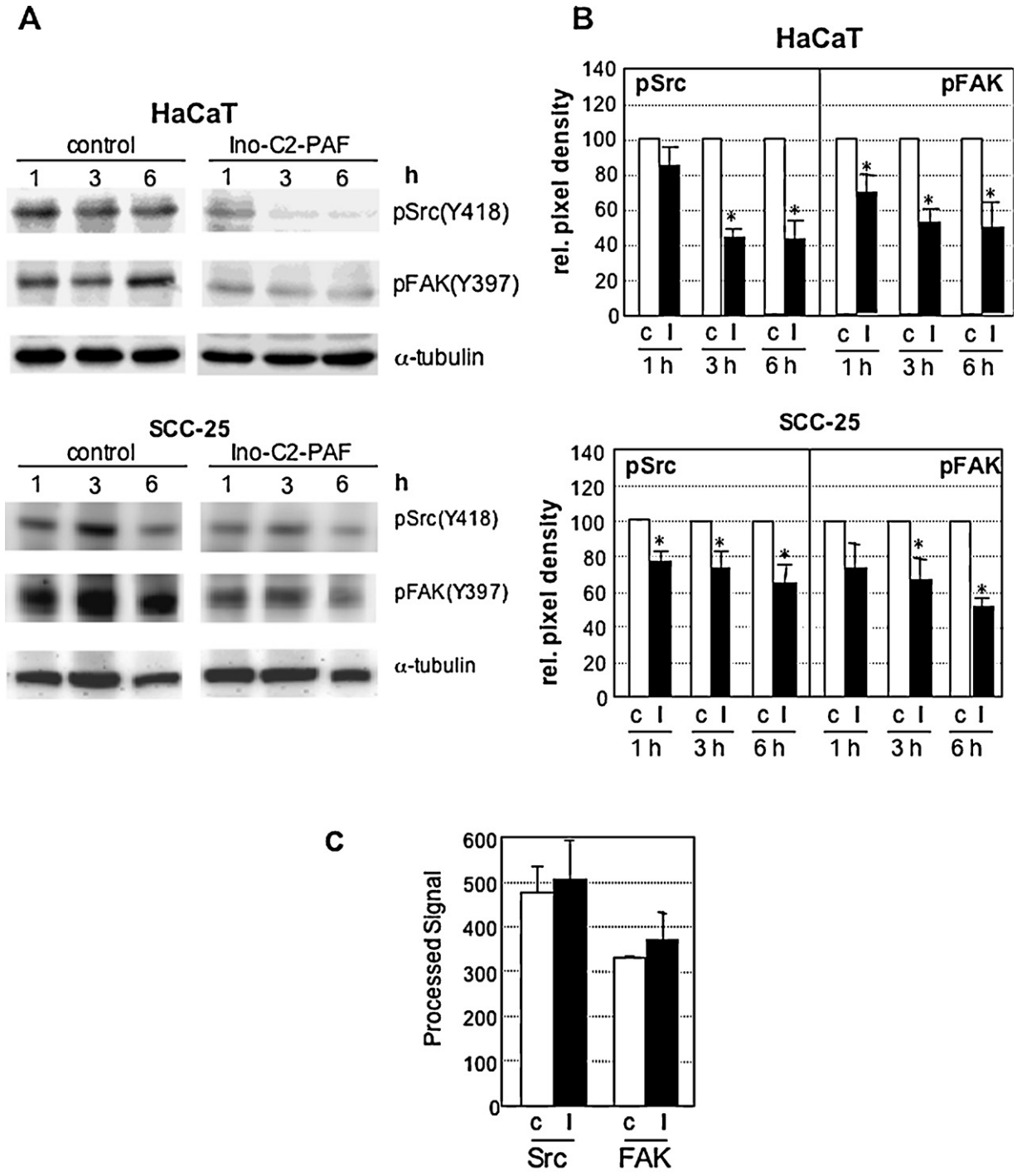

Fig. 4. Ino-C2-PAF attenuates phosphorylation of the cytoplasmic tyrosine kinases FAK and Src. (A) Total cell lysates of untreated cells or cells that were treated with 5 μM Ino-C2-PAF for the indicated amounts of time (1–6 h) were immunoblotted with phosphorylation site-specific antibodies raised against pFAK(Y397) and pSrc(Y418) and the respective secondary antibodies. An α-tubulin antibody was used as a loading control. Bands were visualized using the Digital Imaging System LAS-100. One representative of three independent experiments is shown (left panel). (B) Signal intensities of pFAK(Y397) and pSrc(Y418) of Western blots were quantified using BioRad Quantity One®. Data are the mean of three independent experiments performed. $^{*}p < 0.05$. c: control; I: Ino-C2-PAF. (C) Expression level of FAK and Src were analyzed using microarray technology. HaCaT cells were incubated in serum-free medium with or without 5 μM Ino-C2-PAF. Isolated total RNA was then used for microarray analysis with Agilent whole human genome microarray kit 4 × 44 K arrays. Three independent experiments were conducted. Data are presented as a mean value of processed signals. c: control; I: Ino-C2-PAF. (D and E) An *in vitro* wound with an average width of 475 μm was introduced into confluent cultures of cells as described in Section 2. Three hours after starting wound closure, HaCaT and SCC-25 cells treated with 5 μM Ino-C2-PAF and control cells were stained for pSrc(Y418) (D) and pFAK(Y397) (E). Insets in D show magnification of pSrc(Y418)-containing focal complexes. Additionally, the F-actin cytoskeleton was visualized by Rhodamin-conjugated phalloidin. WB: wound bed.

could partially bypass the effect of Ino-C2-PAF while the transfection of CD2-FAK completely compensated for the inhibitory effect caused by Ino-C2-PAF. In SCC-25 cells, transfection of the cDNAs has also an impact on the inhibitory effect of Ino-C2-PAF but it is less pronounced than in HaCaT cells (Fig. 5).

4. Discussion

In the present study, we showed that the new anti-proliferative synthetic alkyl-phospholipid Ino-C2-PAF was able to reduce the motility of cells at relatively low concentrations. This effect was

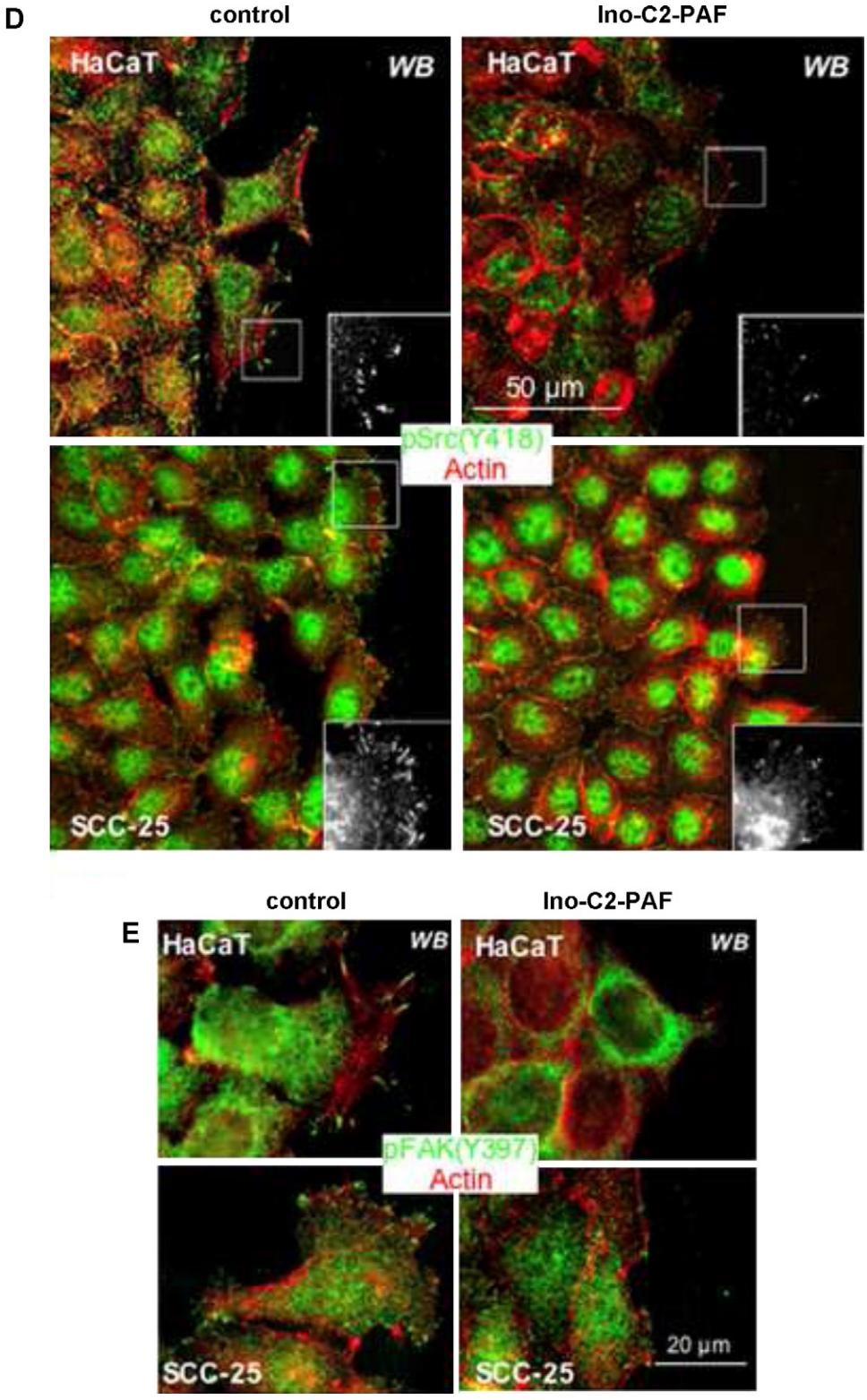

Fig. 4. (*Continued*).

accompanied by altered F-actin cytoskeleton reorganization, increased clustering of integrins, and increased attachment to proteins of the extracellular matrix. Furthermore, Ino-C2-PAF reduced FAK and Src phosphorylation, which is vital in the regulation of cell migration. While migration is inhibited to a similar extent in both cell lines, HaCaT and SCC-25 the impact of Ino-C2-PAF on signaling is stronger in HaCaT cells. This might be due to the fact that in the tumor cell line SCC-25 proto-oncogenes like FAK [34] and Src are *per se* highly phosphorylated.

4.1. Impact of alkyl-phospholipids on cell motility

Other ether lipids with antitumor properties, such as hexadecylphosphocholine and edelfosine, have previously been reported

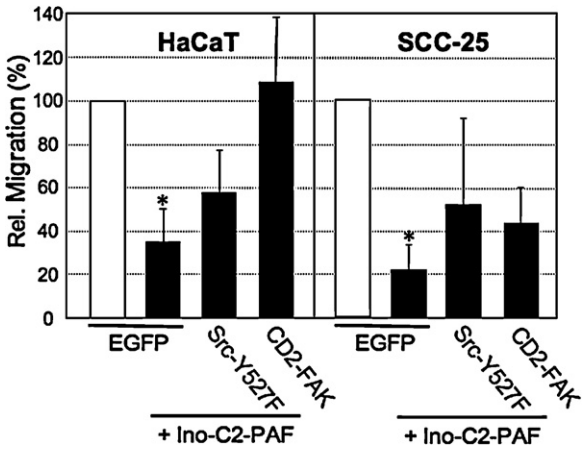

Fig. 5. Constitutively active variants of Src and FAK can in part rescue the inhibition of migration caused by Ino-C2-PAF. 6×10^5 cells that had been incubated in the presence or absence of Ino-C2-PAF were transiently transfected with 2 μg of the respective cDNA and incubated for further 24 h. Subsequently, transfected cells were used for migration assays. The arithmetic mean and standard deviation (SD) are shown. $^*p < 0.05$.

to influence cellular motility. Both hexadecylphosphocholine and edelfosine reduced the capacity of malignant murine MO4 cells to invade precultured heart fragments, an *in vitro* model for cancer cell invasion [24]. At high concentrations, edelfosine reduced both invasion and haptotactic migration of transitional cell carcinoma cells [25]. However, other studies have reported conflicting results, describing stimulated invasion of human MCF-7 breast cancer cells [26] and human HCT-8/S11 colon cancer cells [27] by edelfosine. The latter effect appeared to be linked to the integrin-mediated FAK and Src signaling pathways. The particular effects of edelfosine and the specific differences between edelfosine and Ino-C2-PAF may be explained by a cell type specificity of the compounds. Variations may also arise from the different methodical approaches used in these studies. The structure and stability of the PAF-derived compounds may also be relevant for their impact on migration. Recently, we showed that the glucosidated phospholipid Glc-PAF decreased cell migration in HaCaT cells, while the related compounds Glucose-PC, Glucosamine-PAF, and Glucosimide-PAF increased migration of keratinocytes [28].

4.2. Ino-C2-PAF increases integrin avidity

Integrin-mediated attachment to the ECM is a prerequisite for the controlled movement of cells [29]. Cell attachment to the β_1 integrin ligands collagen IV, laminin-111, and fibronectin was increased with Ino-C2-PAF treatment. Although we did not find evidence of an up-regulation of the β_1 integrin subunit or of α integrin subunits at the protein level, or for increased affinity of β_1 integrins, the Ino-C2-PAF-induced attachment of β_1 integrins was accompanied by increased β_1 integrin clustering and enlarged focal adhesions. The formation of focal contacts was also controlled by transfection of cells with GFP-FAK. Here, increased focal complexes could also be observed in Ino-C2-PAF treated cells. Although integrins are activated and targeting of FAK occurs, subsequent integrin-dependent signaling events such as the FAK/Src pathways are attenuated by Ino-C2-PAF-treatment.

4.3. Role of FAK in skin cells

In skin cells, the non-receptor tyrosine kinase FAK controls cytoskeletal dynamics and focal adhesion disassembly. Accordingly, FAK-null keratinocytes display perturbed motility and fail to emigrate out of skin explants [30]. Furthermore, FAK autophosphorylation is required for efficient recruitment of Src to focal adhesions [31]. Thus, the reduction in cellular motility caused by Ino-C2-PAF may be due to the inhibition of FAK autophosphorylation on tyrosine residue 397 and the consequent reduction in Src activation, which in turn negatively affects the disassembly of focal complexes and results in enlarged focal adhesions. In 2000, Ren and coworkers have shown that cells lacking FAK show more persistent focal adhesions than FAK+/+ cells. This is probably due to disturbed inhibition of the small GTPase Rho A. Increased activity of RhoA resulted in increased focal adhesion lifetime. Accordingly, in FAK+/+ cells co-transfected with V14Rho, focal adhesion turnover was dramatically reduced which leads to the formation of enlarged focal complexes [32].

Taken together, our results suggest that the novel ether lipid Ino-C2-PAF inhibits cell proliferation as well as cellular motility.

Based on data obtained from other alkyl-phospholipids we suggest that Ino-C2-PAF accumulates in the plasma membrane and changes the lipid environment. While focal adhesion targeting of focal adhesion components seems to be unaffected (as shown for GFP-FAK; Fig. 3F) downstream events like tyrosine phosphorylation and formation of the FAK/Src complex are disturbed which in turn results in more persistent and enlarged focal complexes. Furthermore, the balance between small GTPases of the Rho family might be destabilized, which subsequently is responsible for the change in cytoskeletal dynamics and the decrease in cell migration (Fig. 6).

These findings are particularly important because activated FAK is a well-established marker of both transformation and metastasis. Recent studies show that oncogenic transformation is strongly reduced in skin with decreased or abrogated FAK function [33]. Elevated levels of FAK have been detected in head and neck squamous cell carcinoma (SCC), and mice lacking epidermal FAK are more resistant to chemically induced SCC [34].

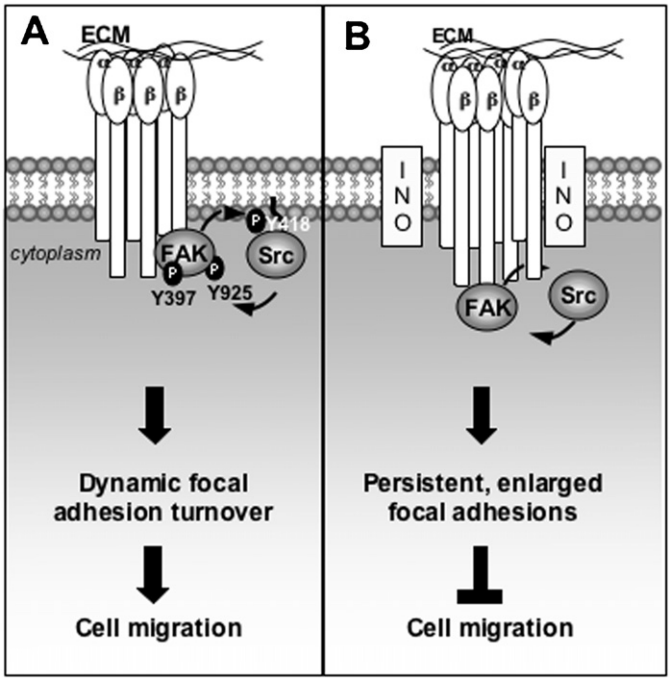

Fig. 6. Schematic representation of the proposed model for the mechanism of action of Ino-C2-PAF. We suggest that Ino-C2-PAF due to its amphiphilic character is incorporated into the plasma membrane. The incorporation leads to a changed lipid environment, which presumably affects membrane targeting of signaling components such as Src and small GTPases. Subsequently, downstream events like tyrosine phosphorylation and formation of the FAK/Src complex are disturbed which in turn might increase the activity of the small GTPases and might result in the formation of more persistent and enlarged focal complexes, which subsequently leads to changes in cytoskeletal dynamics and a decrease in cell migration.

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