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ARTICLE

Ligand-Mimetic Anti-αIIbβ3 Antibody PAC-1 Inhibits Tyrosine Signaling, Proliferation and Lung Colonization of Melanoma Cells

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β3 integrin expression is the hallmark of melanoma and may serve as a potential therapeutic target. While αvβ3 integrin expression seems to be constitutive in melanoma, ectopic expression of platelet-αIIbβ3 is dependent on progression. B16a murine melanoma is a suitable model for studies on αIIbβ3 treatment strategies since αvβ3 is not expressed in this cell line. Here we have used a ligand-mimetic anti-αIIbβ3 monoclonal antibody, PAC-1, to test the biological consequences of αIIbβ3 modulation in melanoma cells. We have previously reported that in B16a cells FAK is constitutively active and tyrosine-phosphorylated. Upon PAC-1 binding to the surface αIIbβ3, which is in the active conformation, FAK became dephosphorylated through a process of PKC-dependent phosphatase activation. Furthermore, PAC-1 binding to B16a cells induced a significant decrease in phosphotyrosine-positive melanoma cells within 30 min. Treatment of B16a cells in vitro with PAC-1 significantly inhibited proliferation by decreasing the mitotic index but not affecting apoptotic rate. Incubation of B16a cells with PAC-1 decreased their lung colonization potential, suggesting a profound alteration in their biological behavior under the effect of this antibody. These preclinical data suggest that the ectopic expression of αIIbβ3 in melanoma cells can be exploited as a novel target of antibody therapy of melanoma. (Pathology Oncology Research Vol 11, No 4, 218–223)

Key words: αIIbβ3, integrin, ligand-mimetic, melanoma

Introduction

Integrins are significant regulators of tumor cell - extracellular matrix interactions.[1-3] On the other hand, integrins at the cancer cell membrane are part of the signaling organelle containing the growth factor receptors as well.[4,5] In malignant melanoma αvβ3 integrins are considered to be the predominant integrin receptor involved in matrix adhesion, degradation and migration, as well as survival.[6,7] The signaling of αvβ3 in melanoma involves the MAPK and PI3K/PKC pathways. Experimental and clinical studies demonstrated that human melanoma may also express the platelet-type αIIbβ3 integrin,[8-10] resulting in a constitutive activation of FAK phosphorylation.[11,12] Interestingly, upon ligation melanoma αIIbβ3 signaled exclusively through the 12-LOX/PKC pathway. Overexpression of αIIbβ3 in melanoma cells in the presence of αvβ3 resulted in a predominant αIIbβ3 integrin expression, involved in adhesion, migration and cell survival, and promoting a more potent angiogenic phenotype by inducing bFGF expression.[13]

Predominant signaling receptors on cancer cells are targeted either by monoclonal antibodies or, in case of tyrosine kinase activity of the cytoplasmic domain, by small molecular inhibitors resulting in new avenues for antitumoral modalities. Cancer cell integrins are long considered to be therapeutic targets. Since they have no intrinsic kinase activity, agents acting on their extracellular domain, such as monoclonal antibodies or small molecular ligand-mimetics, can only be considered.[2] There are two anti-integrin antibodies that have shown clinical activity in humans, both aiming at β3 integrins. LM609-derived anti-αvβ3 antibody (Vitaxin) was shown to act as an anti-angiogenic agent,[14-16] while the anti-β3 integrin antibody ReoPro, aiming at both αIIb- and αvβ3 integrins was shown to have a significant anti-platelet role.[17,18] Interestingly, both agents demonstrated significant anti-

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melanoma activity in preclinical settings.[19] Furthermore, there are available ligand-mimetic small molecular inhibitors targeting αvβ3 or αIIbβ3 integrins, which demonstrated significant antithrombotic activity.[20-25]

Previous preclinical studies on ectopic αIIbβ3 demonstrated that it is expressed in an active conformation,[11] and serves as matrix receptor and migration regulator in human malignant melanoma.[9,10] Along this lane, here we have used a ligand-mimetic anti-αIIbβ3 monoclonal antibody, PAC-1, to test its effect on the proliferation and lung colonization of melanoma cells.

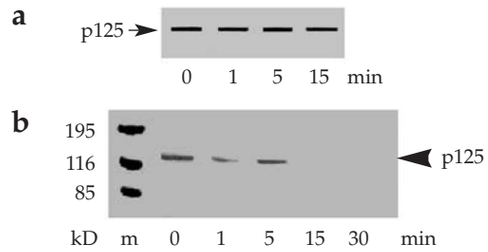

Figure 1. *Effect of PAC-1 treatment on tyrosine phosphorylation of p125/FAK in B16a cells, detected by Western blotting.* ***(a)*** *B16a cells kept in suspension at 37°C for 1-15 min, blotting for tyrosine-phosphorylated proteins visualized by peroxidase/DAB reaction. Note the phosphorylation of p125/FAK band during the observation period, no ligand.* ***(b)*** *Adherent B16a cells incubated with 20 μg/ml PAC-1 antibody for 1-30 min at 37°C. Western blotting of tyrosine-phosphorylated proteins. Note the decreased phosphorylation of p125/FAK band within 15 min. m= molecular weight markers*

Materials and Methods

Murine melanoma cell culture

Murine B16a melanoma cells were obtained from the Animal and Human Tumor Bank of National Cancer Institute (Frederick, MD). Tumor cells were grown as adherent cultures in RPMI 1640 containing 5% FBS and antibiotics (Sigma, St. Louis, MO). Confluent cells were harvested with 0.02% EDTA in PBS.

Antibodies

Mouse monoclonal antibody (mAb) PAC-1 (IgM) which specifically recognizes the high-affinity αIIbβ3 in human platelets[26] and also the murine equivalent of gpIIbIIIa was purchased from Becton Dickinson (San Jose, CA). AB-1 antibody recognizing the α5β1 integrin was from Oncogene Science (Uniondale, NY). An αIIbβ3 ligand, fibrinogen (FBG; Sigma) was also used along with PAC-1. Mouse monoclonal anti-phosphotyrosine (PY20, IgG2b) was purchased from Transduction Laboratories (Lexington, KY). Biotinylated goat anti-mouse IgG and Streptavidin-FlTC were from Amersham Biosciences (Little Chalfont, UK). Non-immune mouse IgG and IgM were obtained from Sigma.

Flow cytometry

Flow cytometry of B16a cells was essentially performed as described.[11,27] Briefly, cells were detached with EDTA, washed with serum-free medium (SFM), and suspended in SFM. Tumor cells (10^6 cells/sample) were fixed in absolute MetOH for 5 min at room temperature, washed with PBS (3x) and labeled for 60 min with anti-phosphotyrosine (10 μ/g/ml) mAb diluted in PBS. Bound antibody was detected by goat anti-mouse IgG-biotin complex (1:100 dilution in PBS) and Streptavidin-FlTC (1:100 dilution in PBS). Substitution of primary antibody with isotype-matched non-immune IgG served as negative control.

Ten thousand cells were measured in a FACStar flow cytometer (Becton Dickinson, Munich, Germany), and the percentage of positive cells was determined by using an established protocol. Briefly, control samples were measured for background fluorescence to establish criteria for positive cells. The gate was set to exclude 90% of the negative population and then the percentage of positive cells was determined. Each experimental point was performed in triplicate, and experiments were repeated at least three times.

Western blotting

Adherent cells. Confluent cell cultures were maintained in PRMI 1640 in a serum-free environment for 48 h before use. Cells were stimulated with 10 μg/ml FBG, 20 μg/ml PAC-1 mAb or non-immune mouse IgM (control) for 1, 5, 15, 30, 60 min at 37°C. At each time point cells were quickly washed with RPMI and the cells were lysed in hot lysis buffer containing 56 mM Tris, 2% SDS and 2 mM Na_3VO_4.

Suspended cells. Cells were harvested from confluent cultures by washing twice with PBS and treating them with 0.02% EDTA at 37°C for 10 min. Cells were washed three times in serum-free RPMI and brought to a final concentration of $5x10^6$ cells/ml. The cells were kept at 37°C for 2 hrs in a shaker, then were stimulated with FBG, PAC-1 or IgM as above. At the end of incubation cells were washed in RPMI, centrifuged and lysed in hot lysis buffer.

Protein concentration was measured by using the protein staining kit of Bio-Rad (Richmond, VA). Equal amount of protein was separated on an 8% SDS-PAGE and electroblotted onto polyvinylidine fluoride membranes (Bio-Rad). The blots were blocked overnight with 5% BSA (Sigma) diluted in PBS, and incubated with mouse anti-phosphotyrosine antibody at a concentration of 2 μg/ml for 1 hr at room temperature. The membranes were then

washed with 0.5% Tween 20 diluted in PBS, followed by HRP-conjugated goat anti-mouse IgG (Amersham) for 45 min at room temperature. Immunoreactive bands were revealed by ImmunoPure Metal Enhanced DAB substrate kit (Pierce, Rochford, IL), and quantitated with an Eagle Eye videodensitometer (Stratagene, San Diego, CA).

Inhibitor treatments

To inhibit PKC activity, pretreatment with Calphostin C (Biomol, Hamburg, Germany) for 15 min was applied at a concentration of 5 μM using light activation according to the manufacturer's protocol. To inhibit tyrosine phosphatases, tumor cells were incubated with a nontoxic concentration (200 μM) of Na_3VO_4 (Sigma) for 5-30 min together with PAC-1 antibody.

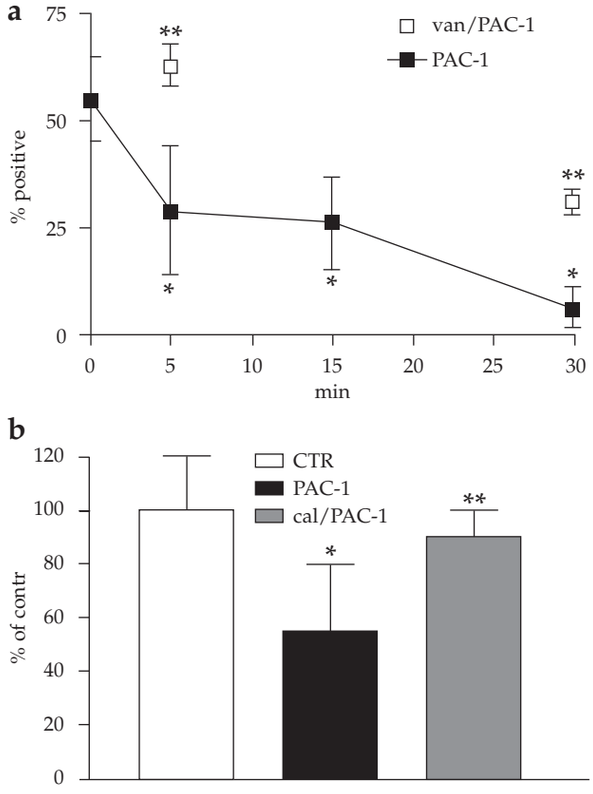

Figure 2. *Effect of PAC-1 treatment on tyrosine-phosphorylated protein (PY) content of adherent B16a cells measured by immunocytochemistry and flow cytometry.* ***(a)*** *Adherent tumor cells were treated by 20 μg/ml PAC-1 alone for 5-30 min and PAC-1 plus 200 μM orthovanadate (van) for 5 and 30 min at 37°C, suspended, fixed in MetOH and labeled with anti-PY mAb revealed by biotinylated anti-mouse Ig and FITC-Streptavidin. Data are expressed as % of positive cells.* ***(b)*** *Effect of PKC inhibition. Adherent B16a cells were preincubated with 5 μM Calphostin-C (cal) for 15 min at 37°C, then incubated with 20 μg/ml PAC-1 antibody for 15 min. PY-positive cells were determined as in Figure 2a. All experiments were run in triplicate and data represent mean±SEM. *p<0.05 compared to control, αp<0.05 compared to PAC-1 alone (ANOVA single factor analysis)*

Cell proliferation test

B16a cells were plated in 96-well tissue culture plates ($5x10^3$/well) in RPMI 1640 medium containing 5% FBS (RPMI/FBS). After overnight incubation at 37°C, adherent cells were treated with 0.2-20 μg/ml PAC-1 or control antibody, or 0.1-10 μg/ml FBG for 48 h in the absence of FBS. At the end of the incubation period, a colorimetric assay (MTT test) was performed. Briefly, 0.5 mg/ml of the tetrazolium dye MTT (Sigma) was added to the wells. After 4 h incubation at 37 °C the medium was gently removed, the plates air-dried, and the formazan crystals formed in viable cells were dissolved in DMSO. The absorbency at 570 nm was measured with a Bio-Rad microplate reader.

Mitotic index and apoptosis

B16a cells were suspended in RPMI/FBS and plated in 24-well tissue culture plates containing coverglass (10^5 cells/well). After overnight incubation at 37°C, adherent cells were treated with 0.2-20 μg/ml PAC-1 or control antibody in triplicates, for 24 h in the absence of FBS. At the end of the incubation period the coverglass was removed, and the cells were fixed in MetOH and stained for nuclei by Propidium iodide. Apoptotic nuclei and mitotic figures have been counted in 100 and 1000 cells respectively on each coverglass under fluorescent microscope (Nikon Eclipse E600, Tokyo, Japan).

Lung colony assay

Single-cell suspension from EDTA-treated B16a cell culture was prepared (10^7 in 0.5 ml Medium 199, Sigma), and treated with 20 μg/ml PAC-1 or control IgM, or 10 μg/ml FBG for 30 min at 37°C. Cells were then diluted in Medium 199, and injected into the tail vein of C57Bl6 mice ($5x10^5$ cells in 100 μl). The experiment was terminated with Nembutal overdose 21 days after tumor cell inoculation, and the number of lung colonies was counted after formaldehyde fixation. The animal protocol was approved by the Animal Committee of the Institution.

Results

Analysis of tyrosine-phosphorylated proteins in B16a cells, both in adherent form (data not shown) as well as in suspension, revealed a predominant protein band at 125 kD (*Figure 1a*), which was stable without any ligand exposure. This band was found to be the FAK protein in previous experiments.[12] We have suggested that the constitutive activation of FAK in B16a cells may be due to the ectopic

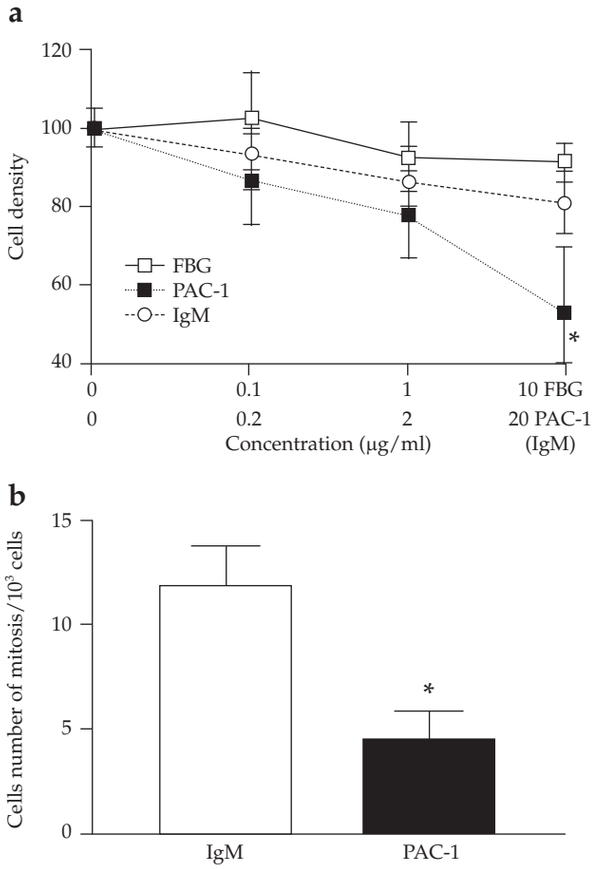

Figure 3. (a) *Effect of PAC-1 on proliferation of B16a cells in vitro. Tumor cells were cultured in serum-free media for 48 hr in the presence of control non-immune IgM, fibrinogen (FBG) or PAC1, and cell density was measured by MTT test. Data are mean±SD, n=6.* ***(b)*** *Incidence of mitotic figures determined under fluorescent microscope by measuring 1000 cells/point. *p<0.05 (ANOVA single factor analysis)*

expression of the αIIbβ3 integrin.[12] In the followings we analyzed the αIIbβ3 signaling by Western blotting, using the mAb PAC-1 recognizing the active conformation of the integrin. Unexpectedly, the tyrosine phosphorylation of p125/FAK protein decreased in a time-dependent manner upon exposure of adherent melanoma cells to 20 μg/ml PAC-1, with maximal effect at 30 min (*Figure 1b*).

Next we applied an alternative measurement of integrin signaling, using anti-phosphotyrosine (PY) antibody labeling of permeabilized B16a cells and determining the percentage of positive cells by flow cytometry. In unstimulated B16a cells more than 50% of the melanoma cell population was positive for phosphotyrosine (*Figure 2a*). Upon PAC-1 exposure of B16a cells a time-dependent decrease was detected in the proportion of PY+ melanoma cells till 30 min (*Figure 2a*), indicating a significant inhibition of the integrin-associated tyrosine phosphorylation cascade.

To determine the signaling components downstream to αIIbβ3 integrin, we have used two inhibitors, orthovanadate and Calphostin C to reveal any role of phosphatases or PKC. Orthovanadate exposure (200 μM) of B16a cells for either 5 or 30 min together with PAC-1 antibody treatment inhibited the loss of PY+ melanoma cells, suggesting the involvement of tyrosine-phosphatases (*Figure 2a*). On the other hand, preincubation of B16a cells for 15 min with 5 μM Calphostin-C before exposure to PAC-1 for another 15 min also prevented the decrease in the PY+ melanoma cells, suggesting the involvement of PKC in the observed phenomenon (*Figure 2b*).

In vitro exposure of B16a melanoma cells to mAb PAC-1 induced a dose-dependent inhibition of cell proliferation within 48 hr with maximal effect at 20 μg/ml (*Figure 3a*). The effect was specific for PAC-1, since treatment with soluble FBG, binding to the similar region of the extracellular domain of the αIIbβ3 complex, was ineffective. Microscopic analysis of the nuclei of B16a cells treated by PAC-1 did not reveal apoptotic figures (data not shown). On the other hand, PAC-1 exposure, unlike FBG, resulted in a significant decrease in the mitotic index of B16a cells (*Figure 3b*).

In the followings, we investigated the effect of PAC-1 pretreatment on lung colony formation by B16a cells. Cells were incubated with PAC-1 antibody, IgM control, or FBG for 30 min, then injected into the tail vein of C57Bl6 mice. The number of lung colonies was determined after 3 weeks. Data indicate that PAC-1-exposed cells had a significantly decreased ability to colonize the lung compared to controls, while FBG had no such effect (*Figure 4*).

Discussion

Here we have demonstrated that a monoclonal antibody, PAC-1, directed to the conformationally active form of αIIbβ3 integrin is capable to induce significant inhibition of integrin signaling through FAK in B16a melanoma cells. In platelets, ligand-activated αIIbβ3 signals through FAK, syk and src, inducing phosphotyrosine cascade.[28-30] The fundamental difference between B16a cells and activated platelets is that B16a cells as well as human melanoma, express αIIbβ3 integrin in a conformationally active form,[9,11] while this integrin is activable in platelets since the cytoplasmic negative regulators are also present. This may explain why the αIIbβ3-associated FAK signaling is active constitutively in B16a cells, similarly to CHO cells transfected with the αIIb chain.[31] We have previously demonstrated that ligation of the ectopic αIIbβ3 in B16a cells induces a serine/threonine phosphorylation cascade dependent on the activation of 12-LOX and PKCα.[12] Meanwhile, FBG, like PAC-1, induced another signaling pathway transiently downregulating and dephosphorylating FAK.[12]

It seems that the ligand-mimetic mAb, PAC-1, is able to activate the same signaling pathway, however, the dissoci-

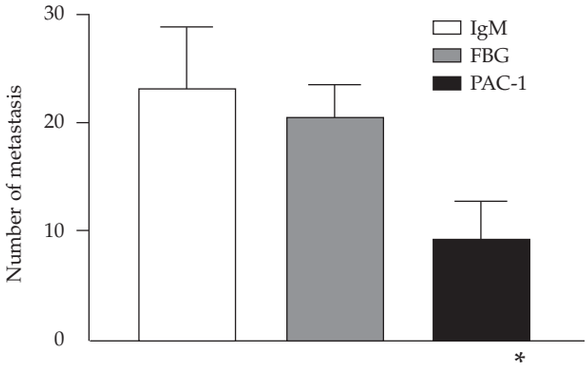

Figure 4. *Effect of PAC-1 preincubation of B16a cells on lung colonization. Tumor cells were incubated in suspension with 20 μg/ml PAC-1 or control IgM, or 10 μg/ml FBG for 30 min at 37°C, then injected iv. into mice. Lung colonies were determined on day 21 after tumor cell injection. Data are means±SD, n=5. *p<0.05 (Mann-Whitney U-test)*

ation from the ligand-binding domain does not occur within a few hours, therefore, the dephosphorylation of FAK lasts for an extended period up to 30-60 min, unlike in the case of the natural ligand, FBG.[12] We have clearly shown here that PAC-1 binding to αIIbβ3 in melanoma cells activates a tyrosine phosphatase through PKCα, responsible for the dephosphorylation of FAK and the inhibition of integrin-mediated tyrosine signaling (*Figure 5*).

Inhibition of tyrosine signaling in cancer cells, usually sustained by the constitutively active oncogenes, is a powerful novel anticancer strategy shown to be clinically successful in case of breast-, colon- and lung cancers, targeting the hyperactive c-erbB2 or c-erbB1. We have postulated that a hyperactive integrin receptor signaling could serve in a similar fashion as target for signaling therapy. Our experimental data indicate the feasibility of this approach. Inhibition of αIIbβ3 signaling in B16a melanoma cells resulted in inhibition of cell proliferation without inducing apoptosis. Furthermore, PAC-1-treated melanoma cells lost their ability to colonize the lung, suggesting that their invasive potential was also abrogated in vivo. Previous studies provided ample of evidences that PAC-1 antibody interferes with in vitro matrix adhesion, spreading and migration of melanoma cells,[9,11] confirmed here by in vivo data.

Integrins are expressed in cancer cells in a low- and high-affinity conformation, the latter being responsible for signaling.[33] Peptide ligands, antibodies or small molecular ligand-mimetics usually do not discriminate between these two functionally distinct states of the integrin receptor. Furthermore, integrins expressed by cancer cells are also expressed by host cells. Accordingly, an inhibitor aiming at an integrin on cancer cells may have unwanted side effects on platelets or endothelial cells. A possible way to overcome the lack of selectivity of integrin inhibitors is to use agents which can specifically recognize the functionally/constitutively active integrins expressed on cancer cells or induced by cancer cells on host cells (endothelial ones: neoangiogenesis, platelets: hemostasis disorders). Recent data indicate that although SMIs can induce transient conformational change in αIIbβ3, this is not the case when the natural ligand (FBG) is present being one potential cause for the reported side effects.[34]

β3 integrin-SMIs are potential novel anticancer agents. αvβ3 mimetics were shown to reduce bone metastasis of breast cancer cells, acting on the target host tissues.[20] Vitaxin, aiming at the predominant integrin, β3, on melanoma cells demonstrated antimetastatic activity.[16] A combination of αIIbβ3 and αvβ3 mimetics reduced breast cancer cell adhesion to endothelium when either SMI alone was inactive.[24] An anti-human β3 antibody acting both at αIIb and αvβ3 integrins, ReoPro, demonstrated remarkable antitumoral and antimetastatic activity in vivo, suggesting that both β3 integrins may be necessary antitumoral targets.[18,19]

A constitutively active integrin, either due to ectopic expression (platelet/stem cell mimicry)[35] or activating mutation on cancer cells, could serve as a selective target for specific antibody therapy or for SMIs recognizing the active conformation. This approach may reduce the unwanted side

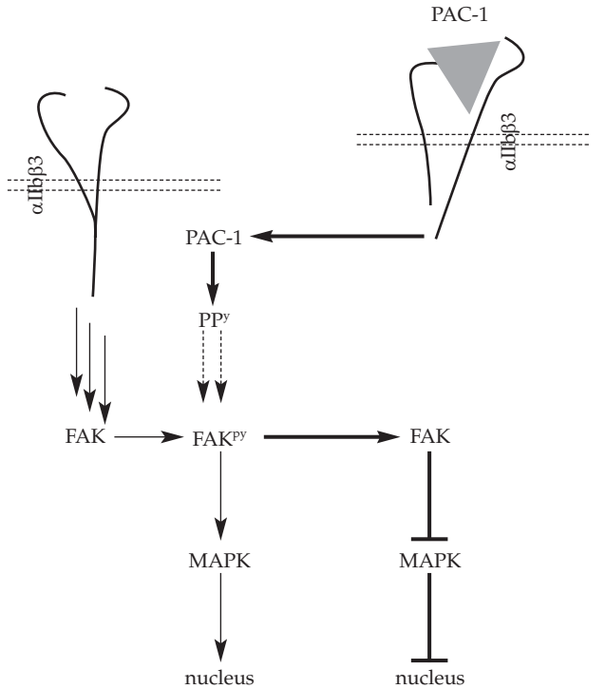

Figure 5. *Schematic representation of signaling events associated with PAC-1 antibody treatment of B16a cells. Arrow= stimulation, line break= inhibition. FAK= focal adhesion kinase, FAK^{y}= tyrosine-phosphorylated FAK, MAPK= mitogen-activated protein kinase, PKC= protein kinase C, PP^{y}= protein tyrosine phosphatase*

effects and provide better cancer selectivity. Overexpression of β3 integrin in human melanoma is the hallmark of the disease which is not exploited therapeutically yet.[2,7] It was demonstrated that the melanoma β3 integrin is activated, contributing to the activation of MAPK signaling pathway.[6] We have shown earlier that human melanoma may express both αvβ3 and αIIbβ3 integrins,[10] leading to a predominant PKC signaling.[12] PAC-1 or a similar ligand-mimetic anti-β3 integrin antibody could well be a feasible novel therapeutic approach for melanoma. The unexpected signaling effect of PAC-1 antibody in murine melanoma may suggest that tyrosine signaling could be inhibited in (melanoma) cells by a carefully selected anti-integrin antibody as well, similar to anti-c-erbB1 or -B2 antibodies.

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