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Cytostatin, an inhibitor of cell adhesion to extracellular matrix, selectively inhibits protein phosphatase 2A

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Abstract

Cytostatin, which is isolated from a microbial cultured broth as a low molecular weight inhibitor of cell adhesion to extracellular matrix (ECM), has anti-metastatic activity against B16 melanoma cells in vivo. In this study, we examined a target of cytostatin inhibiting cell adhesion to ECM. Cytostatin inhibited tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin upon B16 cell adhesion to fibronectin. While the amount of FAK was not affected by cytostatin, electrophoretically slow-migrating paxillin appeared. Alkaline phosphatase treatment diminished cytostatin-induced slow-migrating paxillin. Furthermore, cytostatin increased intracellular serine/threonine-phosphorylated proteins and was found to be a selective inhibitor of protein phosphatase 2A (PP2A). Cytostatin inhibited PP2A with an IC_{50} of 0.09 μg/ml in a non-competitive manner against a substrate, *p*-nitrophenyl phosphate, but it had no apparent effect on other protein phosphatases including PP1, PP2B and alkaline phosphatase even at 100 μg/ml. On the contrary, dephosphocytostatin, a cytostatin analogue, without inhibitory effect on PP2A did not affect B16 cell adhesion including FAK and paxillin. These results indicate that cytostatin inhibits cell adhesion through modification of focal contact proteins such as paxillin by inhibiting a PP2A type protein serine/threonine phosphatase. This is the first report that describes a drug with anti-metastatic ability that inhibits PP2A selectively.

Keywords: Cytostatin; Metastasis; Protein phosphatase 2A; Cell adhesion; Paxillin

1. Introduction

Cell adhesion to extracellular matrix (ECM) involves in the regulation of many biological processes including immunity, development and tumor growth. In the processes of tumor metastasis, cancer cell adhesion to ECM is thought to be one of the targets for anti-metastatic therapy. The cell adhesion to ECM is controlled by many molecules. Integrins act as cell surface receptors for ECM. While integrins themselves have no intrinsic kinase activity, they function through interacting with a number of other proteins. Upon their binding to ECM, they aggregate, assemble several proteins and form a focal contact, in which actin-containing cytoskeleton links structurally [1,2]. The focal contact consists of structural proteins such as vinculin, talin and actin and protein tyrosine kinases such as focal adhesion kinase (FAK) and src, as well as adapter proteins such as paxillin and crk. Phosphorylation and dephosphorylation of these protein components regulate assembly of the focal contact and transduce the cell adhesion signals intracellularly. Among them, tyrosine phosphorylations of FAK and paxillin by cell adhesions are well

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known events for connecting the integrins and actin stress fiber [1,3–5].

Cytostatin (Fig. 1) is originally isolated as a novel inhibitor of cell adhesion to ECM from cultured broth of *Streptomyces* sp. MJ654-NF4 [6,7]. Cytostatin has an anti-metastatic ability and inhibits experimental and spontaneous lung metastasis of B16 cells in mice [6,8]. Furthermore, cytostatin inhibits growth of various tumor cell lines in vitro and induces apoptosis in some cell lines [6,9,10]. To understand the mechanism(s) of cytostatin actions on metastasis and apoptosis, we investigated a primary target of cytostatin in this study focusing on cell adhesion. As a result, we have found that cytostatin selectively inhibits protein serine/threonine phosphatase 2A (PP2A).

2. Materials and methods

2.1. Reagents and antibodies

Cytostatin and dephosphorylated cytostatin were prepared as described [6,7]. Calyculin A was obtained from Gibco BRL (Gaithersburg, MD, USA). PP1 (14-110) and PP2A (14-111) were from Upstate Biotechnology (Lake Placid, NY, USA). PP2B and calmodulin were from Sigma (St. Louis, MO, USA). Calf intestine alkaline phosphatase (CIAP) was from Biozyme (South Wales, UK). Antibodies used for Western blotting were the following: anti-phosphotyrosine (05-321) was from Upstate Biotechnology, anti-FAK (F15020) and paxillin (P13520) were from Transduction Laboratories (Lexington, KY, USA), anti-phosphoserine (61-8100) and phosphothreonine (71-8200) were from Zymed (South San Francisco, CA, USA), anti-vinculin (V4505) and α-actinin (A2534) were from Sigma.

2.2. Cells

Mouse B16 melanoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C with 5% CO_2.

2.3. Cell adhesion to fibronectin (FN)

As reported previously [6,11], FN-coated plates were prepared and the cell adhesion assay was performed. B16 cells were inoculated into the FN-coated plates and incubated with or without test chemicals for 1 h at 37°C. The plates were washed with phosphate-buffered saline (PBS), fixed with glutaraldehyde and stained with crystal violet. In the case of Western blotting, the cells including adhered and unadhered ones were collected after 1 h incubation at 37°C.

2.4. Preparation of cell lysates and Western blotting

Treated cells were washed twice with ice-cold PBS containing 100 μM Na_3VO_4 and then lysed in a lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM Na_3VO_4 and 25 μg/ml each of antipain, leupeptin and pepstatin). Equal protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) was used to visualize the immunoblot signals.

2.5. Immunoprecipitation

Cell lysates were prepared as described above and equal amounts of protein were incubated with anti-FAK (F15020) or anti-paxillin (P13520) from Transduction Laboratories. The immune complexes were collected on protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ, USA) pretreated with anti-mouse IgG and washed four times in IP buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM PMSF and 0.2 mM Na_3VO_4) and once in a buffer containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT. The immunoprecipitates were separated by SDS-PAGE and immunoblotted as described above.

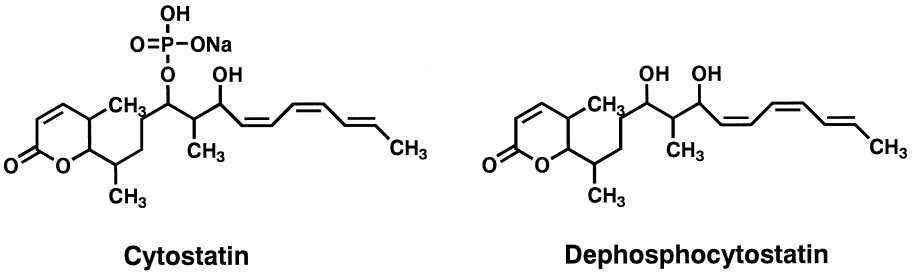

Fig. 1. Structures of cytostatin and dephosphocytostatin.

2.6. Alkaline phosphatase treatment of immunoprecipitated paxillin

Cell lysates were immunoprecipitated with anti-paxillin as described above. The immunoprecipitates were incubated in a phosphatase buffer containing 20 mM HEPES (pH 10), 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml CIAP for 30 min at 37°C and this procedure was then repeated twice. The treated immunoprecipitates were analyzed by immunoblotting.

2.7. Protein phosphatase assay

PP1 (0.05 U), PP2A (0.05 U) or CIAP (10 μg/ml) was incubated with 5 mM *p*-nitrophenyl phosphate (PNPP; Research Organics, Cleveland, OH, USA) as a substrate in a buffer (100 μl) containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT for 1 h. PP2B activity was assessed as described by Honkanen [12]. PP2B (0.5 U) was incubated with 5 mM PNPP in a buffer (100 μl) containing 50 mM

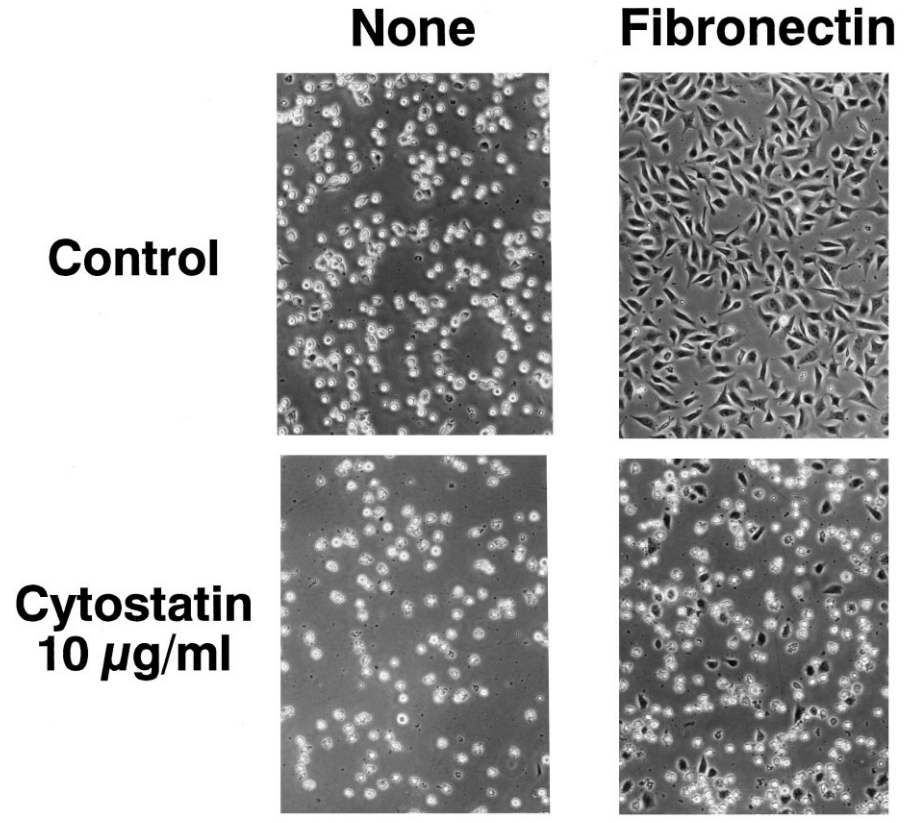

Fig. 2. Effect of cytostatin on B16 cell adhesion to FN. B16 cells were inoculated into a plate pre-coated with none or FN and then incubated with or without 10 μg/ml cytostatin for 1 h at 37°C.

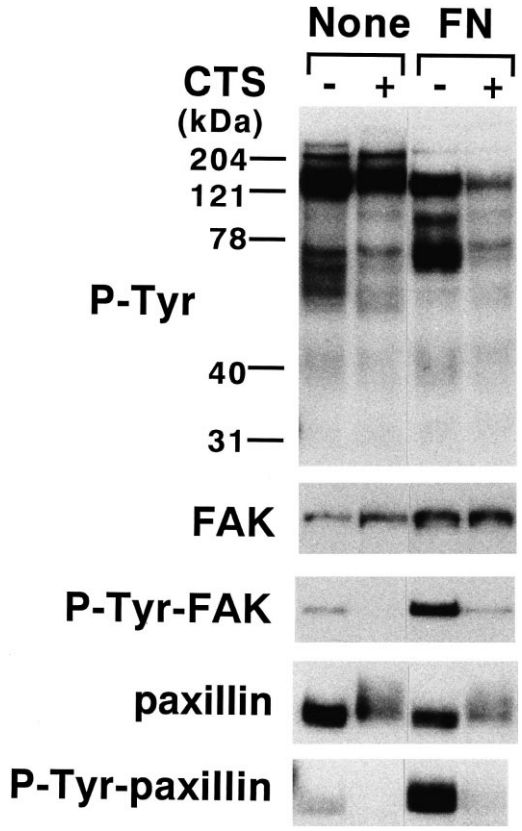

Fig. 3. Effect of cytostatin on cell adhesion signals. B16 cells were inoculated into a plate pre-coated with none or FN and then incubated with or without 10 μg/ml cytostatin (CTS) for 1 h at 37°C. Cell lysates were prepared and Western blotting was done using the indicated antibodies. For tyrosine-phosphorylated FAK and paxillin, the cell lysates were immunoprecipitated with anti-FAK or anti-paxillin antibody and immunoblotted with anti-phosphotyrosine antibody.

Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 0.25 mM DTT, 0.1 mM $CaCl_2$ and calmodulin (50 U). The absorbance of released *p*-nitrophenol was determined at 405 nm in a microtiter plate reader.

3. Results

3.1. Cytostatin inhibits intracellular signals of cell adhesion

When B16 cells were plated on a FN-coated plate, the cells were well spread. But by the addition of cytostatin at 10 μg/ml, the cells were rounded and inhibited to spread out on a plate (Fig. 2). It is well known that focal contact proteins such as FAK and paxillin are phosphorylated at tyrosine residues when cells adhere to FN [3–5]. As shown in Fig. 3, when B16 cells were plated on a FN-coated plate for 1 h, some proteins (~70 kDa) were strongly tyrosine-phosphorylated. Furthermore, when FAK and paxillin were immunoprecipitated, they were found to be also strongly tyrosine-phosphorylated. Cytostatin at 10 μg/ml inhibited the tyrosine phosphorylation of those proteins including FAK and paxillin completely (Fig. 3). These results indicated that cytostatin inhibited the intracellular signals of cell adhesion.

3.2. Modification of paxillin by cytostatin

Although cytostatin did not affect the amounts of FAK, vinculin and α-actinin (Fig. 3 and data not shown), it affected paxillin even in the cells on a

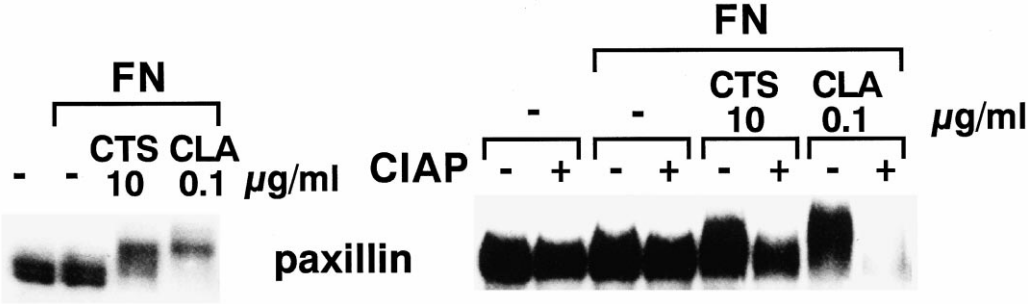

Fig. 4. Phosphorylation of paxillin by cytostatin. B16 cells were inoculated into a plate pre-coated with FN and then incubated with 10 μg/ml cytostatin (CTS) or 0.1 μg/ml calyculin A (CLA) for 1 h at 37°C. (Left) Cell lysates were prepared and immunoblotted with anti-paxillin antibody. (Right) Cell lysates were immunoprecipitated with anti-paxillin antibody, treated with alkaline phosphatase (CIAP) and immunoblotted with anti-paxillin antibody.

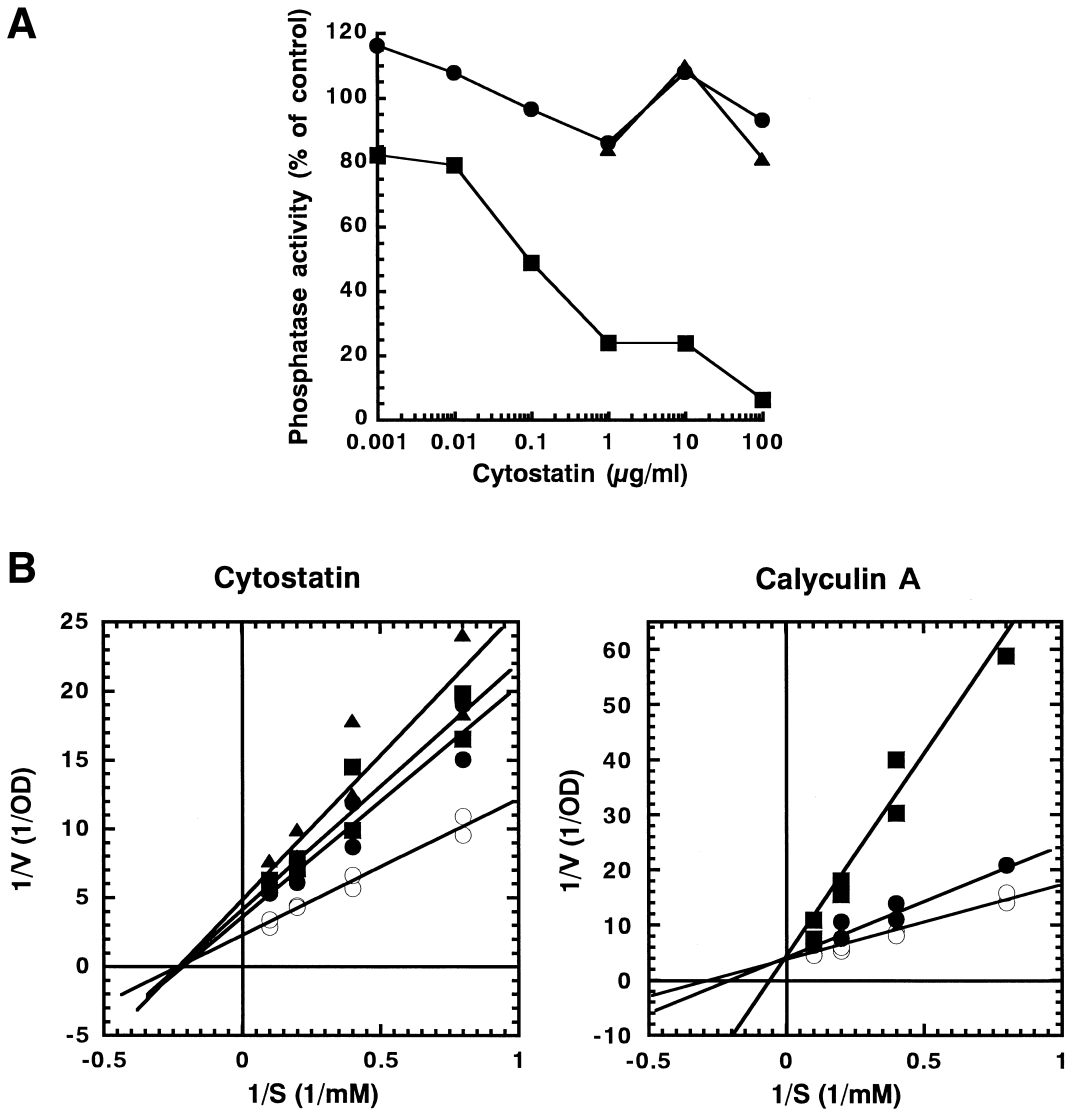

Fig. 5. Inhibition of PP2A by cytostatin. (A) Purified PP1 (●), PP2A (■) or PP2B (▲) was incubated with PNPP in the presence of the indicated concentrations of cytostatin. The phosphatase activities were assessed as described in Section 2. (B) Lineweaver-Burk plot of PP2A. PP2A was incubated with 0 (○), 0.125 (●), 0.25 (■) and 0.5 (▲) μg/ml cytostatin or 0.01 (●) and 0.02 (■) μg/ml calyculin A and the kinetic study was done. K_m for control, 3.4 mM; K_i for cytostatin, 0.3 μg/ml (0.7 μM); K_i for calyculin A, 16 ng/ml (0.016 μM). The values were representative results of three independent duplicate determinations. Each S.E.M. is less than 10%.

non-coated plate. Electrophoretically slow-migrating bands of paxillin on SDS-PAGE appeared by 1 h treatment of cytostatin (Fig. 3).

It is reported that paxillin is retarded electrophoretically by both phosphorylation at serine residues and dephosphorylation at tyrosine residues and dephosphorylation of the slow-migrating paxillin by alkaline phosphatase restores its mobility on SDS-PAGE [13,14]. We looked into the possibility that cytostatin could affect paxillin through serine/threonine phosphorylation comparing a serine/threonine phosphatase inhibitor, calyculin A. As a result, calyculin A at 0.1 μg/ml retarded paxillin on SDS-PAGE more strongly than cytostatin at 10 μg/ml did (Fig. 4). When paxillin was immunoprecipitated and then treated with alkaline phosphatase, both cytostatin- and calyculin A-induced slow-migrating paxillins were disappeared (Fig. 4). Furthermore, a normal

mobility band of paxillin was decreased by both drugs, especially by calyculin A (Fig. 4). These results suggested that cytostatin as well as calyculin A modified paxillin through serine or threonine phosphorylation and decreased it.

3.3. Cytostatin selectively inhibits PP2A non-competitively

To ascertain the possibility that cytostatin could inhibit a serine/threonine phosphatase like calyculin A, the effect of cytostatin on the activities of various protein phosphatases was examined. As shown in Fig. 5A and Table 1, although cytostatin did not affect the activities of PP1, PP2B and alkaline phosphatase even at 100 μg/ml, it inhibited PP2A dose-dependently with an IC_{50} of 0.09 μg/ml (0.21 μM). Compared with other protein phosphatase inhibitors, calyculin A and okadaic acid [15], they inhibited PP2A and PP1 at lower concentrations than that of cytostatin (Table 1). However, cytostatin was found to inhibit PP2A selectively more than calyculin A and okadaic acid and its selectivity was more than 1000 times against PP2A compared with PP1 (Table 1). Thus, cytostatin is rather a selective inhibitor of PP2A. Because of its selectivity, it was suggested that cytostatin inhibited PP2A uniquely. Then, its inhibitory kinetics was examined compared with that of calyculin A. While a Lineweaver-Burk plot represented that calyculin A inhibited PP2A competitively with a substrate, that of cytostatin was revealed to be a non-competitive manner with a substrate (Fig. 5B). These results indicated that cytostatin inhibited PP2A selectively with a unique mechanism.

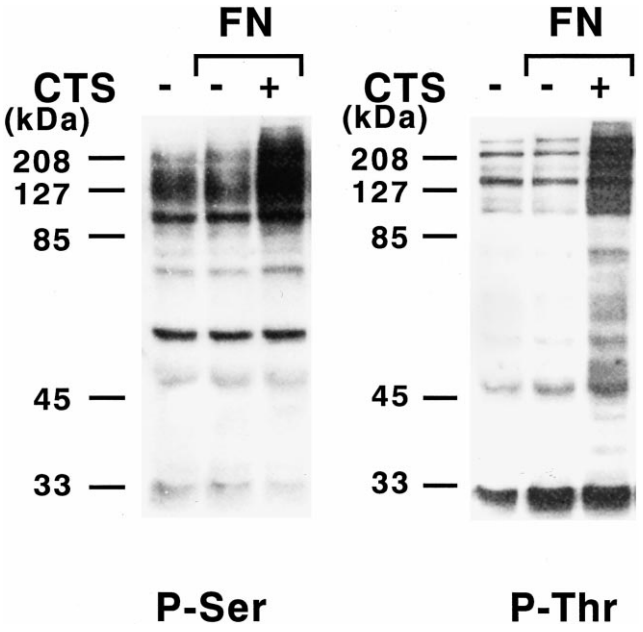

Fig. 6. Increase of serine/threonine-phosphorylated proteins by cytostatin. B16 cells were inoculated into a plate pre-coated with FN and then incubated with 10 μg/ml cytostatin (CTS) for 1 h at 37°C. Cell lysates were prepared and immunoblotted with anti-phosphoserine (P-Ser) or anti-phosphothreonine (P-Thr) antibody.

3.4. Cytostatin accumulates serine/threonine-phosphorylated proteins

Because it is suggested that cytostatin could inhibit intracellular serine/threonine phosphatase, the effect of cytostatin on intracellular serine and threonine phosphorylations of proteins using specific antibodies to phosphoserine and phosphothreonine was examined. Although B16 cell adhesion to a FN-coated

Table 1
Effect of various compounds on protein phosphatases

	PP1 IC_{50} (μM)	PP2A IC_{50} (μM)	PP1/PP2A fold[a]	PP2B IC_{50} (μM)	Alkaline phosphatase IC_{50} (μM)
Cytostatin	>250	0.21	>1 000	>250	>250
DephCTS[b]	n.d.[c]	>250	n.d.	n.d.	n.d.
Calyculin A	0.04	0.004	10	n.d.	n.d.
Okadaic acid	0.005	0.0004	13	n.d.	n.d.

Inhibitory effects of cytostatin, calyculin A and okadaic acid against PP1, PP2A, PP2B or alkaline phosphatase activity were assessed as decribed in Section 2.
[a]IC_{50} in PP1 is divided by IC_{50} in PP2A.
[b]Dephosphocytostatin.
[c]Not determined.

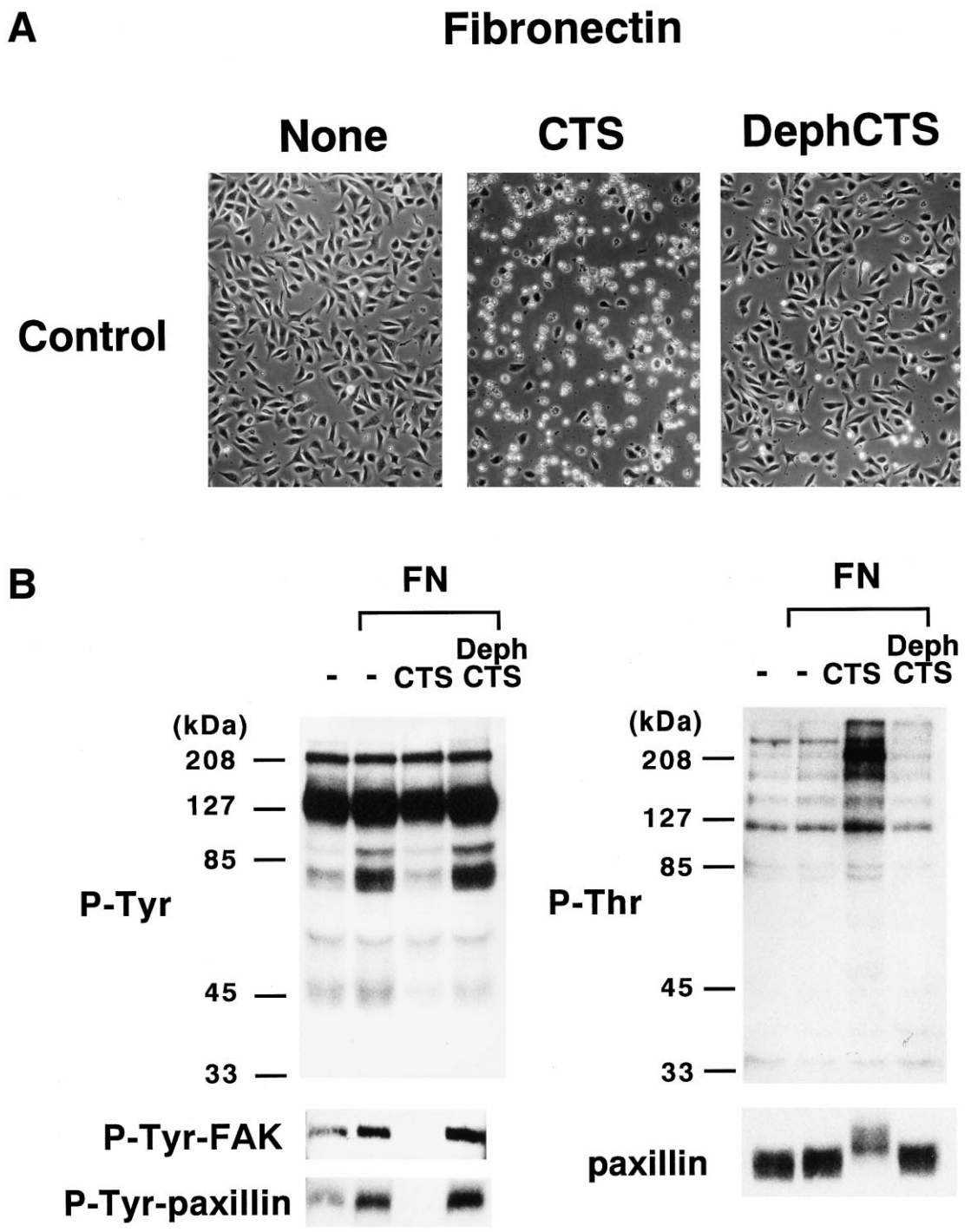

Fig. 7. Effect of dephosphocytostatin on PP2A and B16 cell adhesion. (A) Morphology of B16 cells on a FN-coated plate. The cells were incubated with 10 μg/ml cytostatin (CTS) or dephosphocytostatin (DephCTS) for 1 h at 37°C. (B) Effect of dephosphocytostatin on cell adhesion signals. B16 cells were incubated on a FN-coated plate with 10 μg/ml of cytostatin (CTS) or dephosphocytostatin (DephCTS) for 1 h at 37°C. Western blotting was performed as described in Figs. 3 and 5.

plate did not apparently affect intracellular serine and threonine phosphorylations, cytostatin as expected increased both phosphorylated proteins, especially threonine-phosphorylated ones (Fig. 6). These results indicated that cytostatin inhibited intracellular serine/threonine phosphatase.

3.5. Inhibition of PP2A by cytostatin affects B16 cell adhesion

It is reported that dephosphorylation of calyculin A reduces its phosphatase inhibitory activity [16]. Like calyculin A, a dephosphorylated cytostatin, dephosphocytostatin, lost its inhibitory activity against PP2A (Table 1). To confirm that cytostatin affects B16 cell adhesion through inhibition of PP2A, the effect of dephosphocytostatin on cell adhesion was examined compared with cytostatin. As a result, dephosphocytostatin did not affect cell adhesion and intracellular tyrosine phosphorylation including FAK and paxillin (Fig. 7). Furthermore, it did not increase intracellular serine/threonine phosphorylation and affect paxillin (Fig. 7B and data not shown). Thus, these results indicated that inhibition of PP2A by cytostatin affected the cell adhesion.

4. Discussion

Cytostatin induced cell rounding and inhibited the tyrosine phosphorylation of FAK and paxillin (Fig. 3). However, cytochalasin D, a cytoskeletal actin inhibitor, and herbimycin A, a tyrosine kinase inhibitor, did not induce electrophoretically slow-migrating paxillin (data not shown). Furthermore, cytostatin did not inhibit various protein kinases such as protein tyrosine kinase, protein kinase C and protein tyrosine phosphatase (data not shown). Thus, only actin fiber inhibition or tyrosine kinase inhibition could not affect paxillin. It is reported that during mitosis, paxillin is phosphorylated primarily on serine and dephosphorylated on tyrosine and becomes electrophoretically slow-migrating [13]. Although calyculin A was not reported to affect paxillin [14], both cytostatin and calyculin A were found to modify paxillin through serine/threonine phosphorylation and decrease the protein levels (Fig. 4). Paxillin is known to be important in connecting the integrin with actin fiber, via interacting with various molecules such as vinculin, talin and FAK [1,2]. In fact, it is reported that papillomavirus E6 oncoprotein binds to paxillin and disrupts actin fiber formation, indicating the critical role of paxillin in the maintenance of the actin cytoskeletal network [17]. Thus, changes in phosphorylation and protein levels of paxillin by cytostatin may disrupt the organization of focal contact and then decrease intracellular tyrosine phosphorylation.

In the course of studying the mechanism of cytostatin action, we have found that cytostatin is a selective inhibitor of PP2A. For the interaction between paxillin and PP2A, it is still unknown whether PP2A dephosphorylates paxillin directly in vivo. To understand the role of PP2A in cell adhesion more precisely, we are now studying it in detail. For the inhibition of PP2A by cytostatin, the kinetic study revealed that cytostatin inhibited PP2A non-competitively with a substrate unlike calyculin A (Fig. 5B). This unique inhibitory action on PP2A may result in its selectivity. We have investigated the other related compound to cytostatin in a data base and found that phoslactomycin and fostriecin, structurally similar compounds to cytostatin, also inhibit PP2A [18–21]. Although fostriecin is reported to suppress tumor growth in vivo [22], whether these related compounds also have anti-metastatic activities in vivo has not been reported yet. Thus, this is the first report that describes an anti-metastatic compound that inhibits PP2A selectively. Although we are now studying the precise mechanism by which cytostatin inhibits cancer metastasis in vivo, our results suggest the possible involvement of PP2A in cancer metastasis.

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