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ADHESION-INDUCE PROTEIN TYROSINE PHOSPHORYLATION IS ASSOCIATED WITH INVASIVE AND METASTATIC POTENTIALS IN B16-BL6 MELANOMA CELLS

Yan Chunhong 颜春洪　　Han Rui 韩锐

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050

Objective: The interaction of cancer cell with extracellular matrix (ECM) happens as an earlier and specific event in the invasive and metastatic cascade. To explore the key element(s) in cancer metastasis and observe the cell-ECM interaction and its role. Methods: To interrupt the cell-ECM interaction by suppression of adhesion-induced protein tyrosine phosphorylation with protein tyrosine kinase inhibitor genistein in B16-B16 mouse melanoma cells. Results: When B16-BL6 cells attached to Matrigel, a solubilized basement membrane preparation from EHS sarcoma, a 125 kDa protein increased its phosphotyrosine content dramatically. In contrast, when the cells were pretreated with 20μM or 30 μM genistein for 3 days, it was revealed a less increase in the phosphotyrosine content of this 125 kDa protein in response to cell attachment to ECM was revealed with immunoblot analysis. Accompanied by the lower level of adhesion-induced protein tyrosine phosphorylation the genistein-treated cells exhibited a decrease in their capabilities of adhesion to Matrigel and invasion through reconstituted basement membrane. The potentials of and forming lung metastatic nodules were also shown to be decreased dramatically in these genistein-treated cells. Conclusion: It was suggested that protein tyrosine phosphorylation in cell-ECM interaction might be associated with invasive and metastatic potentials in cancer cells.

Key words: Adhesion, Protein tyrosine phosphorylation, Cell-ECM interaction, Invasion, Metastasis, Genistein

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Metastasis of cancer cells from the local tumor mass to distant site(s) is one of the major causes of death due to cancer. The so-called metastatic cascade includes a large variety of events which are much more complex and explored unclearly.[1] The interaction of cancer cells with extracellular matrix (ECM) seems to be an earlier and specific event during invasion and metastasis.[2] The signaling arisen from cancer cells-ECM interaction was found to involve not only in the migration of cells and expressions of matrix degradative enzymes[3,4] but also in the attachment of escaped cancer cells to the endothelium of microvasculature or lymph follicles.[2] The cell-ECM interaction and associated signal transduction play a pivotal role in the invasive and metastatic cascade.

The molecules that mediate the cell-ECM interaction have already been proved to be integrins. Integrins, a family of cell surface adhesion receptors, are trasmembrane protein heterodimers.[5] Binding of integrins with ECM leads to significant changes of a series of biochemical elements which include tyrosine phosphorylation of cellular proteins.[6] ECM components, such as fibronectin, induced tyrosine phosphorylation of proteins located at focal adhesions when they spread the cells and cluster integrins on the cell surface.[7] Adhesion-induced protein tyrosine phosphorylation was reported in mouse or rat fibroblasts and human KB cells.[7,8] The major tyrosine-phosphorylated protein in response to cell adhesion to ECM is a 125kDa one, which was proved to be focal adhesion kinase (FAK), a protein tyrosine kinase.[7-10]

Phosphorylation of this protein is associated with increase of protein tyrosine kinase activity and v-src transformation of NIH3T3 fibroblasts.[11] Its phosphorylation was also thought to be associated with cytoskeletal assembly and formation of focal adhesions.[8]

Integrins, FAK and other biochemical elements involved in the cell-ECM interaction has already been proven to be associated with invasive and metastatic potentials of cancer cells.[12,13] Protein tyrosine phosphorylation associates all these elements together and affect those elements such as mitogen activated protein kinase kinase (MAPK-K) at the upstream of transcription factors in the signal transduction pathways. It is reasonably assumed that protein tyrosine phosphorylation in the cell-ECM interaction may also be associated with the metastatic potentials of cancer cells. However, untill now, no direct evidence has been accumulated to imply a role of adhesion-induced protein tyrosine phosphorylation in the metastatic cascade. In this study, we suppressed the adhesion-induced protein tyrosine phosphorylation in B16-BL6 mouse melanoma cells with genistein, a potent inhibitor of protein tyrosine kinases. Accompanied by lower level of adhesion-associated protein tyrosine phosphorylation, lower invasive and metastatic potentials were observed in the genistein-treated cells. Therefore, adhesion-induced protein tyrosine phosphorylation might be associated with the invasive and metastatic potentials in cancer cells.

MATERIALS AND METHODS

Cell Culture

B16-BL6 mouse melanoma cells, provided kindly by Prof. I.J. Fidler (M.D. Anderson Cancer Center, Houston, Texas, USA), was routinely cultivated in RPMI 1640 medium supplemented with 10% calf serum. For experiment use, the cells were pretreated with genistein (Wako Pure Chemical Industries, LTD., Japan) for 3 days, dispersed with Cell Dissociation Solution (Sigma Chemical Co.), washed and resuspended in RPMI 1640 medium or PBS.

Immunoblot Analysis of Adhesion-dependent Protein Tyrosine Phosphorylation

The cells were resuspended in RPMI 1640 medium containing 1% BSA, plated onto 60-mm tissue culture dishes which were precoated with Matrigel (1μg/cm^2, Collaborative Research/Becton Dickinson, Bedford, USA), and incubated at 37℃ for 90 min. Adherent cells were lysed in 400 μl modified RIPA buffer (50mM HEPES, pH7.0, 150mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.5 mM sodium orthovanadate, 100mg/ml p-nitrophenyl-phosphate, 10μg/ml aprotinin, 20 μg/ml leupeptin, 1mM PMSF, 0.02%NaN_3).[14] Lysates were collected, centrifuged, and measured with the BSA assay[15] for protein concentrations. Samples were resolved on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, and stained in Ponceaus S. After being blocked in TBS (10mM Tris-HCl, pH7.5, 150mM NaCl) containing 3% BSA, the blot was incubated in 1:2000 dilution of anti-phosphotyrosine antibody, PT66 (Sigma Chemical Co.). Bands of phosphotyrosine-containing proteins were visualized with ProtoBlot Western Blot AP System (Promega Company), according to the manufacture's instruction.

Attachment Assay

Each well of 96-well culture plate was precoated with 0.5μg Matrigel, blocked with 2% BSA for 1 hr at 37℃, and rinsed with 0.1% BSA. B16-BL6 cells were seeded into microwells and incubated at 37℃ for 60 min.[16] Non-adherent cells were washed off with PBS. Followed by being stained with 0.2% crystal violet, the adherent cells were dissolved in 5%SDS/50% ethanol. Absorbance value at 570nm was collected to evaluated the number of adherent cells each well.

Invasion Assay

Chemoinvasion through reconstituted basement membrane was assayed in Transwell Chamber (Costar Scientific Co., Cambridge, USA) as described[17] with little modification. Briefly, polycarbonate filters, 8μM pore size, were coated with 10μg Matrigel (Collaborative Research/Becton Dickinson, Bedford, USA) on the shiny side and 5μg fibronectin (Promega Company) as chemoattractant on the other side. The Matrigel on filters was reconstituted with a drop of serum-free medium at 37℃ for 20 min before use. 2.5×10^5 B16-BL6 cells were resuspended in serum-free RPMI 1640 medium containing 0.1%BSA, seeded

in each chambers and subsequently incubated in CO_2 incubator at 37℃ for 20 hr. Non-invasive cells on the upper side of the filters were wiped off with a cotton swab. The invasive cells on the lower side were fixed, stained with haematoxylin/eosin, and counted. Cell numbers in five randomly selected microscopic fields (×400) were used for statistical analysis.

Assay of Experimental Pulmonary Metastasis

5×10^4 cells were resuspended in 0.2ml PBS and injected into C57BL/6 mouse via the tail lateral vein. The animals were sacrified 21 days later. The tumor nodules on the surface of each lung were counted under a dissecting microscope. Each lung was also weighed to evaluate pulmonary tumor burdon.

RESULTS

Protein Tyrosine Phosphorylation in Response to Adhesion

Matrigel is a solubilized basement membrane preparation extracted from EHS mouse sarcoma, and contains almost all of the ECM components.[18] Immunoblot analysis with an anti-phosphotyrosine antibody PT66 showed that protein tyrosine phosphorylation happened when B16-BL6 cells adhered to Matrigel (Figure 1). Compared with lower phosphorylation level revealed in lysate from cells which were kept in suspension (Figure 1, lane 1), a protein with molecular weight of 125kDa was consistently observed to increase markedly its phosphotyrosine content in response to cell adhesion and spreading (Figure 1, lane 2). However, in lysates from 20 μM or 30μM genistein-treated cells, less increase of phosphotyrosine content in this 125kDa protein was observed (Figure 1, lane 3 and 4). As compared with lysate from control cells, the phosphotyrosine contents in the 125kDa protein were 74.1% or 47.1% in lysates from the cells which were pretreated with 20μM or 30μM genistein for 3 days (Figure 1). The adhesion-induced tyrosine phosphorylation of the 125 kDa protein was suppressed significantly by genistein.

Attachment on ECM

As shown in Figure 2, 20μM and 30μM genistein-treated cells displayed less adherent to Matrigel. The adhesion rate of the genistein-treated cells was decreased about thirteen percent ($P<0.05$) and thirty percent ($P<0.01$) of the control, respectively (Figure 2).

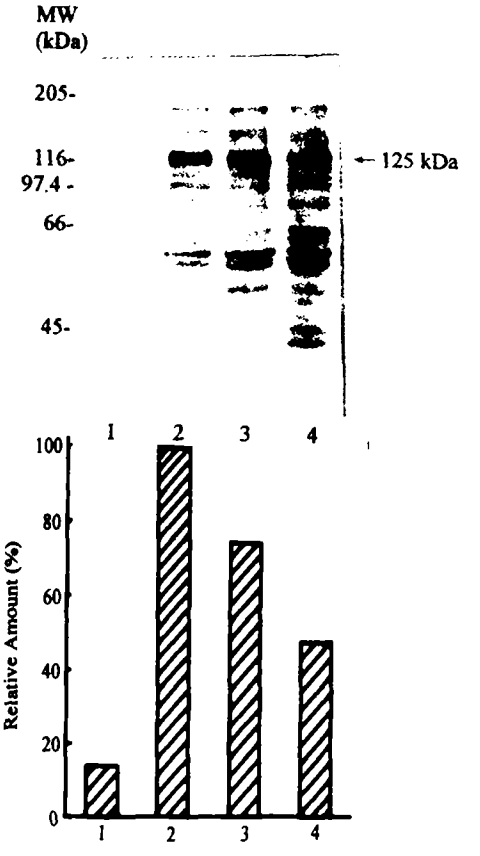

Fig 1. Immunoblot analysis of adhesion-dependent protein tyrosine phosphorylation. Note dramatic increase of phosphotyrosine content in a protein with molecular mass of 125 kDa. The intensity of the 125 kDa band was evaluated with densitometry and presented as relative amount. Lane 1, Lysate from cells kept in suspension. Lane 2, lysate from control adherent cells; Lane 3,4, lysate from cells pretreated with 20μM and 30μM genistein respectively.

Invasive Potentials

Invasion through reconstituted basement membrane prepared with Matrigel was proved to be a useful model to evaluate the invasive potential of tumor cells.[19] In our study, Matrigel was diluted and coated twice. This protocol obtained consistent thickness of Matrigel coating, which was verified by

Coomassie Brilliant Blue staining and light microscopy (data not shown). As depicted in Figure 3, accompanied by the suppression of the adhesion-induced protein tyrosine phosphorylation, invasive potentials of B16-BL6 melanoma cells was decreased dramatically when they were pretreated with 20μM or 30μM genistein for 3 days. Relative to control cells, 63.6% of 20μM genistein-treated cells and 71.5% of 30μM genistein-treated cells were not found to invade through the reconstituted basement membrane.

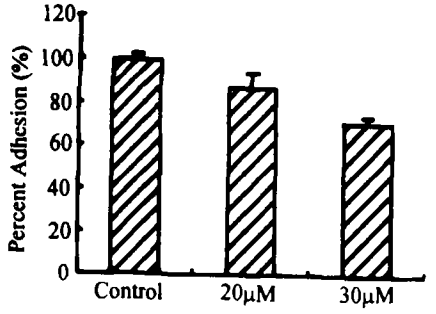

Fig 2. Cell attachment to Matrigel. Values were normalized to the mean value of control and presented as $\bar{\chi}\pm s$ percent adhesion relative to control (n=3).

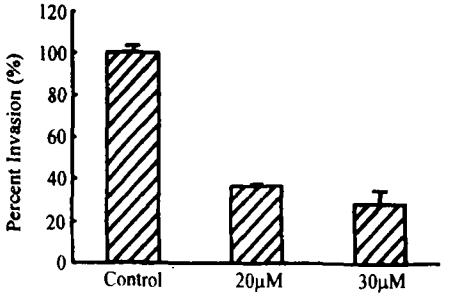

Fig 3. Invasive potentials of B16-BL6 cells. Values were normalized to the mean value of control and presented as $\bar{\chi}\pm s$ percent invasion relative to control (n=3).

Metastatic Potenitals

B16-BL6 melanoma cells preferably form metastases nodules in lung of C57BL/6 mouse. Consistent with lower invasive potentials, genistein-treated cells were observed with lower potentials in production of experimental metastatic lung nodules. Though the incidence of metastasis was just a little lower, the pulmonary tumor burdon was very smaller when the animals were inoculated i.v. the genistein-treated cells. Control cells brought about so heavier a burdon in the animal lungs that the lung weight increased about 3 times as compared with normal value of mouse lung weight. But in the lungs of mice which were injected into the genistein-treated cells, only a few black tumor nodules were observed. The mean number of metastases nodules per mouse was shown to be significant difference between mice injected with control B16-BL6 cells and those injected with genistein-treated cells. The former had a mean number of 154.5±33.6, while the latter with 9.7±11.6 and 12.4±17.8 as the mean metastases number for 20μM and 30μM genistein treatment respectively (Table 1).

Table 1. Metastatic potentials of genistein-treated cells[1]

Cells	Incidence of metastasis	Mean No. of Tumor Nocules each Lung	Lung Weight (g)
Control	10/10	154.5± 33.6	666.8±189.0
Genistein			
20 μM	9/10	9.7± 11.6**	175.9± 27.8**
30 μM	8/10	12.4± 17.8**	193.3±35.3**

[1] The cells were pretreated with genistein for 3 days, and injected i.v. into C57BL/6 mice. Metastatic potentials of the cells were evaluated 21 days later.

[2] Significant difference ($P<0.01$, Student's t-test), compared with control cells.

DISCUSSION

Metastatic potential of tumor cells is thought to be associated with one or more of many different cellular traits, such as adhesion, migration, secretion or activation of matrix metalloproteinases, and angiogenesis.[2] It is exceedingly difficult to attribute metastasis exclusively to any one trait or gene product. However, because cell-ECM interaction happens so often in metastasis cascade, we believe that one key biochemical element involved in cell-ECM interaction would be revealed to be necessity in metastasis formation. Therefore, we treated B16-BL6 melanoma cells with protein tyrosine kinase inhibitor genistein to interrupt the signalings in the cell-ECM interaction and observe the invasive and metastatic behaviors of treated cancer cells.

As our results showed, with immunoblot analysis genistein-treated cells were revealed a lower level of tyrosine phosphorylation of proteins, especially a 125 kDa one, when they were allowed to attach to ECM. It is worth to notice that genistein was not included in the cell suspension when the cells were plated onto ECM-coated dishes, which means that lower level of protein. Our other study showed that at concentrations lower than 100μM, genistein in the cell suppension could not disrupt the ECM signalings directly (manuscript submitted). Genistein is an effective inducer or silencer of gene expression.[20] Maybe genistein affected expression of proteins such as integrins or FAK in the signaling cascade associated with the cell-ECM interaction. In our accompanied studies, genistein was not found to affect the expression of FAK with immunoblot analysis (data not shown). No matter what cause may really ascribe to, the cells, which were revealed with lower phosphotyrosine content in response to adhesion, were found to be lower in not only their potentials to invade through reconstituted basement membrane, but also their lung colonization potentials. Indeed, lower adhesion capability due to disruption of the cell-ECM interaction might be a part of the causes that the invasive and metastatic potentials decreased in the genistein-treated cells.

The major phosphorylated protein in response to adhesion was proved to be FAK, a 125kDa one located at focal adhesion sites.[7-10] Phosphorylation of this protein was associated with cytoskeletal assembly and formation of focal adhesions.[8] FAK was also believed to play an important role in cancer cell dissemination.[19,20] In this study, when B16-BL6 cells were allowed to attach to ECM, a protein with molecular mass of 125kDa increased its phosphotyrosine content. Our preliminary study showed that the phosphorylation level of this protein increased dramatically with the time of cell plating on fibronectin, but increased little higher when the cells were allowed to spread on poly-L-lycine (unpublished data). Immunofluorescence microscopy also showed that adhesion-dependent protein tyrosine phophorylation happened at cell periphery, where focal adhesion contacts formed (data not shown). All these imformation persuaded us to presume that the 125kDa protein in this study is FAK. Lower phosphorylation level of FAK do harm to ECM signal transduction and the cell-ECM interaction, so cytoskeletal assembly and formation of invadopodia was affected, which may ascribe to lower invasive and metastatic potentials of the genistein-treated cells.

In summary, it was found in this study that lower invasive and metastatic potentials were accompanied by the lower level of adhesion-induced protein tyrosine phosphorylation in B16-BL6 melanoma cells treated with genistein. We concluded that protein tyrosine phosphorylation involved in the cell-ECM interaction might be associated with the invasive and metastatic potentials in cancer cells.

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REFERENCES

1. Liotta LA, Steeg PS, Stetler-Stevenson WG, et al. Cancer metastasis and angio-genesis: an imbalance of positive and negative regulation. Cell 1991; 64: 327.
2. Stetler-Stevenson WG, Azanvoorian S, Liotta LA, et al. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9:541.
3. Werb Z, Tremble PM, Behrendtsen O, et al. Signal transduction through the fibronection receptor induces collagenases and stromelysin gene expression. J Cell Biol 1989; 109:877.
4. Stack MS, Gray RD, Piozzo SV, et al. Modulation of murine B16-F10 melanoma plasminogen activator production by sythetic peptide derived from the laminin A chain. Cancer Res 1993; 53: 1998.
5. Dedhar S. Integrin mediated signal transduction in oncogenesis: an overview. Cancer Metast Rev 1995; 14: 165.
6. Clark EA, Brugge JS. Integrins and signal transduction pathways: the road taken. Science 1995; 268: 233.
7. Kornberg L, Earp S, Parsons JT, et al. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. J Biol Chem 1992; 267: 23439.
8. Burridge K, Turner CE, Romer LH, et al. Tyrosine phosphrylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol 1992; 119: 893.

9. Hanks SK, Calab MB, Harper MC, et al. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronection. Proc Natl Acad Sci USA 1992; 89: 8487.
10. Schaller MD, Borgman CA, Cobb BS, el al. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 1992; 89: 5192.
11. Guan J-L, Shalloway D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 1992; 358: 690.
12. Weiner TM, Liu ET, Craven RJ, et al. Expression of focal adhesion kinase gene and invasive cancer. Lancet 1993; 342: 1024.
13. Owens, LV, Xu L, Craven RJ, et al. Overexpression of the focal adhesion kinase (p125^{FAK}) in invasive human tumors. Cancer Res 1995; 55: 2752.
14. McCune BK, Earp HS. The epidermal growth factor receptor tyrosine kinase in liver epithelial cells: the effect of ligand-dependent changes in cellula location. J Biol Chem 1989; 264:15501.
15. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Analyt Biochem 1985; 150:76.
16. Hiscox S, Hallett MB, Puntis MCA, et al. Inhibition of cancer cell motility and invasion by interleukin-12. Clin Exp Metastasis 1995; 13: 396.
17. Saiki I, Murata J, Wataneve K, et al. Inhibition of tumor cell invasion by ubenimax (bestatin) *in vitro*. Jpn J Cancer Res 1989; 80:873.
18. Kleiman HK, McGarvey ML, Hassell JR, et al. Basement membrane complexes with biological activity. Biochemistry 1986; 25: 312.
19. Albini A, Iwamoto Y, Kleinman HK, et al. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 1987; 47: 3239.
20. Zwiller J, Sassone-Corsi P, Kakazu K, et al. Inhibition of PDGF-induced c-jun and c-fos expression by a tyrosine protein kinase inhibitor. Oncogene 1991; 6: 219.