Soluble Fibronectin Activates RhoA and Causes Cytoskeleton-related Changes of Cancer Cells

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Abstract It is well known that fibronectin within extracellular matrix can ligate membrane receptor integrin and induce RhoA mediated signals leading to reorganization of the cytoskeleton and regulation of cell migration. However, a big portion of fibronectin is dissolved in the plasma. Whether soluble fibronectin has similar effect needs to be elucidated. Here we show that adding soluble fibronectin to cell culture medium caused change of RhoA protein from inactive GDP-binding form to active GTP-binding form, with increased association with its substrate. Antibody against $\alpha_5\beta_1$ integrin prevented the activation of RhoA protein. Soluble fibronectin induced F-actin formation of the cells. In human prostate cancer cell line PC-3, soluble fibronectin caused morphological change from polygonal to round and antibody against $\alpha_5\beta_1$ integrin prevented the change. The results revealed that soluble fibronectin could bind $\alpha_5\beta_1$ integrin and induce RhoA mediated signal transduction.

Key words soluble fibronectin; RhoA; integrin

Integrins are membrane receptors that anchor cells to extracellular matrix proteins. They mediate cellular adhesion and motility and play a central role in regulating cell growth and apoptosis [1-3]. Integrin can transduce signals between cells and extracellular matrix (ECM). The interaction between ECM component and integrin leads to reorganization of the cytoskeleton and regulation of cell migration, and activates many intracellular signaling pathways. Such events have been most extensively studied in fibroblasts and linked to activation of small GTPases belonging to the Rho family of small GTP-binding proteins [4]. Rho family of small G proteins acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state, and plays important role in regulating cellular activities [5]. RhoA is a chief member of Rho family of small GTP-binding proteins and mediates signaling relating to cytoskeleton arrangement, migration, proliferation and gene expression. Research data have addressed the significance of the association between integrin and RhoA [6-8].

Fibronectin is a secreted glycoprotein with molecular size of 450 kDa. It is distributed in both unsolvable and soluble forms. The former is mainly in extracellular space or extracellular matrix and the latter is mainly in the plasma. The fibronectin within extracellular matrix is involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape. It can bind integrin on the surface of the cells attached to the matrix and activate integrin-mediated signal transduction [9]. It has been reported that RhoA was activated in cells attached to fibronectin-coated surface [10]. However, it was not clear whether soluble fibronectin could activate RhoA or not. This experiment was designed to investigate the effect of soluble fibronectin on RhoA activity and the consequent cytoskeleton-related changes in human gastric cancer cell line SGC-7901 and human prostate cancer cell line PC-3.

1 Material and Methods

1.1 Cell lines

Human gastric adenocarcinoma cell line SGC-7901 was provided by Institute of Biochemistry and cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Human prostate cancer

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cell line PC-3 was a kind gift from Dr. Renate Pilz in University of California, San Diego, USA.

1.2 Reagents

Dulbecco's modified eagle media (DMEM) culture medium was from Gibco (Grand Island, NY). Fetal calf serum (FCS) and newborn calf serum (NBCS) were from Minhai Bio-engineering Co. Ltd (Lanzhou, China). Mouse monoclonal antibody against RhoA was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against integrin $\alpha_5\beta_1$ was from Chemicon International (Temecula, CA). Goat anti mouse and rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immuno-Research Laboratories (West Grove, PA). Lysophosphatidic acid (LPA) and fibronectin (from bovine plasma) were from Sigma (St. Louis, Missouri). Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Buckinghamshire, England).

1.3 Preparation of Rhotekin-GST

The plasmid DNA encoding RhoA binding domain (RBD) of Rhotekin fused to glutathione-S-transferase (GST) was transfected into *E.coli*. The bacteria were cultured at 37 ℃ over night (o/n) and then induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) at 30 ℃ for 2 h to express proteins. The bacteria cells were lysed with lysis buffer containing 50 mmol/L Tris-HCl pH 7.4, 1% NP-40, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mmol/L phenylmethan-sulfonyl fluoride (PMSF). The lysate was centrifuged and the supernatant was incubated with glutathione beads at 4 ℃ for 2 h. The beads were washed several times with washing buffer containing 50 mmol/L Tris-HCl pH 7.4, 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$ and 1 mmol/L DTT. After the final washing, the beads were suspended in washing buffer containing 10% glycerol and kept in –70 ℃ until use.

1.4 RhoA-GTP pull down assay

Rho activity was measured according to the method from Ren *et al.* [7]. Briefly, 3×10^6 cells were seeded on 10 cm dish. After different treatment, the cells were washed with Tris-buffered saline (TBS) and lysed with 400 μl lysis buffer containing 50 mmol/L Tris-HCl pH 7.4, 1% NP-40, 1% CHAPS, 200 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mmol/L PMSF. The cell lysate was centrifuged to get rid of the cell debris. Ten microliters of the supernatant was kept for loading control and the rest of the supernatant was incubated with GST-Rhotekin-glutathione beads at 4 ℃ for 45 min, with continuous shaking. The beads were washed three times with a buffer containing 50 mmol/L Tris-HCl pH 7.4, 2% NP-40, 200 mmol/L NaCl, and 10 mmol/L $MgSO_4$. After the final washing, 20 μl of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added to the beads and the beads were boiled for 5 min to release proteins.

1.5 Western blotting

SDS-PAGE gels of different concentrations were cast according to the molecular size of target proteins. Samples proteins were accumulated with voltage of 8 V/cm and then separated with voltage of 15 V/cm on gel. After electrophoresis, the proteins on the gel were transferred onto PolyvinylideneFluioride (PVDF) membrane and the membrane was blocked with 3% bovine serum albumin (BSA) in TBS-T for 1 h at room temperature (RT). The incubation with primary antibody was over night at 4 ℃ and the incubation with secondary antibody was 50 min at RT, with three times of washing after each incubation. ECL reagents were used to visualize the positive bands on the membrane. Briefly, same volumes of solution A and solution B were mixed and added onto the protein side of the membrane. The incubation was 1 min at RT. The exposure time of the first film was 15 s. The exposure time of the second film was adjusted according to the extensity of the signals on the first film.

1.6 Fluorescent microscopy

The cells grown on cover slips were fixed with fresh prepared 2% paraformaldehyde in phosphate-buffered saline (PBS). After being penetrated with 0.3% Triton X-100 and washed with PBS, cells were stained with 50 ng/ml rhodamine-conjugated phalloidin for 1 h at RT to visualize filamentous actin. The formation of F-actin of the cells was observed with fluorescent microscope.

2 Results

2.1 Soluble fibronectin activated RhoA in SGC-7901 cells

Adding fibronectin to culture medium of SGC-7901 cells increased RhoA activity in a dosage dependent manner (Fig.1). Pre-incubating the cells with the antibody against integrin $\alpha_5\beta_1$ partially inhibited the RhoA activation induced by soluble fibronectin (Fig.2).

2.2 F-actin formation in SGC-7901 cells treated with soluble fibronectin

To examine cytoskeletal changes induced by fibronectin, we stained F-actin with rhodamine-conjugated phalloidin. SGC-7901 cells growing on glass cover slip showed few F-actins (Fig.3A). Soluble fibronectin dose-dependently increased the formation of F-actin. (Fig.3B, Fig.3C, Fig.3D).

2.3 Soluble fibronectin caused morphological changes of PC-3 cells

Since PC-3 cells changed their configuration much more obviously when they were treated with RhoA activator LPA, the morphological experiment was carried out with these cells. Treating PC-3 cells with soluble fibronectin changed cell configuration from polygonal to round (Fig.4A, Fig.4B). Antibody against integrin $\alpha_5\beta_1$

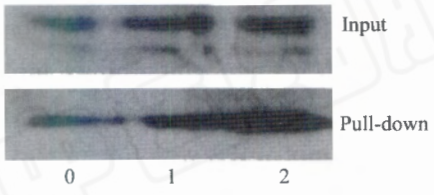

Fig. 1 RhoA activity in SGC-7901 cells treated with soluble fibronectin (FN)

Cell lysate input (2.5% of total lysate) and pull-down of GTP-RhoA were analyzed by Western blotting with an antibody against RhoA. 0: cells without treatment; 1: cells treated with 0.2 μg/ml FN for 15 min; 2: cells treated with 1.0 μg/ml FN for 15 min. The result shown was a representative of three experiments.

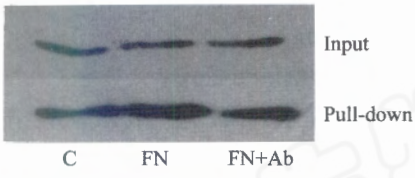

Fig. 2 RhoA activity in SGC-7901 cells treated with integrin antibody followed by soluble fibronectin (FN)

Cell lysate input (2.5% of total lysate) and pull-down of GTP-RhoA were analyzed by Western blotting with an antibody against RhoA. C: cells without treatment; FN: cells treated with 1.0 μg/ml FN for 15 min; FN+Ab: cells treated with integrin antibody (1∶500) for 30 min followed by 1.0 μg/ml FN for 15 min; The result shown was a representative of four experiments.

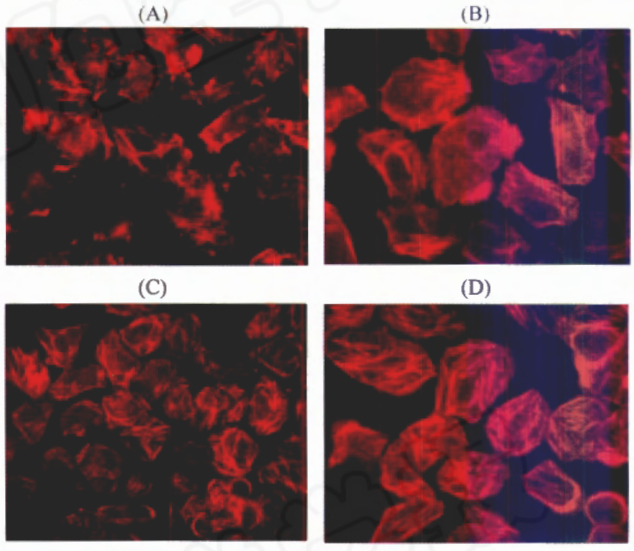

Fig. 3 F-actin formation in SGC-7901 cells treated with fibronectin (FN)

The cells were fixed with fresh prepared 2% polyformaldehy, penetrated with 0.3% Triton X-100 and incubated with rhodamine-conjugated phalloidin for 1 h at RT. (A) SGC-7901 cells without stimulation, nearly no F-actin could be seen. (B), (C), (D) SGC-7901 cells treated with 1 μg/ml FN, 5 μg/ml FN and 10 μg/ml FN respectively for 30 min. Formation of F-actin increased dose-dependently.

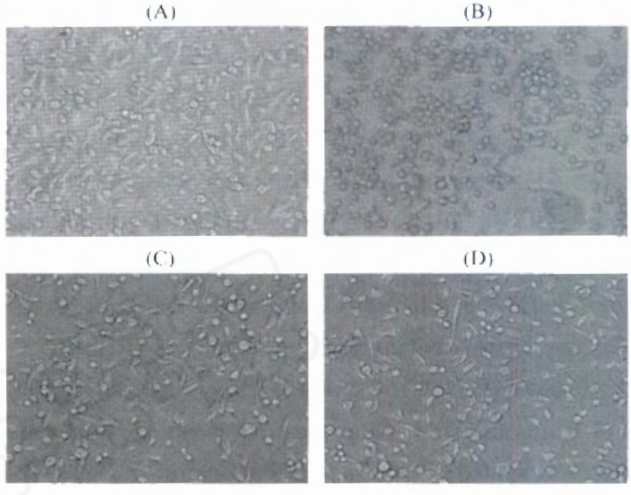

Fig. 4 Morphological changes of PC-3 cells treated with fibronectin, integrin antibody plus fibronectin, and Y27632 plus fibronectin (FN)

Phase contrast image of live PC-3 cell. (A) PC-3 cells without treatment. (B) PC-3 cells treated with 1 μg/ml FN for 15 min. (C) PC-3 cells treated with antibody against integrin $\alpha_5\beta_1$(1∶500) for 30 min and then with 1 μg/ml FN for 15 min. (D) PC-3 cells treated with Rock inhibitor Y27632 (1 μmol/L) for 30 min and then with 1 μg/ml FN for 15 min.

partially blocked the effect of soluble fibronectin (Fig. 4C). The blocking effect was similar to that of Y27632, an inhibitor of RhoA related protein kinase Rock (Fig. 4D).

3 Discussion

Integrin consists of one α and one β subunit. So far, 8 β subunits and 18 α subunits have been found and the subunits may assemble into 24 instinct integrins[11]. Among them, integrin $\alpha_5\beta_1$ is the receptor of fibronectin [12]. When cells attach to extracellular matrix containing fibronectin, integrin $\alpha_5\beta_1$ is activated. The activated integrin then associates with G protein and causes the switch of the protein from GDP-binding to GTP-binding. The GTP-binding G protein can trigger signal transduction and cause alternation of cell activities. There has been a lot of research data about the fibronectin-integrin mediated signal transductions and nearly most of them are based on the interaction between fibronectin and integrin through attachment of the cells on extracellular matrix containing fibronectin [13–15]. However, a big portion of fibronectin is soluble within the body [16]. Soluble fibronectin exists mainly in the blood plasma and can reach each part of the body along the blood stream. There is no research data addressing whether the soluble fibronectin can activate integrin-mediated signal transduction or not. Our results showed for the first time that adding soluble fibronectin to culture medium caused the activation of RhoA, one of the most important events of integrin-mediated signal transduction. This indicated that soluble fibronectin can also bind integrin and activate consequent signal events.

As membrane receptors that anchor cells to extracellular matrix, integrins not only mediate cellular adhesion and motility, but also play a central role in regulating cell growth and apoptosis. It has been proved that engagement of integrins $\alpha_5\beta_1$ can lead to prolonged RhoA activation [7] and many cellular activities induced by integrin ligation appear dependent on RhoA activity, including integrin-induced activation of the MAP kinase pathway, cytoskeletal changes and cell migration[1,3,8,10]. To address the effect of soluble fibronectin further, we observed the formation of F-actin and the morphological change of the cells treated with soluble fibronectin. The results showed that soluble fibronectin increased the formation of F-actin and caused the change of cellular shape from polygonal to round. This indicated that soluble fibronectin could stimulate cell activities such as changes of cytoskeleton and cellular configuration.

Integrin $\alpha_5\beta_1$ and its ligand fibronectin are highly expressed in many cancers including prostate cancers and may influence tumor cell growth, invasion, and metastasis [3]. RhoA is also highly expressed in cancer cells and is pivotal in migration and metastasis of the cells. Our results revealed the association between soluble fibronectin-integrin and RhoA related cell signaling and activity. This will provide available clue and help to illuminate the complicated signaling pathways of cancer cells.

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可溶性纤维连接蛋白可活化 RhoA 并引起癌细胞骨架相关改变

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摘要 细胞外基质中的纤维连接蛋白可以使细胞表面的整合素受体聚集起来, 引起 RhoA 介导的信号通路的活化, 从而导致细胞骨架的重组和细胞迁移的调节。然而, 大部分纤维连接蛋白以可溶形式存在于血浆中, 这些可溶性纤维连接蛋白是否有相似的效应仍有待于进一步的研究。实验发现, 向细胞培养液中加入可溶性纤维连接蛋白, 可使胃癌细胞系 SGC-7901 中的 RhoA 由 GDP 结合的非活性形式转变为 GTP 结合的活性形式, 与其底物结合的量增加, 而 $\alpha_5\beta_1$ 整合素的抗体可以阻断这一活化过程; 可溶性纤维连接蛋白可诱导细胞聚合体形式的肌动蛋白 (F-肌动蛋白)的形成。在人前列腺癌细胞系 PC-3 中, 可溶性纤维连接蛋白可引起细胞从多角形向圆形的形态改变, $\alpha_5\beta_1$ 整合素的抗体可阻断这一改变。以上结果显示可溶性纤维连接蛋白能与 $\alpha_5\beta_1$ 整合素结合并诱导 RhoA 介导的信号转导。

关键词 可溶性纤维连接蛋白; RhoA; 整合素

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