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ORIGINAL PAPER

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Expression of FAK-related non-kinase (FRNK) coincides with morphological change in the early stage of cell adhesion

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Abstract Focal adhesion kinase (FAK), a protein tyrosine kinase, has recently been suggested to play a role in signal transduction through integrins. In fact, FAK is involved in cell proliferation and cell motility by performing signal transduction through integrins. FAK-related non-kinase (FRNK) has been found to be an inhibitor of FAK. As the expression level of FRNK in the cell is very low, the study of FRNK has been preferentially performed by gene overexpression, up to the present, and the role of constitutive FRNK in cells remains unclear. We hypothesized that FRNK is involved in the adhesion of cells to the extracellular matrix (ECM) and investigated the expression of FRNK by time kinetic analysis shortly after cell seeding. We found that FRNK expression was significantly increased in the cells during the early stage of cell adhesion to the ECM. These data indicated that FRNK plays an important role in cell adhesion during the very early stages of cell culture.

Key words Focal adhesion kinase · FAK-related non-kinase · Early stage of cell adhesion · Squamous cell carcinoma

Introduction

The adhesive interaction between a cell and the extracellular matrix (ECM) mediates cell growth, differentiation, and metabolism, all of which are indispensable in maintaining the organism. Cell adhesion is closely related to the phenomenon of signal transduction as well as cell structure formation, cell proliferation, and cell differentiation. Integrin, especially, acts as a representative adhesion receptor in the cell's adhesion to the ECM.[1–3] Integrin controls intracellular information transfer and plays a role in cell differentiation, growth, and migration.

Focal adhesion kinase (FAK) is the most prominent of the tyrosine-phosphorylated proteins acting in cell response to integrin clustering or integrin-mediated adhesion and is a main constituent in the signal transduction of focal adhesion.[4–7] The occurrence of focal adhesion triggers the self-phosphorylation of FAK. FAK contains a proline-rich region in the C-terminal domain, the tyrosine phosphorylation site, and expresses kinase activity. It has been shown that the Tyr397 causing tyrosine phosphorylation is important for the signal transduction mechanism of FAK. Tyrosine phosphorylation is induced, accumulating the signal transduction molecules to form the cytoskeleton.

In cancer invasion and metastasis, two kinds of signal transduction take place in the cancer cell. One involves cytoskeleton proteins and is related to cell metastasis; the transduction acts in focal adhesion with a cytoskeleton protein, which receives the tyrosine phosphorylation. The other type involves a molecule that affects cell motility through the signal transduction system by externally stimulating the growth factor. In cancer cells with high invasion and motility, tyrosine phosphorylation is observed. It has been reported that the FAK signal is increased by Tyr397 phosphorylation in cancer growth and in cancer cell migration and invasion.[8–12] This action causes changes in the adhesion between a cancer cell and the ECM or between cancer cells themselves, generating desorption from the primary lesion and the possibility of metastasis.

In many studies, endogenous expression of FAK-related non-kinase (FRNK) has been utilized as a dominant-negative mutant to inhibit FAK signaling.[13,14] A promoter of FRNK exists in the intron of FAK. On the nucleotide level the proper FRNK arrangement exists on the 5′-side, with the protein translation-starting region located on its back side (FRNK's proper arrangement). A similar arrangement is seen at the amino acid level on the C-terminal side of FAK. A FAT domain is included, which is the integrin binding site. However, FRNK does not have kinase activity.

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Significantly, it has been proven that FRNK reduces the Tyr397 phosphorylation of FAK and that it suppresses cell adhesion, growth, migration, and invasion. It has been reported that FRNK also affects the proliferation of tumor cells.[15–21] Because the expression level of FRNK in the cell is very low, this subject has been researched using FRNK overexpression. The resulting reports have shown that FRNK provides important information regarding the role of FAK in promoting tumor growth, invasion, and metastasis.[22–24] However, they have not revealed the FRNK mechanism; furthermore, none of the studies have been performed without FRNK overexpression.

The present study focused on elucidating the mechanism of cell adhesion during the initial stages of cancer invasion and metastasis and investigating the role of FRNK in squamous cell carcinoma without its overexpression.

Materials and methods

Cell culture

SAS cells, a human squamous cell carcinoma cell line,[25] were cultured in Dulbecco's modified Eagle medium nutrient mixture F-12 ham (Sigma) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere, 5% CO_2, at 37°C.

Cells were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded in 150-mm plasma-coated polystyrene tissue culture dishes or non-plasma-coated culture dishes.

Adhered cells and unattached cells were separated as follows. The dishes were washed two times in phosphate-buffered saline (PBS) to remove the medium. The cells remaining on the culture dishes were regarded as adherent cells while those floating in the medium were regarded as unattached cells. Cells cultured on non-plasma-coated culture dishes also resulted in unattached cells.

Chemical treatments

Actinomycin D was purchased from Sigma-Aldrich, Japan, and Z-VAD-FMK was purchased from R&D Systems (USA). Actinomycin D was kept as a 1.25 μg/μl solution in dimethylsulfoxide (DMSO) at 4°C, and Z-VAD-FMK as a 20 mM solution in DMSO at 4°C.

SAS cells at semiconfluent states were treated with these chemicals (final concentration: actinomycin D, 0.2 μg/ml; Z-VAD-FMK, 5 μM) at the desired final concentration and duration. Control cells were treated with the vehicle (DMSO) used to dilute actinomycin D and Z-VAD-FMK.

RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from cultured cells by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

Reverese transcription (RT) was performed with the Omniscript Reverse Transcription kit (Qiagen, Germany). Polymerase chain reaction (PCR) was performed with the QuantiTect SYBR Green PCR (Qiagen). The PCR conditions were as follows: initial denaturation at 95°C for 15 min, 50 cycles at 94°C for 15 s, 58°C for 30 s, 72°C for 30 s.

The primers were as follows:

FAK (GenBank accession no. L13616): 5′-CTTCTGCAG TTTCCCCAGAG-3′,5′-CCAGGTGGTTGGCTCACT AT-3′

FRNK (GenBank accession no. H38320): 5′-GTGGATGA TCTCCTGCCTTC-3′,5′-GCCTCTTGCACTCCTC ACTC-3′

GAPDH (GenBank accession no. NM002046): 5′-CGACC ACTTTGTCAAGCTCA-3′,5′-AGGGGTCTACAT GGCAACTG-3′

Standard curves were generated using serial dilutions (1–0.0001) for known quantities of SAS cell cDNA.

The mRNA expression level of FAK and FRNK normalized to those of GAPDH were determined by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) and ABI Prism 7000 Software version 1.0.

Western blot analysis

After being incubated for each time period, culture cells were divided into adhesion cells and unattached cells and lysed with RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/m leupeptin, 1 mM Na_3VO_4, and 1 mM NaF]. Cell lysates were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by boiling in SDS buffer at a concentration of 25 μg. (The concentration had been adjusted by freeze-drying, if necessary.)

After electrophoresis, the gel was blotted to nitrocellulose membranes. Membranes were treated with Qentix signal enhancer (Pierce, Rockford, IL, USA) and incubated in blocking buffer [Tris-buffered saline, pH 7.4 (TBS), 1% (w/v) milk (skim milk powder; Wako)] for 1 h at room temperature and then incubated for an additional 1 h in primary antibody [FAK (c-20); Santa-Cruz Biotechnology, Santa Cruz, CA, USA], antiphospho-FAK (Tyr397) (upstate), and monoclonal anti-βb-actin (Sigma) diluted 1:1000 in blocking buffer.

After washing three times in TBS for 5 min each time, membranes were incubated for 1 h at room temperature in secondary antibody (Amersham Bioscience) diluted 1:3000 in blocking buffer. Membranes were again washed three times in TBS for 5 min each. Detection was performed using enhanced chemiluminescence (ECL system for Amersham), Light-Capture (ATTO), and CoolSaver (ATTO).

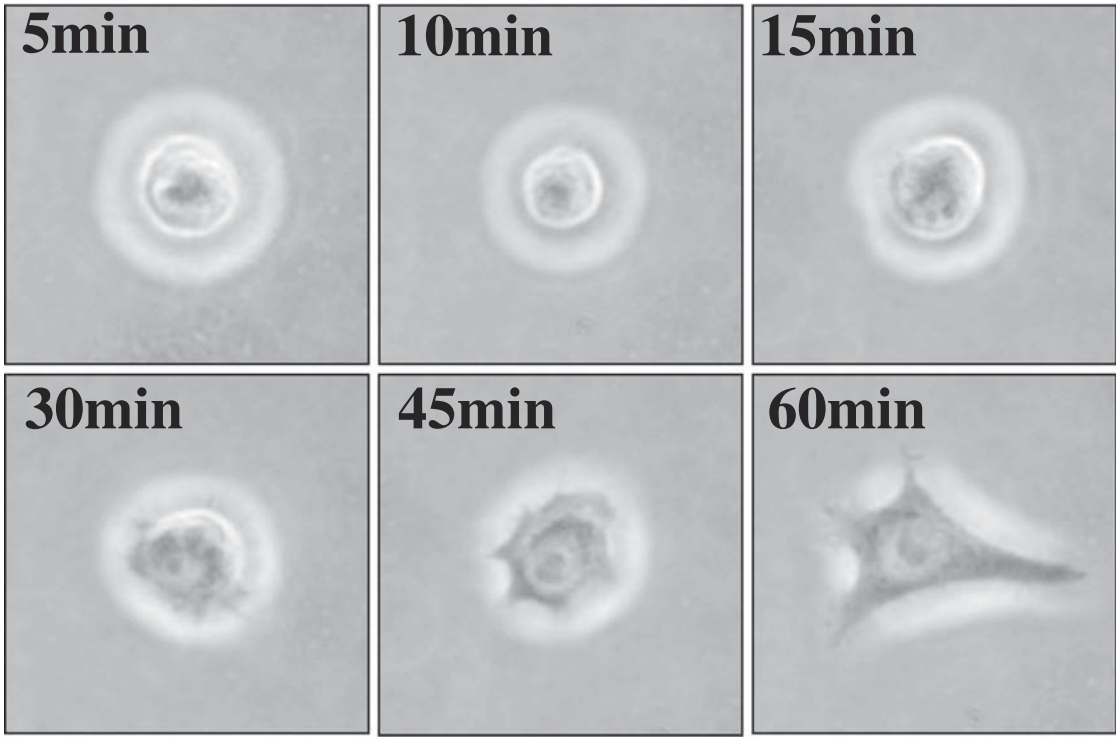

Fig. 1. Morphological change in SAS cells at 5, 10, 15, 30, 45, and 60 min after seeding. The cells were washed by phosphate-buffered saline (PBS) and observed with a phase-contrast microscope

Results

Morphological findings

After culturing the cells for 5, 10, 15, 30, 45, 60, and 120 min, morphological changes were observed (Fig. 1). At 5–15 min, the cell showed a spheroid shape, and the cell adhesion process could not yet be detected. However, after 30 min, there was a tendency of slight expansion to the margin of the cell, and the cell seemed to become attached to the culture dish.

Expression of FAK and FRNK gene transcription in SAS cells during the time course

FAK and FRNK gene transcription products at each period after cell seeding were investigated in the SAS cell line. Cultured cells at semiconfluent density, which stabilizes cell proliferation, were used for the control (Fig. 2).

In 15-min cultivated cells, FRNK gene transcription products were significantly increased by 6.2 times compared to the control ($P < 0.01$). There was a significant decrease in the FRNK gene transcription product of 60-min cultivated cells in comparison with 15-min cultivated cells ($P < 0.05$). No significant difference was seen among the FAK gene transcription product in cells cultivated for 5 or 60 min and the control.

Protein expression of FAK and FRNK

FAK and FRNK proteins produced by the culture cells were extracted after culturing for 0, 5, 10, 15, 30, 45, 60, and 120 min. Protein analysis was performed using the antibody to the C-terminal site of FAK similarly to FRNK by SDS-PAGE and Western blotting (see Fig. 4B). It has been reported that the FRNK protein is generally present in only small amounts in culture cells. Therefore, the detection of FRNK is very difficult. However, in this study, we focused on the early stage of cell adhesion, and FRNK protein could be detected in mRNA expression.

Thus, a band of the FRNK protein was detected in the cultivated cells after culture for 5 min. However, it was detected neither in cells cultured for 30 min nor in the control (Fig. 3).

Distinction between the FRNK and FAK fragments

Because FAK is cut off by caspase 3 and caspase 6, the structure of the C-terminal fragment of cleaved FAK, which resembles FRNK, was examined (see Fig. 4B).[26] We distinguished the degradation products of FAK from FRNK in cells cultured for 15 min by treatment of actinomycin D and Z-VAD-FMK. Actinomycin D induces apoptosis, and Z-VAD-FMK functions as an inhibitor of caspase. Two bands were confirmed after the actinomycin D treatment, and the

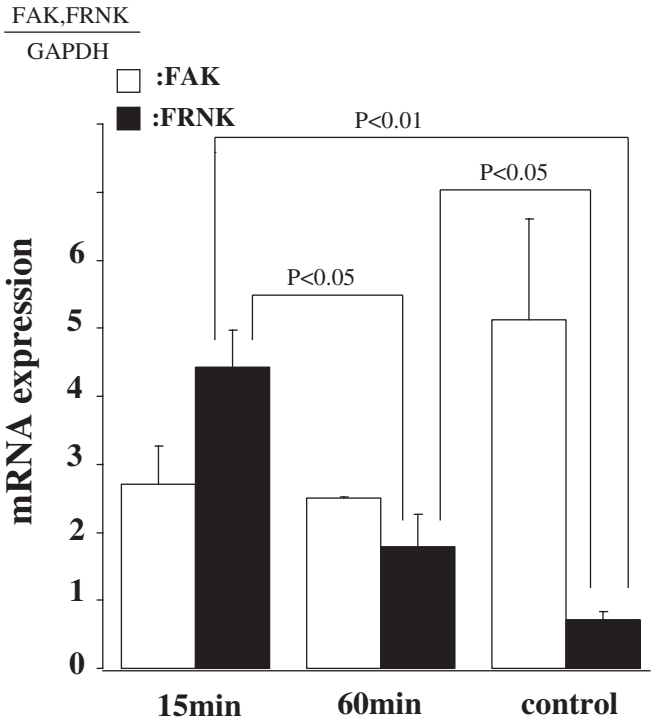

Fig. 2. Focal adhesion kinase (FAK) and FAK-related non-kinase (FRNK) gene transcription products in a time course after cell seeding were searched by real-time polymerase chain reaction (PCR). Semiconfluent culture cells, which stabilize cell proliferation, were used for the control. The *vertical line* of the graph is the relative expression level: i.e., the expression level of FAK or FRNK divided by the expression level of glyceraldehyde phosphate dehydrogenase (GAPDH). □, FAK; ■, FRNK. FRNK gene expression was increased in cells cultured for 15 min in comparison with those cultured for 60 min and the controls. FRNK gene expression decreased with the passage of time

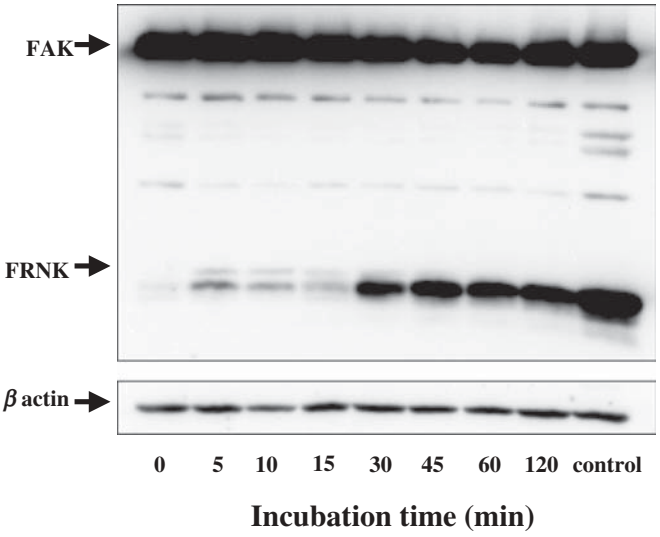

Fig. 3. Western blot analysis of FRNK and FAK. FRNK was detected in three lanes that had been cultured for 5–15 min and was not detected in those cultured for 30 min or more. FAK was detected equally in all

FAK cleavage was confirmed by the decrease of the band by the Z-VAD-FMK treatment. The band of the cell cultured for 15 min expressed at a different position compared with the band of the FAK fragments. A band of FRNK was detected in the cultivated cells after culture for 15 min by treatment of actinomycin D and Z-VAD-FMK (data not shown). Thus, we could conclude that the band expressed in the cells cultivated for 15 min after seeding was the FRNK band and not a product of FAK (see Fig. 4A).

FRNK protein expression in adhered cells and unattached cells

SAS cells were divided into adhered cells and unattached cells; at the same time, the cells were incubated for 15 min without adhesion by the use of a non-plasma-coated culture dish, and their protein was extracted. Afterward, the expression of FAK, FRNK, and the phosphorylation of FAK using the C-terminal side and FAK phosphorylation antibodies was investigated using SDS-PAGE and Western blotting. As a result, the FRNK protein was detected in both the adhered cells and the unattached cells cultivated for 15 min after seeding.

Neither the adhered cells nor the unattached cells cultured for 60 min expressed the FRNK protein. FAK phosphorylation was detected only in the adhered cells (Fig. 5).

Discussion

FAK is a protein tyrosine kinase that is involved in signal transduction through integrin and plays a role in the process of focal adhesion formation during cell migration. FRNK is thought to suppress the activity of FAK. As the expression level of FRNK in the cell is very low, it is difficult to detect without gene overexpression. It has been reported that FRNK overexpression is associated with suppressed cell adhesion and decreased cell chemotaxis in some cell lines.[12,19,24] Therefore, we assume that FRNK is involved in cell adhesion. In this study we investigated the expression of FRNK by time kinetics analysis after cell seeding. In culture for 5–15 min, the cells showed a spherical shape. After 30 min in culture, there was a tendency toward slight expansion at the margin of the cells, and the cells began to attach to the culture dish. After that, adhesion was shown over the entire cell margin, along with focal adhesion formation.[27]

We investigated FRNK gene expression in 15-min cultivated cells showing spherical configuration and compared it with that in 60-min cultivated cells forming focal adhesions. The expression level of the FRNK gene transcription product was significantly higher in the cells after cultivation for 15 min than that in both the control cells under the semiconfluent condition and in the 60-min cultured cells. These results suggested that FRNK is expressed in the cells immediately after seeding but soon disappears with the formation of focal adhesion.

It has been reported that the protein expression level of FRNK is closely related to that of FRNK at the mRNA

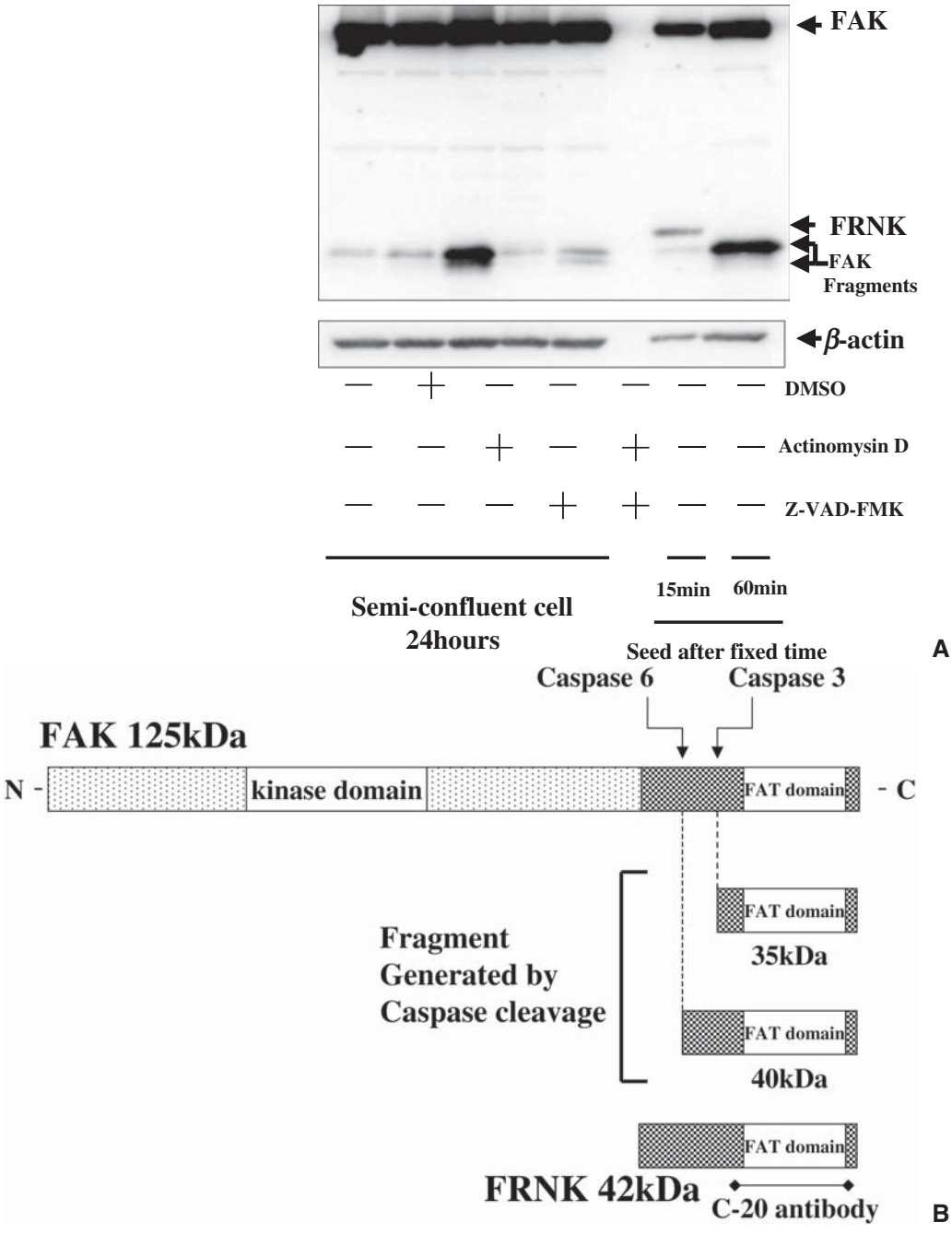

Fig. 4A,B. Comparison between FRNK and degradation products of FAK by apoptosis. **A** Two bands were detected in the lane treated with actinomycin D and decreased by Z-VAD-FMK. Therefore, FRNK was different from the cleavage product of FAK induced by apoptosis. **B** Schematic representation of FAK and FRNK. The positions of the kinase domain and focal adhesion targeting domain (FAT) are illustrated. Also shown are the regions encompassing the epitopes recognized by the C-20 antibody: comparison of the proteolytic fragments generated by caspase cleavage of FAK with the natural competitive inhibitor of FAK and FRNK

level.[15] Immunohistochemical analysis and the Western blotting method are commonly used to investigate protein expression. However, as the protein structure of FRNK is almost identical to part of the C-terminal of FAK, it is difficult to distinguish FRNK from the C-terminal of FAK using the antibody. In this study, a new finding was obtained without FRNK overexpression: We found the expression of FRNK protein was elevated in cells shortly after cultivation. The FRNK band gradually attenuated and finally disappeared after a culture duration of 30 min. This result was obtained without the technique of gene transfection; hence, we consider this the essential action of FRNK.

FAK is cleaved by caspases 3 and 6 at two different sites. The degradation products formed by the C-terminal cleavage of FAK by caspases are 35 kDa and 40 kDa. Importantly, the 40 kDa product of FAK is similar to FRNK in molecular weight and structure. Hence, we treated the cells with actinomycin D and Z-VAD-FMK and changed the expression of caspase in the cells to distinguish FRNK from the cleavage products of FAK. Consequently, we could detect a band that did not change by caspase. This result demonstrated that the band recognized in the cells cultured for 5–15 min was surely FRNK.

It has been reported that FRNK suppresses Src kinase and MAP kinase by inhibiting the activity of FAK phosphorylation and that cell invasion and migration were decreased by FRNK. There is also the question of what kind of culture conditions affect FRNK. In our study, the

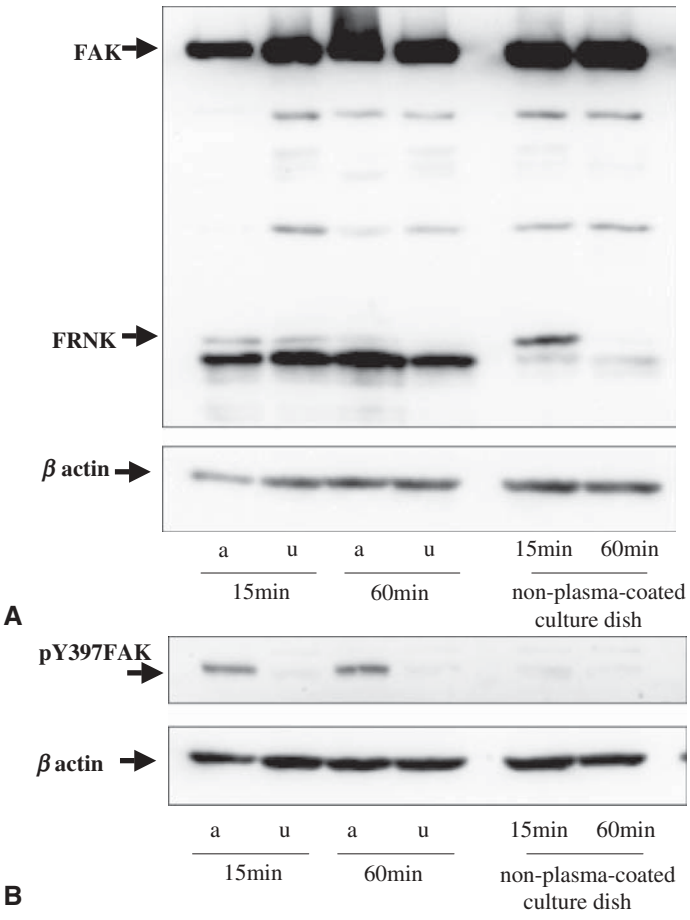

Fig. 5A,B. FRNK protein expression in adhered cells and unattached cells. **A** After incubation for a fixed time, cells were separated into adhered cells and unattached cells, and the protein was extracted. *a*, adhered cells; *u*, unattached cells. *Lane 1*, 15-min culture, adhered cells; *lane 2*, 15-min culture, unattached cells; *lane 3*, 60-min culture, adhered cells; *lane 4*, 60-min culture, unattached cells; *lane 5*, 15-min culture in non-plasma-coated culture dish, unattached cells; *lane 6*, 60-min culture in non-plasma-coated culture dish, unattached cells. FRNK was detected in adhered cells, unattached cells, and unattached cells in a non-plasma-coated culture dish cultured for 15 min. **B** FAK phosphorylation under common condition. FAK phosphorylation was detected only in adherent cells

expression of FRNK was investigated in the early stage of culture for adhered cells and unattached cells. There was no difference in the expression of FRNK between the adhered cells and unattached cells; however, FRNK expression was strongly increased in cells prevented from adhering by the use of a non-plasma-coated culture dish. In addition, the phosphorylation of FAK was recognized only in the adhered cells regardless of culture duration.

In light of the foregoing findings, we focused on the mechanism that determines whether the cells connect to the ECM. Actually, it was reported that cell adhesion decreased with the overexpression of FRNK and that FRNK plays some role in this process until the cells begin to adhere after seeding.[28,29] It has been indicated that FRNK suppresses adhesion and signal transduction involving FAK by inhibiting phosphorylation of FAK. In this study, phosphorylation of FAK was detected in cells at the same time as FRNK, at 15-min culture. However, FAK phosphorylation was not shown in the unattached cells at this stage. FAK phosphorylation was recognized in adhered cells until 60 min, but FRNK expression was not detected at this point; the same pattern was shown in the unattached cells.

The mechanism by which FRNK suppresses the phosphorylation of FAK has not yet been clarified. However, the results of this study suggest that FRNK might not completely inhibit the phosphorylation of FAK in the cell immediately after seeding and that FRNK might have a temporary role until the cell has adhered to various ECM proteins in the initial stage of culture. From these results, the following hypotheses about the function of FRNK can be drawn.

First, it is possible that FRNK adjusts the function of FAK until the cell obtains adequate adhesion to the ECM. Cells combine with the ECM through integrin. When the cell is forming focal adhesions through FAK, the cell's form has a tendency to expand to the margin of the cell; this occurs 30 min after seeding. Before that, the cell shows a spherical shape. The timing of the cell's spheroid shape coincides with the expression of FRNK. It is likely that FRNK regulates cell and ECM adhesion through the action of FAK. The cell's adhesion mechanism is not determined by FAK alone. However, it was reported that FRNK overexpression caused loss of cell adhesion strength. That finding suggests that it is one of the FRNK mechanisms that FRNK is expressed only in the early stage of cell adhesion and plays a role in the cell's adhesion and change in form.

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