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著者	Kawahara Ei, Tokuda Ryouko, Nakanishi Isao
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MIGRATORY PHENOTYPES OF HSC-3 SQUAMOUS CARCINOMA CELL LINE INDUCED BY EGF AND PMA: RELEVANCE TO MIGRATION OF LOOSENING OF ADHESION AND VINCULIN-ASSOCIATED FOCAL CONTACTS WITH PROMINENT FILOPODIA

EI KAWAHARA [1]*, RYOUKO TOKUDA [2], and ISAO NAKANISHI [2]

[1]*Department of Laboratory Sciences, School of Health Sciences, Faculty of Medicine, Kanazawa University, Kodatsuno, Kanazawa 920-0942 and* [2]*Department of Pathology, School of Medicine, Faculty of Medicine, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan*

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* To whom correspondence should be addressed

TEL +81-76-265-2598

FAX +81-76-234-4228

EMAIL kawahara@med.kanazawa-u.ac.jp

ABSTRACT

Cell migration is involved in carcinoma cell invasion and wound healing. We examined motogenic cytokines that potentiated migration of human HSC-3 carcinoma cells. To assess migratory activity, modified Boyden chambers were used. Among a variety of potential motogenic cytokines, EGF enhanced migration of HSC-3 cells both on collagen and fibronectin. PMA also enhanced migration. Inhibitors of protein kinase C completely inhibited PMA-induced migration, but only partly inhibited EGF-induced migration. Protein kinase A was also involved in the EGF-induced signaling pathway for migration. Although the signaling pathways were independent, and the cell shape on collagen was different from that on fibronectin, migratory cells stimulated by EGF or PMA showed common morphology on different ligands. The cells were polygonal or round in shape and the loss of long cytoplasmic extensions was noted. Migratory HSC-3 cells stimulated by EGF or PMA became less adhesive to collagen and fibronectin. Since both EGF- and PMA-stimulated migration did not require de novo protein synthesis, the signaling pathways possibly lead to assembly and disassembly of an actin cytoskeleton. Immunofluorescence for vinculin was concentrated into focal contacts in EGF- and PMA-stimulated HSC-3 cells, whereas the fluorescence signal was hardly detected in non-stimulated cells. Talin and β1 integrin were immunolocalized at focal contacts in non-stimulated cells, and it remained unchanged in stimulated cells. Numerous filopodia visualized with actin immunofluorescence were formed around stimulated HSC-3 cells, whereas filopodia were short and sparse around elongated cytoplasms in non-stimulated cells. Thus, shortening of cytoplasmic extensions with numerous filopodia, loosening of adhesion, and vinculin-associated focal contacts were regarded as migratory phenotypes.

INTRODUCTION

Cell migration is a crucial phenomenon in cancer invasion and wound healing that requires dynamic adhesive interactions between the internal motile structures and the external substratum with adhesion receptors such as integrins serving as the transmembrane link. The signals mediated by integrins regulate the motile machinery and the subsequent directed movement (Clark and Brugge, 1995; Felsenfeldt *et al.*, 1996; Ruoslahti and Giancotti, 1989). In addition to ligand binding, motogenic cytokines are responsible for cell migration when cells have the corresponding receptors (Stoker and Gherardi, 1991). It is now widely accepted that the main target of the signals for gliding and crawling locomotion is the actin cytoskeleton and the adhesive apparatus. Adhesion of protrusion, traction leading the cell body and nucleus to move forward, and tail retraction take place in cooperation with these cytoskeletal components (Lauffenburger and Horwitz, 1996; Mitchison and Cramer 1996; Schmidt *et al.*, 1993). Forward protrusions of the membrane at the front of the cells, referred to as lamellipodia and filopodia, are formed by the reorganization of the actin cytoskeleton and are important structures for cell movement.

Focal contacts are adhering apparatus which represent areas of very close interaction between the cell and the substrate on which actin stress fibers focus (Burridge *et al.*, 1988; Jockush *et al.*, 1995). Integrins also anchor to the apparatus with the linking proteins, vinculin, talin, and α-actinin (Horwitz *et al.*, 1986; Otey *et al.*, 1990). Along with the structural linking proteins, there are signal-transducing proteins including tyrosine kinases, serine/threonin kinases and the corresponding substrates in the focal contacts (Burridge, 1986; Burridge *et al.*, 1988). Focal contacts represent a tightly-adhesive apparatus, the formation of which has been interpreted as a static phenotype rather than a motile one (Couchman and Rees, 1979; Duband *et al.*, 1988). Cell-substrate interactions are required for migration, but strong cell-substrate adhesion inhibits migration (Ruoslahti and Giancotti, 1989). Thus, focal contacts do not seem to be required for migration. However, adhesive forces are needed for locomotion in certain cell sites; the cell-substrate interactions at the cell front must be

stable enough to transmit force to generate traction. Previous studies showed that tyrosine kinases regulating cell motility existed in the protruding structure (Lewis *et al.*, 1996) and microinjection of antibodies against talin inhibited the migration of fibroblasts (Nuckolls *et al.*, 1992). Taken together, focal contacts are considered to play an important role in cell migration. Thus, in order to understand the mechanism of cell motility, it is important to address the question how focal contacts are implicated in cell migration.

Epidermal growth factor (EGF) elicits migration in a wide variety of cells including keratinocytes, columnar epithelial cells, carcinoma cells, Swiss 3T3 cells, fibroblasts, glial cells and endothelial cells (Chinkers *et al.*, 1979; Westermark and Bromquist, 1980; Westermark *et al.*, 1982; Grotendorst *et al.*, 1989; Ridley *et al.*, 1992). It promotes the formation of filopodia, lamellipodia and membrane ruffling (Chinkers *et al.*, 1979; Schlessinger and Geiger, 1981). EGF potentiates the signal-transduction pathway for cell proliferation, which differs from the pathway for migration (Chen *et al.*, 1994; Chen *et al.*, 1994; Klemke *et al.*, 1997), but the responses do not necessarily follow simultaneously after EGF-stimulation (Blay and Brown, 1985). Furthermore, it is well-known that cells alter their shape on different ligands mediated by different integrins. Due to these complex factors, it is difficult to determine which is the migratory phenotype. In the present study, we found that EGF and phorbol ester enhanced migration of HSC-3 carcinoma cells, and they were mediated by independent signal-transduction pathways. Phenotypes in common with migratory cells on different ligands were analyzed by comparing the stimulated cells that showed strong migratory activity with the non-stimulated cells.

MATERIALS AND METHODS

Drugs. EGF (Genzyme, Cambridge, MA, USA), platelet derived growth factor (PDGF, Becton Dickinson, Bedford, MA, USA), transforming growth factor (TGF)-β1 (Genzyme), tumor necrosis factor (TNF)-α (Genzyme), interleukin (IL)-8 (Bachem, USA), fibroblast growth factor (FGF)-1, keratinocyte growth factor (KGF)(Upstate Biotechnology Incorporated, Lake Placid, NY, USA), insulin-like growth factor (IGF)-

1 (Genzyme), and hepatocyte growth factor (HGF, R&D Systems, USA) were used. Phorbol 12-myristate 13-acetate (PMA), cycloheximide and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calphostin C, staurosporine and KT5720 were purchased from Biomol (Plymouth Meeting, PA, USA).

Cell line. Human oral squamous carcinoma cell line HSC-3 obtained from the Human Science Research Resource Bank (Osaka, Japan) were used (Kawahara *et al.*, 1995). Cells were maintained in Eagle's minimum essential medium (MEM, Flow Laboratories, Irvine, Scotland) containing 10% fetal bovine serum (FBS, Gibco BRL., Grand Island, NY, USA) on plastic dishes 10 cm in diameter. The cells of subconfluent cultures were harvested with 1 ml of 0.25% trypsin for 3 min at 37℃ and the trypsin was inactivated by mixing with 4 ml of 0.1% soybean trypsin inhibitor (Sigma) in MEM. The cells were washed with serum-free MEM containing RIA grade 0.5% bovine serum albumin (Sigma, BSA/MEM) and was used for all the experiments in the present study.

Adhesion assay. Polystyrene, 24-well cluster plates (Falcon Products, Oxnard, CA, USA) were coated with 10 μg/ml fibronectin purified from human plasma as described earlier (Kawahara *et al.*, 1989), or collagen type I purified from human placenta (Minamoto *et al.*, 1988) in phosphate-buffered saline (PBS, pH 7.4) overnight at 4℃. The wells were then blocked with BSA/MEM overnight at 4℃. The 50,000 cells, in quadruplicate determinations, were then loaded on ligands or BSA and incubated for different time intervals within 4 hours. Non-adherent cells were removed by gentle washing and remaining adherent cells were quantified by MTT assay (Mossman, 1983).

Migration assay. Cell migration assay was performed on the 24-well Transwell™ chamber containing a polycarbonate membrane with 8 μm-pores (Costar, Cambridge, England). The undersurface of the membrane was coated with 10 μg/ml of human plasma fibronectin or human placental collagen type I in PBS at 4 C overnight. After the ligand solution was removed, the membranes were overcoated with BSA/MEM. Cells were treated with one of the potential motogenic factors for 30 min at 37 C.

After washing by centrifugation, the resuspended cells (50,000 cells) in BSA/MEM, in quadruplicate determinations, were loaded on a membrane and incubated 18 to 24 hours. Cells which had migrated onto the surface of the substrate-coated membrane were fixed and stained with 2% crystal violet in 2% ethanol. After washing dyes with distilled water, dyes staining the cells were eluted with acetic acid. Optical density of the eluate at 495 nm was measured. Background optical density was evaluated on BSA-coated membranes and subtracted from all data points. When cells were sparse, the cell numbers were counted directly under four high-power fields of a microscope.

Immunofluorescence microscopy. Indirect immunofluorescence was performed to examine the phenotypic alteration of actin, vinculin, talin and β1-integrin. Cells treated with EGF or PMA for 30 min at 37°C were loaded on ligand-coated chamber slide glasses (Nalgen Nunc International, Naperville, IL, USA) and incubated for 90 min at 37°C. Cells were fixed with 3% paraformaldehyde for 10 min and cell membranes were permeabilized with 0.1% triton X for 3 min at room temperature. Monoclonal antibodies anti-α-, β- and γ- actin (JLA 20, Calbiochem, Cambridge, MA, USA), anti-vinculin (VIN-11-5, Sigma), and anti-talin (8d4, Sigma) were reacted for 60 min at room temperature. After washing with PBS, FITC-conjugated anti-mouse IgG antibodies (Cappel, West Chester, PA, USA) or FITC-conjugated anti-mouse IgM antibodies were reacted for 60 min at room temperature. Expression of integrins using anti-α2 (P1E6, DAKO Corporation, Carpinteria, CA, USA), -α3 (P1B5, DAKO Corporation), -α5 (P1D6, Chemicon International Inc., Temecula, CA, USA), -α5 (SAM1, Cosmo-Bio CO., Tokyo, Japan), and -β1 (4-145, Sumitomo, Osaka, Japan) was also examined with or without a membrane permeabilizing agent. As a negative control, FITC-conjugated anti-mouse IgG or FITC-conjugated anti-mouse IgM was reacted with cells without primary antibodies, and then non-specific fluorescence was observed around cell nuclei especially in permeabilized cells. As a positive control for α5, an osteogenic sarcoma cell line, OST, was used (Kawashima *et al.*, 1994). The dilutions of JLA20, VIN-11-5, 8d4, PIE6, P1B5, P1D6, SAM1, and 4-145 were 10, 10, 50, 50, 50, 50, 50, and 100, respectively.

RESULTS

EGF potentiates migratory activity of HSC-3 cell on fibronectin and collagen

In migration assays using Transwell™, HSC-3 cells migrated in small numbers both on fibronectin and collagen, but did not migrate on BSA (Fig. 1A). PMA, an activator of protein kinase C (PKC), is known to induce migration [33], on collagen and fibronectin in a dose-dependent manner (1A, C). Among a variety of motogenic cytokines, EGF markedly enhanced migration in a dose-dependent manner at concentrations between 1 and 10 ng/ml both on collagen and fibronectin with more effective activity of migration than observed with PMA (Fig 1B, D). Neither PMA nor EGF induced HSC-3 cell migration on BSA. PDGF (0.2-5 U/ml), TGF-β1 (1-20 ng/ml), TNF-α (1,000-30,000 U/ml), IL-8 (2-50 ng/ml), KGF (0.1-10 ng/ml), IGF-1 (10-1,000 ng/ml) and HGF (1-100 ng/ml) failed to enhance migration. Migratory activity remained unchanged at concentrations over 10 ng/ml of EGF and 5 ng/ml of PMA. We used 10 ng/ml of EGF and 5 ng/ml of PMA in the following experiment.

Signal transduction pathways of PMA- and EGF-induced migration are independent

It has been reported that de novo protein synthesis was required for EGF-induced carcinoma cell migration (Klemke *et al*., 1994; Yebra *et al*., 1995). To examine if there was a time lag for protein synthesis to initiate migration, HSC-3 cells were stimulated with EGF for 30 min, and then allowed to migrate for various time intervals. Migration was measurable within 1 hour both on collagen and fibronectin. Cycloheximide mixed with EGF or PMA was incubated with cells. After washing, cycloheximide was added again in cell suspension and the cells were allowed to migrate. Cycloheximide did not inhibit HSC-3 cell migration at all on collagen or fibronectin (Fig. 2A). Since PMA enhanced cell migration, involvement of PKC in EGF-induced migration was investigated using PKC inhibitors, staurosporine and calphostin C. Staurosporine, which inhibits the kinase domain of PKC, was incubated with cells 30 min prior to the addition of EGF or PMA, mixed with EGF or PMA, and incubated for 30 min at 37° C. After washing, staurosporine was mixed with cells again. Similarly, calphostin C, which inhibits the regulatory domain of PKC, was

reacted with cells 30 min prior to the addition of EGF or PMA, mixed with EGF or PMA, and incubated for 30 min. After washing, the migration assay was performed. The results showed that both PKC inhibitors inhibited PMA-induced migration completely, but 50 n*M* calphostin C inhibited EGF-induced migration by only about 20 % and 5 n*M* staurosporine inhibited EGF-induced migration by about 50 % (Fig. 2B, C). Staurosporine in this concentration reportedly inhibits protein kinase A (PKA) (Schächtele *et al.*, 1988), thus, a specific inhibitor of PKA, KT5720, was used (Kase et al., 1987). KT5720 partly inhibited migration in a dose-dependent manner (Fig. 2D).

Diminution of adhesive ability to ligands coincides with migratory activity

Adhesion assays were performed at 0.5, 1, 2 and 4 hours. Photomicroscopic inspection at 30 min revealed that almost all cells had a rounded morphology and adhered to ligands loosely. After several gentle washings, the number of adhered were decreased, and the remaining adhered cells were then counted by MTT assay. Cells started to spread at one hour, and all the cells adhered tightly by four hours. Cells treated with EGF or PMA had adhered more loosely and showed no spreading even at one hour. This indicated a diminished adhesive ability in the cells treated with EGF and PMA. Through the MTT assay, cells treated with EGF showed diminished adhesive ability at 30 and 60 min (Fig. 3A, B). PMA did not show decreased adhesion within this time course, thus we examined adhesion earlier than 30 minutes. The results showed decreased adhesion of cells treated with PMA on fibronectin at 10 and 20 minutes (Fig. 3C).

Morphological changes induced by EGF and PMA in association with migration

We further examined morphological changes in association with enhancement of migration using phase contrast microscopy. Cells were reacted with EGF or PMA for 30 min at 37°C, loaded on the ligand-coated chamber slide glasses, and incubated for 90 min at 37°C. When HSC-3 cells were not treated with any agent, cells were bipolar or tripolar with elongated cytoplasmic extensions on collagen (Fig. 4a). Cytoplasms were expanded in small areas at the ends of and along the cytoplasmic extensions, and they looked tightly adhered. Cells treated with EGF lost the

cytoplasmic elongation and became round to polygonal (Fig. 4b). Cells treated with PMA were also rounded to polygonal with some spreading, and short, lamellipodia-like projections were seen around cells (Fig. 4c). Cells on fibronectin have several cytoplasmic extensions showing asteroid features with tight attachment to the ligand (Fig. 4d). EGF and PMA on fibronectin altered the morphology of HSC-3 cells in a manner similar to those on collagen (Fig. 4e, f). Common features for migration of HSC-3 cells were shortening of cytoplasmic extensions and loosened attachment to ligands. A small number of cells were found showing wide and round cytoplasms on collagen and fibronectin when cells were treated with PMA or EGF.

Appearance of vinculin-associated focal contacts and filopodia induced by EGF

The signaling pathways for migration mentioned above did not require de novo protein synthesis. These pathways may possibly direct the assembly and disassembly of focal contacts, at least some of which are known to be composed of integrins, actin and a series of focal adhesion proteins. Anti-vinculin and -talin antibodies were used for assessment of focal contacts. Vinculin was hardly found in elongated cells (Fig. 5a), while talin was immunolocalized in focal contacts in a spear-tip shaped appearance at the periphery of focal cytoplasmic expansions at the tip of elongated cytoplasmic extensions (Fig. 5b, c). A few cells were found which were non-polarized large, well-spread, and which showed strong immunofluorescence for vinculin and talin that seemed to stud the cells at the periphery (Fig. 5d). EGF- or PMA-stimulated polygonal cells without long cytoplasmic extensions on collagen and fibronectin had clear vinculin-immunofluorescence at the periphery of the cells (Fig. 5e, f, g). Talin was also found at the periphery of polygonal cells stimulated by EGF or PMA (Fig. 5h). Under high visual fields of an immunofluorescence microscope, numbers of the cells having vinculin- or talin-positive cells in 100 cells were counted in triplicate, and the significance of differences was analyzed by student's t test. EGF- or PMA-stimulated cells showed significantly higher positivity for vinculin than non-stimulated cells did, and there was no significant difference in talin-positive cells (Fig. 6A, B). Using anti-actin antibody, short filopodia were seen around the small focal expansion

of cytoplasmic extensions, and actin fibers were seen along the cell membrane of the extensions (Fig. 7a, c). EGF-stimulated migratory cells had numerous long filopodia around them that were not seen by phase contrast microscope. Numerous filopodia were also seen around PMA-stimulated cells on collagen and fibronectin (Fig. 7b, d).

Localization of integrins

Localization of potential adhesion receptors for collagen or fibronectin were examined using anti-α2, -α3, -α5, and -β1 antibodies. When cells were fixed without permeabilization, punctate immunofluorescence of α2, α3 and β1 subunits was shown diffusely in HSC-3 cells on collagen or fibronectin. The expression was prominent at the periphery of the cells and along the short filopodia (Fig. 8a). α5 was not visualized in HSC-3 cells using anti-α5 antibodies, P1D6 nor SAM1, whereas positive control cells showed positivity with SAM1. EGF- or PMA-induced polygonal cells with numerous filopodia showed α2, α3 and β1 immunofluorescence in a punctate appearance at the periphery of the cells and sparsely at the root of filopodia (Fig. 8b). β3 was weakly stained in a punctate pattern diffuse in the cytoplasm, but it was not shown at the periphery. There were no differences among α2-, α3- and β1-distibution between cells on fibronectin or collagen. A few large, well-spread cells, which were determined to be tightly-adhered stationary cells, revealed not only α2-, α3- and β1-positivity in a punctate manner diffusely in the cytoplasms, but also a spear-tip-shaped fluorescence at the periphery of the cells (Fig. 8c) in a pattern similar to the immunofluorescence of talin or vinculin (Fig. 5d). When cells were permeabilized with triton X 100, immunofluorescence in a punctate pattern disappeared and α2, α3 and β1 integrins were immunolocalized in focal contacts of unstimulated, PMA-stimulated, and EGF-stimulated cells (Fig. 8d, e).

DISCUSSION

The morphology of EGF-induced migratory cells was small, and round to polygonal. This stands in contrast to that of non-stimulated cells with their elongated cytoplasmic extensions. This was an unexpected result as migrating cells have been described as having elongated cytoplasms (Couchman and Rees, 1979). It is important

to identify migratory cells in a cell population which contains cells with various shapes. Our method of evaluation seems reasonable because we observed changes not in individual cells, but in the majority which had increased total migratory activity. Further examination by actin staining revealed numerous filopodia, which have been reported to be important for cell migration, in the round or polygonal cells, as well as lamellipodia, both of which are formed in the movement front (Chinkers et al., 1979). In non-stimulated, less motile cells, small cytoplasmic expansions with a few filopodia were formed at the tips of cytoplasmic extensions similar to the growth cone in neurite outgrowths (Varnum-Finney and Reichardt, 1994). Whereas focal cytoplasmic expansions at the tip and along the cytoplasmic extensions are considered as a tightly-anchored phenotype, this could be an incomplete form of lamellipodia and filopodia since even non-stimulated cells had some migratory activity.

The signaling pathways which lead to cell motility are actualized by the differential activation of effector molecules (Chen *et al.*, 1994; Chen *et al.*, 1994). Our findings suggest that both PKC and PKA are involved in the EGF receptor-mediated signaling pathways which lead to motility in HSC-3 carcinoma cells. PKC activation is reportedly associated with $\alpha v\beta 5$-dependent migration. PKC has been found to be required for cell spreading, formation of focal adhesions, and cell migration (Woods and Couchman, 1992; Yebra *et al.*, 1995). PKC-dependent cell migration is regulated by cytoskeletal association and focal adhesion kinase phosphorylation (Lewis *et al.*, 1996). However, although PKC activator alone efficiently induced migration, PKC was only partly involved in the present migration-directed signaling pathways. PKA was also a minor pathway, and there may be other effector molecules. Previous studies showed that PKC, PKA and/or mitogen-activated protein kinase were required for cell migration. PKA-mediated signaling exclusively directs actin cytoskeletal assembly with or without focal adhesion signal proteins (Leader *et al.*, 1983; Lamb *et al.*, 1988; Morton and Tchao, 1994; Han and Rubin, 1996). Mitogen-activated protein kinase and myosin light chain kinase have recently been found to be involved in EGF receptor-mediated signaling pathways. Thus, there is more than one pathway which

lead to migration, and the primary pathway among one cell population may differ from that in another cell population.

It is another problem whether migration-directed signal transduction pathway needs a transcriptional event. In the present study, EGF-induced migration did not require de novo protein synthesis whereas it has been reported that it was required. Differences in integrin species may bear different signaling, as when Klemke *et al.* (1994) showed that αvβ5-dependent migration required PKC activation, which induced a transcriptional event (Yebra *et al.*, 1995), whereas α2β1-dependent migration in the same cells did not. Although the precise mechanisms which cause the difference between them still remain unclear, the formation of focal contacts may play a key role in migration. In contrast to the classic model, αvβ5 integrins do not localize in focal contacts, spread, nor migrate without stimulation (Carter *et al.*, 1990; Wayner *et al.*, 1991). Many other integrins including α2β1, α3β, α5β1, and αvβ3, concentrate into focal contacts (Carter *et al.*, 1990; Carter *et al.*, 1990; Wayner *et al.*, 1991), as was shown in the present study. Subsequently, those integrins bind to talin, and then appear to function in cell spreading and migration (Carter et al., 1990).

Talin and vinculin, structural protein components of focal contacts, did not colocalize in the non-stimulated cells. Talin was concentrated into focal contacts in the non-stimulated cells and it remained unchanged in the stimulated cells. In contrast, vinculin was seen only in the stimulated cells. It was noted that vinculin was hardly found in cells anchored tightly to the ligand and was, rather, aggregated in focal contacts in the EGF-stimulated cells which were motile and weakly adhesive to the ligand, in spite of the fact that focal contacts were thought to be a tightly-adhesive apparatus. Talin binds to vinculin, integrin, actin (Horwitz *et al.*, 1986) and phospholipids of the cell membrane (Kaufmann *et al.*, 1992), and vinculin binds to talin and actin, and both are thought to be linking proteins focusing actin and integrins on focal contacts. There has been debate as to whether vinculin interacts directly with actin ever since a close connection between them was found (Geiger *et al.*, 1980). Recently, Johnson and Craig (1995) have clarified the regulatory mechanism of direct

actin binding to vinculin and they showed that vinculin did not bind to actin when the F-actin binding site was masked by the intramolecular association of head and tails (Johnson and Craig, 1994; Johnson and Craig, 1995; Gilmore and Burridge, 1995). Taken together with the present study, it appears that once vinculin was activated by EGF, vinculin was able to bind to actin and talin. Thus, EGF regulated the assembly of vinculin into the organized focal adhesion, which elicited migration in cooperation with actin rearrangement.

Vinculin is synthesized in migrating cells (Zieske *et al.*, 1989; Bellas *et al.*, 1991), indicating the importance of vinculin in migration. In this study, de novo synthesis of vinculin was likely as transcription of the vinculin gene reaches its peak value within 60 minutes (Bellas et al., 1991). However, examination using cycloheximide did not show that migration required de novo protein synthesis. Thus, EGF-stimulated vinculin reorganization to focal contacts may be more important than vinculin transcription. Furthermore, a report that a vinculin-deficient cell line extended unstable lamellipodia and filopodia (Varnum-Finney and Reichardt, 1994) supports the important role of vinculin in cell motility. On the other hand, vinculin was found to disappear from adhesion plaques in response to PDGF stimulation, which also induces filopodial formation and the subsequent chemotaxis in 3T3 cells (Herman and Pledger, 1985; Herman *et al.*, 1986; Bornfeldt *et al.*, 1995). Vinculin transfection studies using cDNA and antisense cDNA showed that vinculin correlated with a stationary phenotype (Fernández et al., 1993). By our observations, vinculin positivity was also detected in a few large spreading cells which were regarded as stationary cells. Thus, the importance of vinculin in cell migration may depend on the structure, localization, and its number.

Integrin aggregates which did not colocalize with talin and vinculin were also found when they were immunostained without cell membrane permeabilization. The loss of the immunofluorescence pattern with cell membrane permeabilization could be due to the effect of the surfactant, triton X. Although integrin distribution without permeabilization have been scarcely reported, they have described that there

were numerous integrin aggregaes diffusely on the cell surfaces (Duband *et al.*, 1988; Samuelsson *et al.*, 1993). The integrin aggregates which form small punctate spots on filopodia (Rosen *et al.*, 1990) may indicate a structure which is not linked to talin nor vinculin and they may be necessary in filopodia to engage the force-generating machinery of the cytoskeleton. Furthermore, weakening of adhesion is ubiquitously accepted to be the migratory phenotype, and it may be caused by diminution of integrin receptors or lowered cytoskeletal tension (Crowley and Horwitz, 1995). In the present study, this was probably due to integrin redistribution subsequent to the rearrangement of the actin cytoskeleton (Ali and Hynes, 1977; Horwitz *et al.*, 1986).

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Figure legend

Fig. 1. Stimulatory effects of EGF and PMA on migration of HSC-3 cells. PMA enhances migration on fibronectin (FN, A) and collagen (COL, C). HSC-3 cells do not migrate on BSA (A). EGF enhances migration of HSC-3 cells on fibronectin (B) and collagen (D) in a dose-dependent manner. NT, no treatment. OD, optical density.

Fig. 2. Inhibitory effects of cycloheximide (CHX), staurosporine (SP), calphostin C (CP) and KT5720 on migration. HSC-3 cells were allowed to migrate toward collagen for 18 to 24 hours. CHX does not inhibit EGF-enhanced migration suggesting that the migration does not require de novo protein synthesis (A). An inhibitor of PKC, SP inhibits EGF-induced migration about 50%, whereas it inhibits PMA-induced migration almost completely (B). A specific inhibitor of PKC, CP, inhibits EGF-induced migration only about 20% (C). A specific inhibitor of PKA, KT5720 also inhibits EGF-enhanced migration in part (D).

Fig. 3. Adhesion of HSC-3 cells to fibronectin. EGF diminishes adhesion to collagen (A) and fibronectin at 30 min and 1 hour (B). Early adhesion within 30 min shows diminution of adhesion in PMA- and EGF-treated cells on fibronectin (C).
NT, no treatment.

Fig. 4. Morphological change of HSC-3 cells on collagen (a-c) and fibronectin (d-f). Non-stimulated HSC-3 cells (a, d) have long thin cytoplasmic extensions with focal cytoplasmic spannings at the edge. When HSC-cells are treated with EGF (b, e) or PMA (c, f), both of which have increased migratory activity, shapes are polygonal or round. X 650.

Fig. 5. Immunofluorescence for vinculin and talin. Vinculin is not or scarcely shown in non-stimulated elongated cells on collagen (a). Talin is shown in HSC-3 cells at the periphery of the elongated cytoplasm on collagen (b) and fibronectin (c). A small

number of well-spread, large cells appeared under non-stimulated (d) or stimulated condition on collagen (d) and fibronectin, and spear-tip shaped fluorescence of vinculin at the periphery is marked. Vinculin is concentrated into focal contacts at the periphery of EGF-treated HSC-3 cells on collagen (e) and fibronectin (f), and PMA-treated HSC-3 cells on collagen (g) and fibronectin. Talin is also seen in EGF-treated HSC-3 cells at the periphery of the cell on collagen (h) and fibronectin. Non-specific immunofluorescence is observed around nuclei. X 850.

Fig. 6. Percentages of vinculin- or talin-positive cells. Numbers of vinculin- or talin-positive cells were counted in 100 cells in triplicate. The percentage of vinculin positive cells in EGF- or PMA-stimulated cells were significantly higher ($p<0.05$) than that in non-stimulated cells (A), although there was no significant difference in talin-positive cells (B).

Fig. 7. Immunofluorescence for actin. Non-stimulated cells have short filopodia sparsely around focal cytoplasmic spannings on collagen (a) and fibronectin (c). When cells are treated with EGF, numerous long filopodia appear around cells on collagen (b) and fibronectin (d). X 850.

Fig. 8. Immunofluorescence for integrin β1 in HSC-3 cells on collagen. Cells were fixed without cell membrane permeabilization (a, b, c) and with permeabilization (d, e). HSC-3 cells without stimulation (a) show densely dotted β1 on the cell body, cell processes, and short filopodia. The punctate immunofluorescence are shown sparsely along the long filopodia when treated with EGF (b). In a small number of stationary, well-spread cells showing vinculin-positive focal contacts, β1 concentrates into focal contacts, as well as punctate diffuse immunofluorescence in non-stimulated cells and EGF-stimulated cells (c). When cells were permeabilized, immunofluorescence is observed in focal contacts in non-stimulated cells (d) and EGF-stimulated cells (e). When cells were fixed with permeabilization, immunofluorescence of β1 in non-

stimulated cells (d) and stimulated cells (e) are localized to focal contacts. Non-specific perinuclear fluorescence is observed in permeabilized cells. X 850

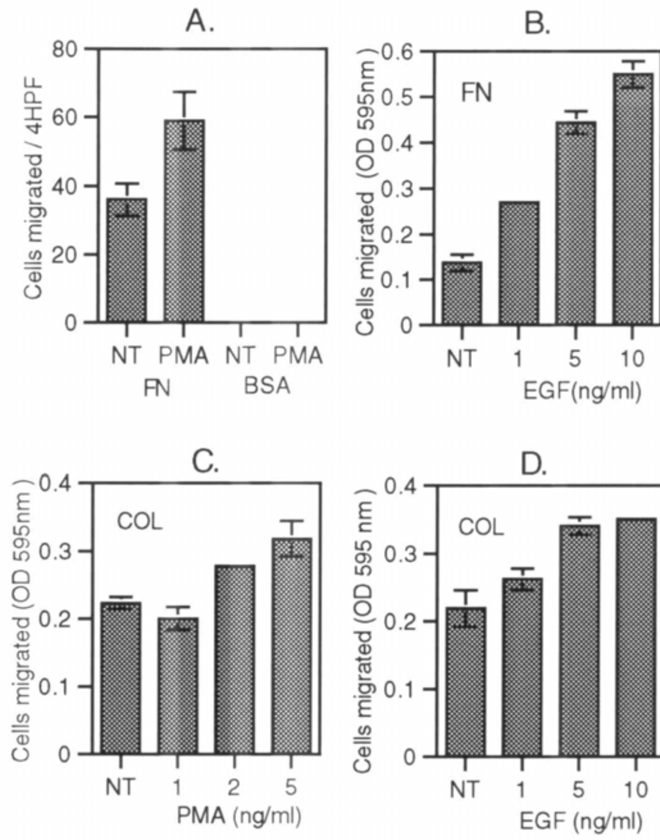
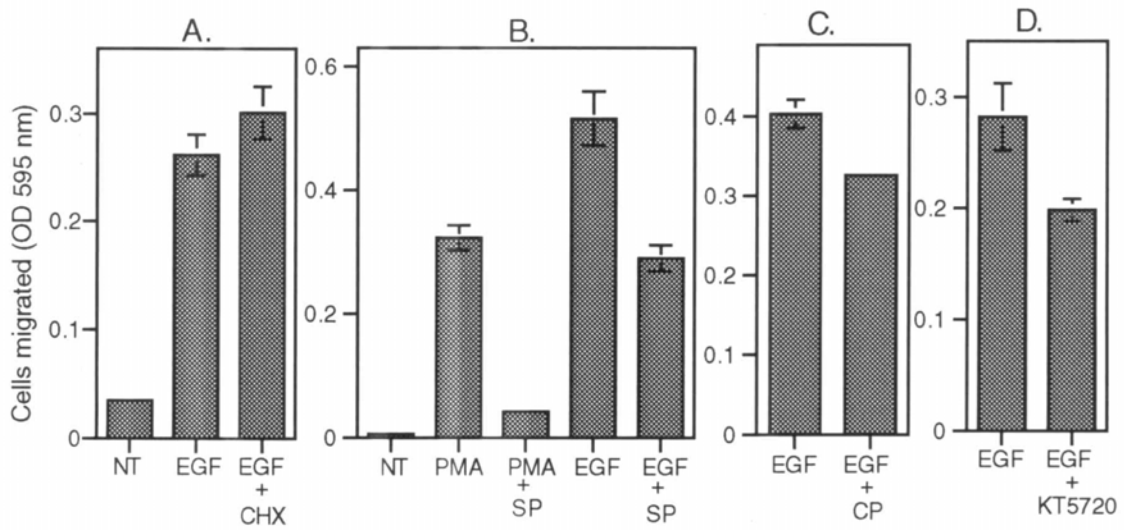


Fig. 1



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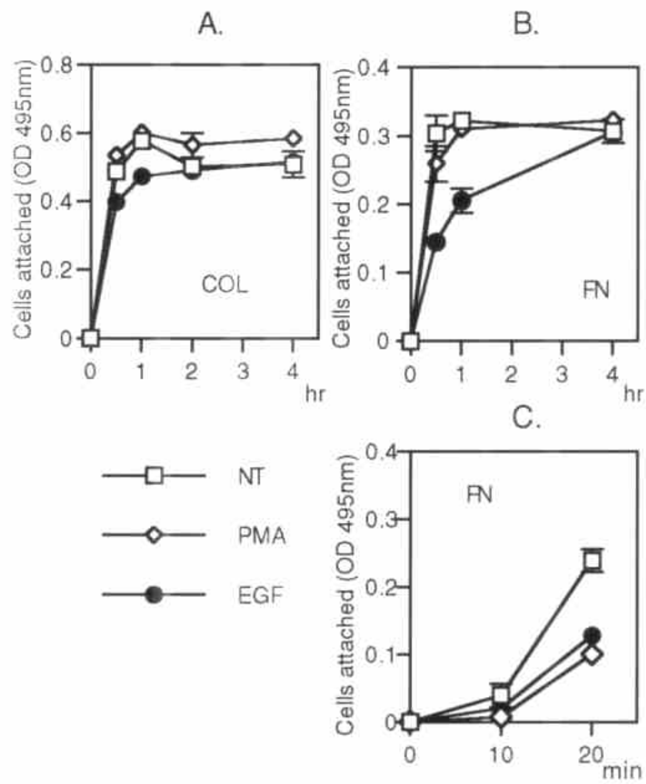
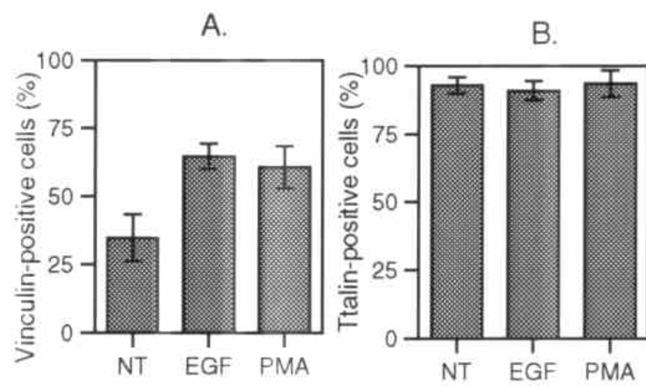


Fig. 3



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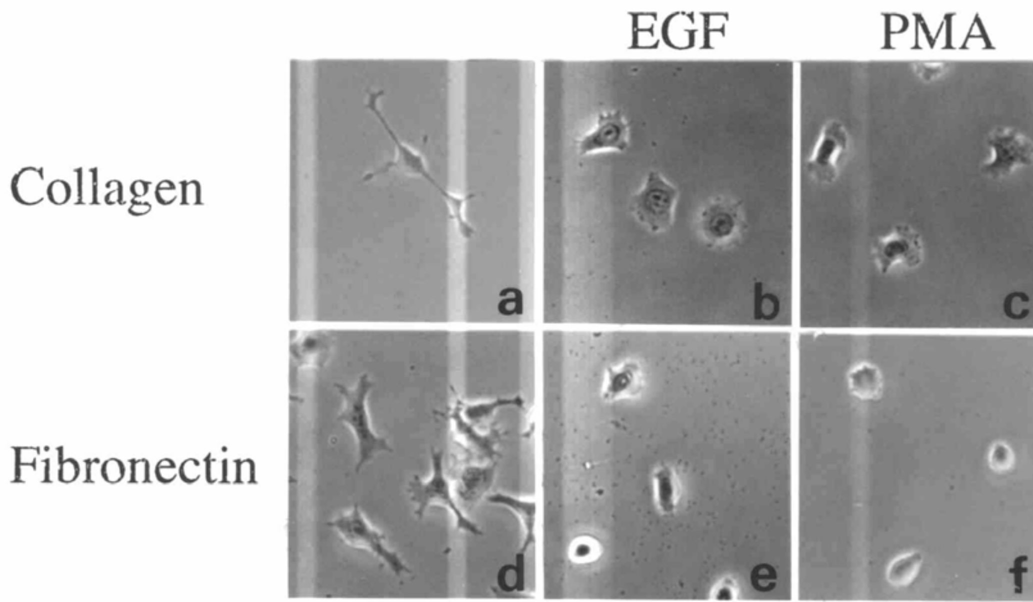

Fig. 4

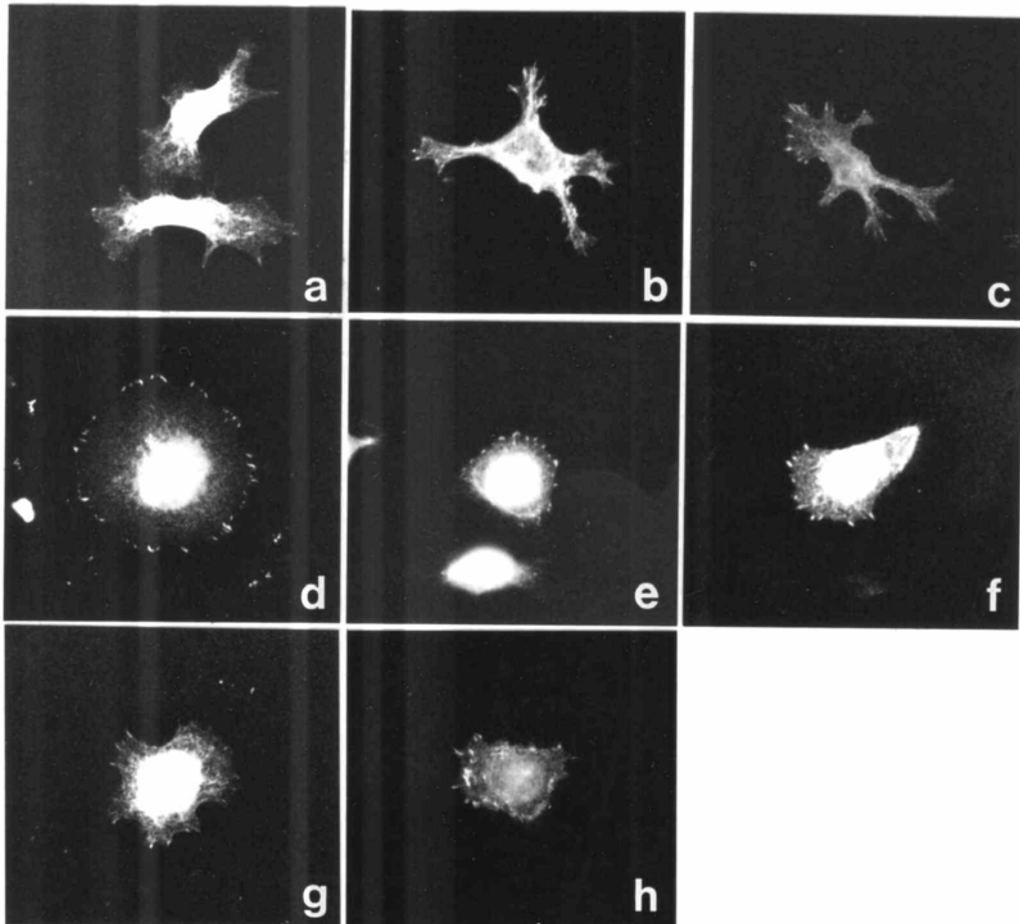

Fig. 5

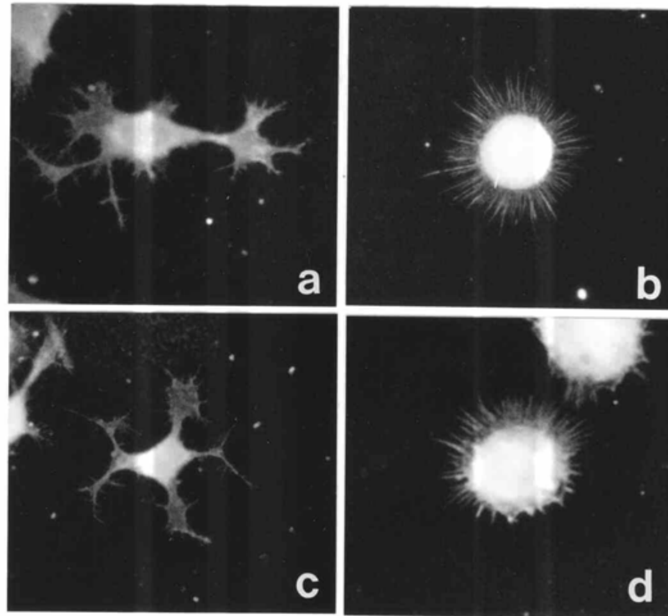

Fig ~~6~~7

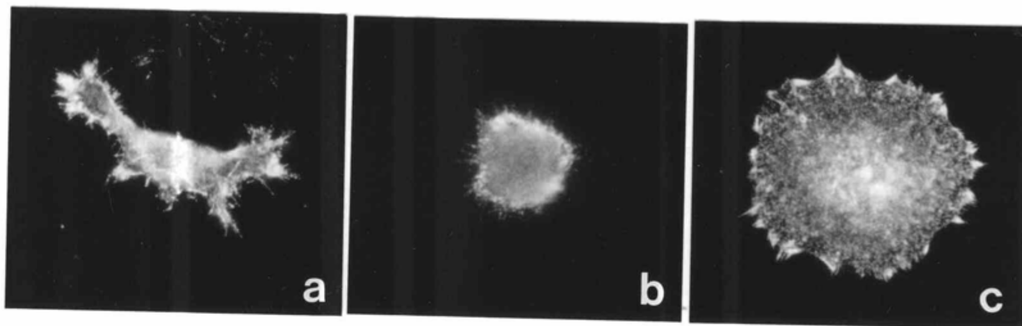

Fig. ~~7~~8