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INVESTIGATIVE REPORT

Activation of Focal Adhesion Kinase in Detached Human Epidermal Cancer Cells and Their Long-term Survival Might be Associated with Cell Surface Expression of Laminin-5

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While normal epithelial cells are anchorage-dependent, cancer cells are anchorage-independent. To elucidate the mechanism underlying the anchorage-independence of cancer cells, we cultured detached cells in medium containing elastase. Detached human epidermal cancer cells (DJM-1) survived for at least 3 weeks and focal adhesion kinase was still phosphorylated. In contrast, most detached keratinocytes underwent rapid apoptosis and focal adhesion kinase was not phosphorylated while the cells were alive. Thus, discontinuation of the phosphorylation of focal adhesion kinase preceded cell death. Immunostaining showed laminin-5 expression on the surface of detached DJM-1 cells, but not on detached keratinocytes. Receptors for laminin-5 (i.e. integrins) were detected on both detached DJM-1 cells and keratinocytes. Laminins are secreted proteins, so we speculated that laminin-5 adhered to the surface of secreting DJM-1 cells via integrins and evoked activation of focal adhesion kinase, with the resultant signalling cascade promoting cellular survival. If this hypothesis is correct, cell surface expression of laminin-5 may be used to explain the characteristics of cancer, immortality, tumour formation, metastasis and angiogenesis. ***Key words: cell surface laminin-5; detached cancer cells; focal adhesion kinase; immortality; integrins*.**

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Apoptosis that occurs after cells have become detached is known as anoikis (1, 2). While normal epithelial cells cannot survive without adhesion to the appropriate extracellular matrix (ECM), detached cancer cells are able to remain viable. Upon adhesion to the ECM, cell surface receptors for ECM proteins, known as integrins (2), form clusters on the cell membrane and promote the assembly of actin filaments. This assembly stimulates further clustering of integrins to form aggregates consisting of ECM proteins, integrins, and actin stress fibres, which are known as focal adhesions. Focal adhesion kinase (FAK) (3–5) is a cytoplasmic protein that localizes at sites of adhesion. FAK interacts with integrin β subunits and its tyrosine 397 is autophosphorylated. Src binds to the autophosphorylation site of FAK and promotes the phosphorylation of other tyrosine residues on FAK. Finally, Src phosphorylates FAK at tyrosine 925 to generate a binding site for the SH2 domain of Grb2. This is one mechanism by which FAK activates the Ras/MAPK pathway. Because of the important role of FAK in the regulation of cell viability and the fact that detached cancer cells can survive, unlike normal cells, we investigated FAK in the present study. We cultured a human epidermal cancer cell line (DJM-1) (6, 7) and its normal counterpart (human keratinocytes) under detached conditions by adding elastase to the culture medium (6, 8). DJM-1 is a cell line established at our laboratory from a malignant trichilemmal cyst, which is a type of epidermal cell carcinoma cell line with intercellular bridges. Previous studies on anchorage-independent survival of cancer cells have employed soft agar, which is a semi-solid medium. In our serum-free liquid cultures, detached DJM-1 cells survived for at least 3 weeks and FAK was kept phosphorylated. In contrast, most of the keratinocytes died within 3 days, leaving a small number of differentiated cells, and FAK showed no tyrosine phosphorylation even when the cells were still viable. It was a surprising finding that FAK was activated in detached DJM-1 cells. Immunostaining showed the expression of both laminin-5 (laminin-332) and its receptor integrins on the surface of detached DJM-1 cells. On the basis of these findings, we suggest that FAK activation in detached DJM-1 cells may be related to surface expression of laminin-5. Cells with laminin-5 expression seem to possess the 4 major characteristics of cancer: immortality, tumour formation, metastasis and angiogenesis.

MATERIALS AND METHODS

Reagents

Several of the reagents used may no longer be available because this study was conducted more than 10 years ago, apart from immunostaining with anti-laminin-5 γ2 chain and the experiment using HeLa cells. Elastase derived from human sputum was purchased from Elastin Products Inc. (Owensville, MO, USA) and Pansorbin was from Calbiochem Inc. (San Diego, CA, USA). Monoclonal anti-FAK antibody (clone 2A7) was obtained from

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Upstate Biotechnology Inc. (Lake Placid, NY, USA); monoclonal anti-phosphotyrosine antibody (clone PY20) from ICN Biomedicals Inc. (Costa Mesa, CA, USA); monoclonal anti-human laminin-5 γ2 chain antibody (clone D4B5) and monoclonal anti-human integrin α3 chain (clone ASC-1), β1 chain (clone LM534) and β4 chain (clone ASC8) antibodies from Chemicon International Inc. (Temecula, CA, USA); peroxidase-conjugated anti-mouse IgG and the immunostaining reagents (ENVISION+ System) from Dako Cytomation (Kyoto, Japan); anti-human integrin α6 chain antibody (clone 450-30A) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); FITC-conjugated rabbit anti-mouse IgG from Zymed Laboratories (Carlsbad, CA, USA). The bromodeoxyuridine (BrdU) uptake assay was performed using "Cell proliferation kit, RNP20", (Amersham Inc., Amersham, UK). Other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

DJM-1, a human epidermal cancer cell line, was cultured in serum-free KGM medium or HuMedia-KG2 medium (Kurabo Inc., Osaka, Japan), which was modified MCDB153 medium supplemented with human epidermal growth factor (0.1 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml) and bovine pituitary extract (0.4%). Human keratinocytes (from neonatal foreskins) were purchased from Kurabo Inc. and were cultured in the same medium as DJM-1 cells. Both types of cells became attached to Petri dishes and proliferated vigorously when cultured in serum-free medium at 37ºC in 5% CO_2/95% humidified air.

We tried several methods to prevent attachment of cells to the dishes during culture for a prolonged period, including coating the Petri dishes with poly(2-hydroxyethyl methacrylate) (9), rotation culture, and culture in the presence of elastase. The last method was found to be most satisfactory, and elastase-mediated cell detachment has already been reported (6, 8). Cells prior to confluence in Petri dishes were detached and dissociated into a single-cell suspension by overnight incubation in the presence of elastase at 10 μg/ml (8.8 units/ml) for keratinocytes and 7 μg/ml (6.1 units/ml) for DJM-1 cells. A higher concentration of elastase was needed for keratinocytes than for DJM-1 cells, which implied that the former cells were more firmly attached than the latter, through an elastase-sensitive anchorage. Detached cells were inoculated into uncoated Petri dishes and cultured in serum-free medium in the presence or absence of elastase at the same concentrations as above. The medium was changed every second day. Viable cells were counted by using a haemocytometer after trypan blue staining. Cells attached to the dishes were counted after dissociation into a single-cell suspension by trypsin treatment.

Immunoprecipitation and immunoblotting

Cells were washed with phosphate-buffered saline (PBS) containing 0.1 mM Na_3VO_4 and lysed at 4ºC in a solution containing 50 mM Tris/HCl (pH 7.5), 4 mM EDTA, 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM Na_3VO_4, 0.1% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, and 1% nonidet P-40 (lysis buffer) (10). Insoluble materials were removed by centrifugation for 10 min at 2000 g and then 10 min at 10,000 g at 4ºC. The same amounts of proteins were used for immunoprecipitation in each experiment. Lysates were pre-cleared by incubation for 2 h with rabbit anti-mouse IgG and then incubation with Pansorbin (a 10% suspension of fixed *staphylococcus aureus* in PBS) (11) for 2 h at 4ºC. The pre-cleared lysates were incubated with anti-FAK antibody (5 μg/ml) overnight at 4ºC. Immune complexes were recovered by incubation with rabbit anti-mouse IgG for 2 h and then with Pansorbin for 2 h at 4ºC. Then the immunoprecipitates were subjected to electrophoresis (7% SDS-PAGE) after boiling in sample buffer with mercaptoethanol and were electro-transferred to nitrocellulose membranes. The proteins were subsequently probed with anti-FAK antibody (1 μg/ml) or anti-phosphotyrosine antibody (1 μg/ml). Immunoreactive bands were visualized by incubation with peroxidase-conjugated anti-mouse IgG and then with 3-amino-9-ethyl-carbazol dissolved in N,N-dimethylformamide.

Immunostaining

Cells that had either been detached for 48 h or were attached and growing exponentially were incubated with anti-laminin-5 γ2 chain antibody overnight at 4ºC, followed by incubation with peroxidase-conjugated anti-mouse IgG for 2 h at room temperature, and reaction products were visualized by incubation with DAB-chromogen solution. Cells were studied with or without fixation in 1% paraformaldehyde in PBS for 10 min, and similar results were obtained. Integrin staining was carried out similarly using FITC-conjugated secondary antibodies and reaction products were visualized with an Olympus BX-FLA fluorescence microscope.

Bromodeoxyuridine uptake

DJM-1 cells cultured under detached conditions for 7 days were incubated with a mixture of 5-bromo-2'-deoxyuridine and 5-fluoro-2'-deoxyuridine at a 10:1 volume ratio for 38 h at 37ºC, centrifuged onto slide glasses using a Cytospin 3 (Shandon Inc., Astmoor, UK), and stained with anti-bromodeoxyuridine antibody according to the manufacturer's instructions.

RESULTS

DJM-1 cells survived in detached culture and retained their proliferative capacity

When keratinocytes and DJM-1 cells (Fig. 1a) were cultured in serum-free medium, both types of cells became attached to the surface of polystyrene Petri dishes

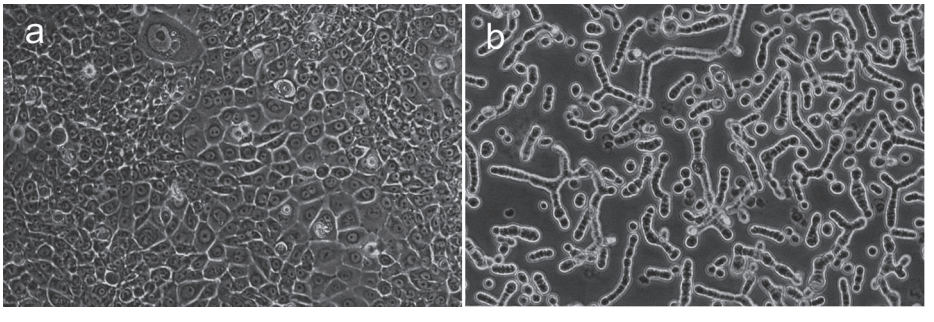

Fig. 1. (a) DJM-1 cells attached and growing exponentially in elastase-free medium. (b) DJM-1 cells that have been detached from the substratum for 2 weeks by culture in the presence of elastase (7 μg/ml). Cells are connected to each other and form linear structures with some branches. Magnification ×200.

and proliferated vigorously. When keratinocytes and DJM-1 cells were cultured in the presence of appropriate concentrations of elastase, attachment to the dishes was completely prevented. Under these conditions, the number of viable keratinocytes decreased rapidly and only about 8% of the seeded cells remained after one week (Fig. 2). In addition, keratinocytes that were incubated for more than 3 days in the presence of elastase lost the ability to attach to the culture dish or to proliferate, i.e. no cell growth was observed after transfer to fresh Petri dishes containing elastase-free medium (data not shown). This indicates that the remaining viable keratinocytes were differentiated cells. In striking contrast, the number of viable DJM-1 cells decreased gradually in detached culture and reached approximately 30% of the seeded cells after 3 weeks. Also, the ability of DJM 1 cells to proliferate was not impaired after culture for 3 weeks in the presence of elastase. As shown in Fig. 2, DJM-1 cells proliferated vigorously when transferred to fresh Petri dishes containing elastase-free medium.

Detached DJM-1 cells made new connections

DJM-1 cells subjected to overnight culture with elastase mostly existed as single cells floating in the medium. However, these cells formed connections with each other to produce lines of cells that showed branches in some places (Fig. 1b). These lines became longer every day and included the majority of the cells in each culture dish from 2 weeks onward. Superficially, these structures resembled the hyphae of fungi. Cell-cell bonds were strong and pipetting could not dissociate the linear structures into single cells.

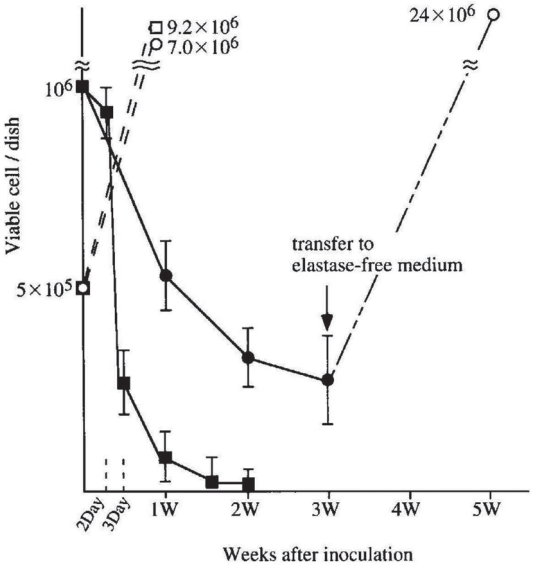

Fig. 2. Survival of detached DJM-1 cells and keratinocytes. DJM-1 cells and keratinocytes were detached and dissociated into single-cell suspensions by overnight culture in the presence of elastase. Dissociated cells were inoculated into Petri dishes and cultured in medium containing elastase at 7 μg/ml for DJM-1 cells and 10 μg/ml for keratinocytes. The medium was changed on alternate days so the cells did not re-attach to the culture dish. Some of the cells were re-suspended in elastase-free medium and were cultured in parallel as a control (attached cells). After 3 weeks, detached DJM-1 cells were transferred into fresh dishes containing elastase-free medium (*arrow*). These cells became attached to the dishes again and proliferated rapidly. Viable cells were counted with a haemocytometer after trypan blue dye staining and results were expressed as the mean±SD (n=3). ●: detached DJM-1 cells; ○: attached DJM-1 cells; ■: detached keratinocytes; □: attached keratinocytes; SD: standard deviation; DJM-1: detached human epidermal cancer cells.

Tyrosine phosphorylation of FAK was sustained in detached DJM-1 cells

As shown in Fig. 3, there was strong tyrosine phosphorylation of FAK in attached keratinocytes (Fig. 3a) or DJM-1 cells (Fig. 3b). Tyrosine phosphorylation of FAK became undetectable in detached keratinocytes after overnight culture in the presence of elastase. FAK protein expression was hardly detectable in these detached keratinocytes, although their viability was above 95%. In contrast, tyrosine phosphorylation of FAK was still prominent in detached DJM-1 cells after culture in the presence of elastase for one week, although the level of tyrosine phosphorylation was lower than in attached DJM-1 cells. FAK protein expression was also reduced in detached DJM-1 cells by comparison with that in attached cells.

Detached DJM-1 cells expressed laminin-5 and integrins

The γ2 chain and β3 chain of laminin-5 are only found in laminin-5 itself. Thereby we used a monoclonal anti-laminin-5 γ2 chain antibody to detect the existence of laminin-5. This antibody clearly stained the surface of detached DJM-1 cells (Fig. 4a), as well as

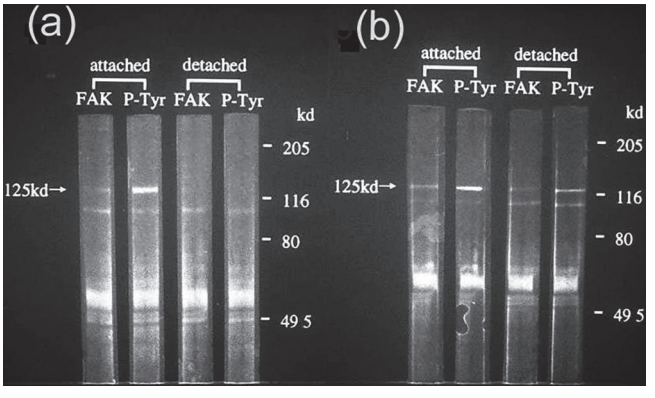

Fig. 3. FAK tyrosine phosphorylation in (a) keratinocytes and (b) DJM-1 cells. FAK was immunoprecipitated with anti-FAK antibody and the immunoprecipitates were further analysed using anti-FAK and anti-phosphotyrosine antibodies. (a) "Attached" indicates keratinocytes attached to Petri dishes and growing exponentially, while "detached" means keratinocytes detached by overnight culture in the presence of elastase. (b) "Attached" indicates DJM-1 cells attached to Petri dishes and growing exponentially, while "detached" means DJM-1 cells cultured for 7 days in the presence of elastase. FAK is denoted by the arrow at 125 kD.

attached DJM-1 cells (Fig. 4b). In contrast, detached keratinocytes were not stained at all (Fig. 4c), Attached keratinocytes showed 2 types of staining (Fig. 4d, 4e). Keratinocytes that were growing exponentially (Fig. 4d) were stained membranously. Some of the keratinocytes cultured for a further 7 days (Fig. 4e) showed dense cytoplasmic perinuclear staining. These cells may be the differentiating cells. Integrin subunits, α3, α6, β1 and β4 chains, were detected on the surface of both kinds of cells, whether they were attached or detached, indicating the presence of receptors for laminin-5 (i.e. integrin α3 β1, α6 β4 and α6 β1) (Figs 5 and 6). No reduction in the staining intensity of the detached cells was apparent.

Bromodeoxyuridine (BrdU) uptake by detached DJM-1 cells

BrdU uptake was only seen in some of the detached DJM-1 cells. Among these cells, uptake was stronger for single cells (Fig. 7a) than for cells connected to each other in lines (Fig. 7b).

DISCUSSION

The number of viable keratinocytes decreased rapidly after detachment with remaining small number of differentiated cells (Fig. 2). Rapid decrease of detached keratinocytes was mediated by the detachment and not by the toxicity of elastase because keratinocytes cultured in the presence of elastase at a concentration that did not evoke the detachment (3 μg/ml) continued to proliferate for at least 4 days (data not shown). Furthermore, keratinocytes cultured in the presence of elastase at 46 μg/ml, a far higher concentration than 10μg/ml, together with ONO-5046, an elastase specific inhibitor, were not detached from the substratum and their viability was not impaired (8). Thus the rapid decrease of detached keratinocytes was anoikis. Prior to the occurrence of anoikis in keratinocytes, FAK showed no tyrosine phosphorylation (Fig. 3). In contrast, DJM-1 cells survived for a prolonged period in detached culture and FAK showed persistent tyrosine phosphorylation. Thus prolonged survival of detached DJM-1 cells seemed to be associated with FAK tyrosine phosphorylation.

It was a surprising phenomenon that FAK remained active in detached DJM-1 cells. FAK is normally activated when cells become attached to appropriate matrix proteins (2–5). In the case of keratinocytes, the matrix protein that directly binds to these cells *in vivo* and activates FAK is laminin-5 (12–15), which binds to keratinocytes via laminin receptors on the cell membrane known as integrins α3β1, α6β4, and α6β1. BP180 (typeXVII collagen), another major components of anchoring filaments, interacts with the β4 integrin subunit through its intracellular amino-terminus (16), but the involvement of BP180 in FAK activation is unknown. Because detached DJM-1 cells became connected to each other in lines (Fig. 1b), we initially thought that such cell-cell contact might have been the cause of FAK activation. If laminin-5 was expressed on the surface of a cell, integrins on the neighbouring cell could bind to it and activation of FAK would occur. Laminins (17–19) are secreted proteins (20, 21) that are not thought to be associated with the cell surface. However, our immunostaining experiment clearly demonstrated the expression of laminin-5 on the surface of detached DJM-1 cells (Fig. 4a). In contrast,

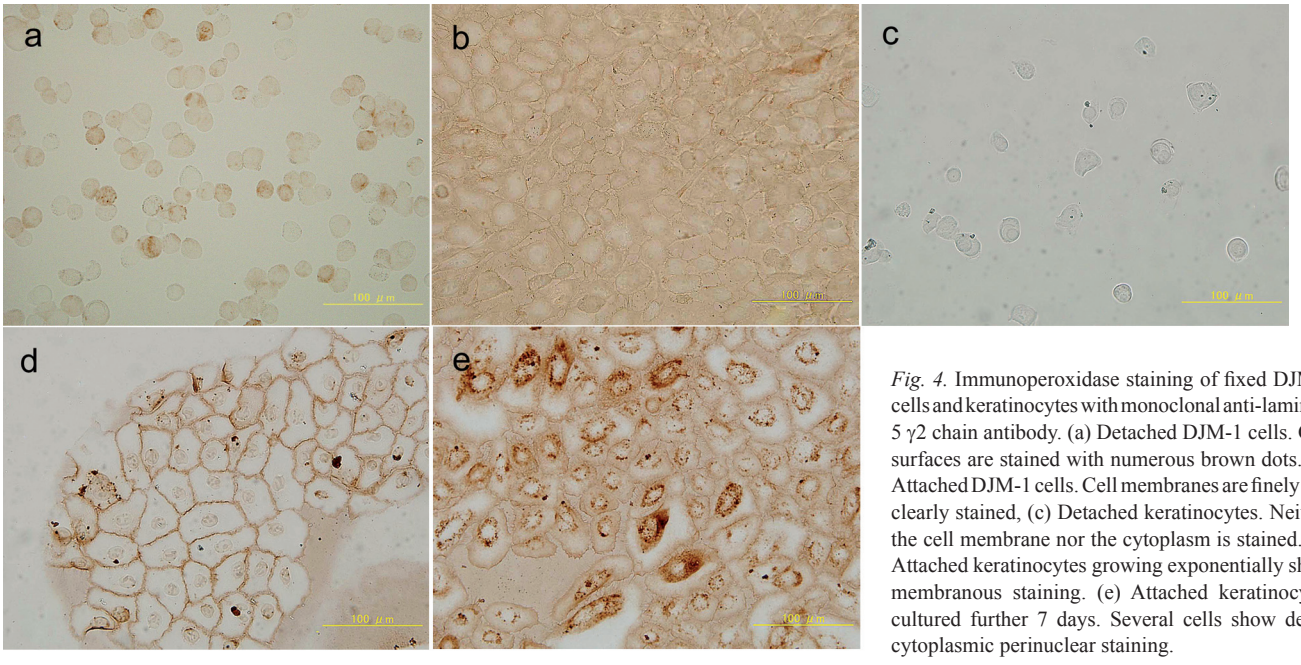

Fig. 4. Immunoperoxidase staining of fixed DJM-1 cells and keratinocytes with monoclonal anti-laminin-5 γ2 chain antibody. (a) Detached DJM-1 cells. Cell surfaces are stained with numerous brown dots. (b) Attached DJM-1 cells. Cell membranes are finely and clearly stained, (c) Detached keratinocytes. Neither the cell membrane nor the cytoplasm is stained. (d) Attached keratinocytes growing exponentially show membranous staining. (e) Attached keratinocytes cultured further 7 days. Several cells show dense cytoplasmic perinuclear staining.

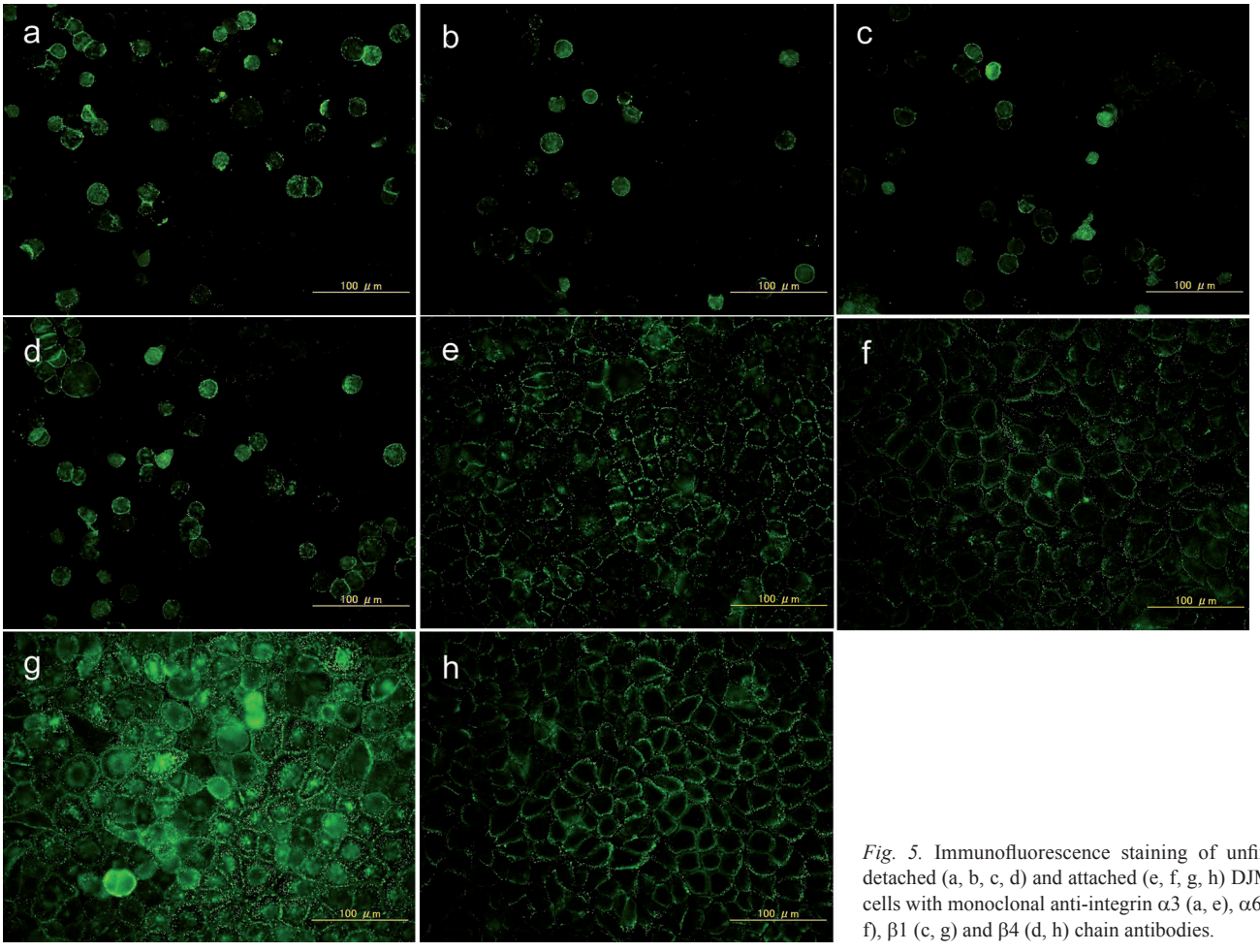

Fig. 5. Immunofluorescence staining of unfixed detached (a, b, c, d) and attached (e, f, g, h) DJM-1 cells with monoclonal anti-integrin α3 (a, e), α6 (b, f), β1 (c, g) and β4 (d, h) chain antibodies.

laminin-5 was not detected on the surface of detached keratinocytes (Fig.4c). Immunostaining also indicated the expression of integrin α3β1, α6β4 and α6β1 on both kinds of cells, whether they were detached or attached. Accordingly, our initial speculation seemed to be reasonable. However, 2 findings suggested that the survival of detached DJM-1 cells did not depend on cell-cell contact and the formation of linear structures: (*i*) a few floating single cells remained among the linear and branched structures, and these single cells showed greater BrdU uptake than cells in the structures (Fig. 7), which indicated that the single cells had higher proliferative activity; and (*ii*) HeLa cells, a human uterine cervical cancer cell line, also remained viable for at least 3 weeks under the same detached culture conditions and the majority of the viable cells were single cells. We then developed another hypothesis to explain how detached cancer cells remain viable. The cells could secrete laminin-5 that immediately adheres to the cell surface via integrins. Binding of laminin-5 to integrins would activate FAK and promote the survival of detached cancer cells, since it is known that interaction of laminin-5 with integrins, α3β1 or α6β4 induces intracellular signal transduction (2, 19), that supports cellular viability and proliferation. Globular domain 3 (LG3) of the laminin-5 α3 chain (present at the C-terminus) should mediate this binding, since the major integrin-binding sites are located here (22).

This hypothesis depends on the existence of cell surface laminin-5. Marinkovich et al. (20) detected 2 types of laminin-5, a cell-associated form and a medium-form, in their immunoprecipitation experiment. Laminin-5 is initially synthesized by cultured keratinocytes as a cell-associated form (estimated to be 460-kDa). After secretion, this is degraded to a 440-kDa molecule by extracellular processing of the α chain from 200-kDa to 165-kDa and further to a 400-kDa molecule by processing of the γ chain from 155-kDa to 105-kDa. A significant portion of the cell associated-form of laminin-5 persists in the cellular fraction, because this form adheres to cell membrane components, the culture dish, or other extracellular matrix molecules after secretion, while the medium-forms (440-kDa and 400-kDa) lose this affinity and diffuse into the culture medium. The cell-associated form presumably performs biological functions, such as attachment and continued adherence of cultured keratinocytes. Hence, our "cell surface laminin-5" probably corresponds to the cell-associated form.

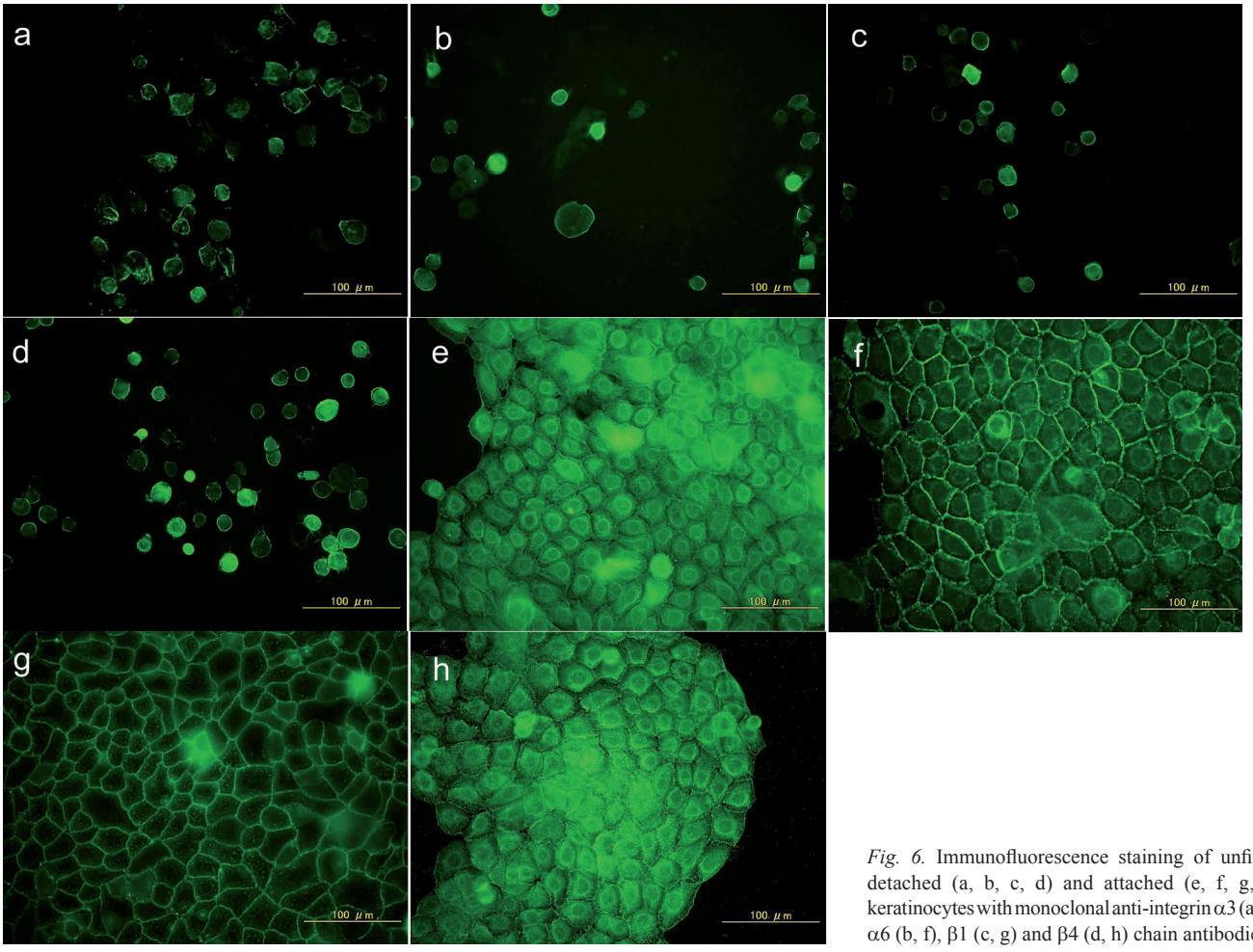

Fig. 6. Immunofluorescence staining of unfixed detached (a, b, c, d) and attached (e, f, g, h) keratinocytes with monoclonal anti-integrin α3 (a, e), α6 (b, f), β1 (c, g) and β4 (d, h) chain antibodies.

Laminin-5 is synthesized by keratinocytes (14, 20) and other epithelial cells (23), so cancer cells originating from epithelial structures may be able to synthesize laminin-5. Because laminin-5 was present on the surface of detached DJM-1 cells but not on the surface of keratinocytes, cell surface expression of laminin-5 might be a characteristic of cancer cells. Such surface expression of laminin-5 could explain the following 4 major characteristics of cancer: (*i*) immortality, (*ii*) tumour formation, (*iii*) metastasis, and (*iv*) angiogenesis.

Immortality

Surface expression of laminin-5 promotes continuous activation of FAK and thus endows cancer cells with immortality.

Tumour formation

Laminin binds to receptors such as integrins and dystroglycan at its C-terminus, polymerizes through short arm interactions at the N-terminus (LN domains), and

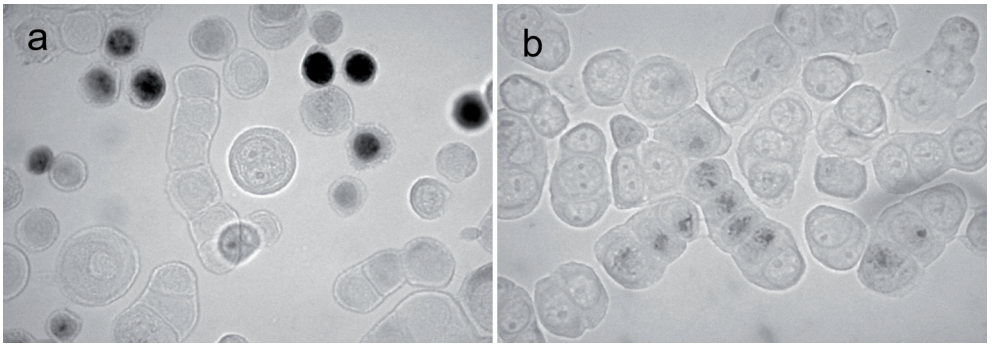

Fig. 7. Bromodeoxyuridine uptake by DJM-1 cells after 7 days of detached culture. Only some of the cells show uptake, which is stronger for single cells (a) than for cells connected to each other in lines (b).

thereby reorganizes the receptors (24). Laminin also self-polymerizes upon secretion and this polymerization is crucial for formation of the basement membrane (25). Studies using laminin-1 (26, 27) led to the theory that laminin network assembly depends on the formation of a heterotrimeric complex between LN domains derived from the α, β and γ chains of different molecules with insignificant homologous interactions. A recent study (25) has, however, indicated that lack of homologous interactions is an exception, because homologous interactions occur for LN domains derived from all α chains, as well as from β2 and β3 subunits. Thus, laminin isoforms other than laminin-1 can also self-polymerize. This applies to laminin-5, which contains truncated N-terminal short arms. Consequently, it is likely that cells with surface expression of laminin-5 bind to each other through polymerization of laminin-5 on different cells via short arm interactions at the N-terminus, resulting in the formation of a tumour mass. In our cell culture system, however, mass formation was not seen, probably because surface laminin-5 was continuously degraded by elastase in the culture medium. There is also the question of how cells with surface laminin-5 that is continuously degraded could maintain FAK activation. It is possible that FAK activation occurs immediately after a cell binds to secreted laminin-5, and that degradation of this laminin-5 by elastase occurs afterward. Despite the continuous degradation of surface laminin-5 by elastase in our culture system, DJM-1 cells presumably synthesized and secreted laminin-5 in a constitutive fashion and this led to FAK activation in detached cells.

Metastasis

FAK has been implicated as a key regulator of cell migration because it mediates the assembly of integrin-associated focal adhesions. Disassembly of these focal adhesions is necessary to cellular migration and FAK also controls this process (28). After phosphorylation by Src, FAK functions as an adaptor molecule that mobilizes both extracellular signal-regulated kinase 2 (ERK2) and calpain-2 to focal adhesions. This mobilization accelerates ERK2-induced activation of calpain-2 and calpain-2-mediated truncation of focal adhesion components, resulting in disassembly of the adhesions. Matrix metalloproteinases (MMPs) are efficient stimulators of cell migration that degrade the ECM around cancer cells. FAK-mediated signalling also induces the expression of genes encoding MMPs. Thus, FAK activation by cell surface laminin-5 could stimulate tumour cell migration and therefore induce metastasis.

Angiogenesis

It is thought that the majority of epithelial cells synthesize laminin-5 (23), so cancer cells originating from epithelial tissues would also synthesize laminin-5 and may possibly secrete it constitutively and abundantly. Secreted laminin-5 not only adheres to the cell membrane but also to ECM molecules. Thus the ECM around cancer cells could be enriched with laminin-5, which would induce haptotactic migration of vascular endothelial cells, since laminin-5 possesses endothelial cell binding sites (29). Laminin-5 also regulates vessel stability. Basic fibroblast growth factor (bFGF), a potent angiogenic factor, may also be involved. Laminins have binding sites for heparan sulphate proteoglycan (HSP) (30) and HSP has the ability to bind to various growth factors, including bFGF (31). Thus HSP bound to surface laminin-5 could act as a reservoir for bFGF, which would also induce angiogenesis around cancer cells.

If the hypotheses described above are confirmed, cancer cells might be defined as cells with surface expression of laminin-5.

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