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TPA-induced cohort migration of well-differentiated human rectal adenocarcinoma cells: cells move in a RGD-dependent manner on fibronectin produced by cells, and phosphorylation of E-cadherin/catenin complex is induced independently of cell-extracellular matrix interactions

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Abstract We have already presented a two-dimensional cell motility assay using a highly metastatic variant (L-10) of human rectal adenocarcinoma cell line RCM-1 as a motility model of tumour cells of epithelial origin. In this model, L-10 cells showed locomotion as a coherent sheet when stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and we called this type of movement "cohort migration". Electron and immunoelectron microscopic study of the migrating cell sheets demonstrated localized release from cell–cell adhesion only at the lower portion of the cells with loss of E-cadherin immunoreactivity, and this change was associated with increased tyrosine phosphorylation of the E-cadherin–catenin complex, including β-catenin. Cell–extracellular matrix (ECM) interactions involved in this TPA-induced cohort migration and their effect on tyrosine phosphorylation of the E-cadherin-catenin complex have now been investigated. L-10 cell cohort migration was almost completely inhibited by addition of Arg-Gly-Asp (RGD) peptide into the medium, and thus RGD dependent. Cohort migration was stimulated on type I and IV collagens, fibronectin (FN)- and laminin-coated substratum, but was inhibited by RGD only on FN-coated surface. By using immunofluorescent techniques, FN was demonstrated preferentially around migrating cells, and a protein synthesis inhibitor, cycloheximide, inhibited the migration by about 75%. FN produced by L-10 cells were found to be mostly EDA+ FN when analysed by RT-PCR. Moreover, anti-FN antibody, but not anti-vitronectin antibody, inhibited the TPA-induced cohort migration almost completely. Thus, it was likely that L-10 cells produced FN themselves and moved on the FN substrate in an RGD-dependent manner. However, stimulation of migration by type I collagen coating and inhibition by RGD treatment did not affect the tyrosine phosphorylation of the E-cadherin–catenin complex induced by TPA, indicating that cell–cell interactions were adjusted to suit cell migration, irrespective of the condition of cell–ECM adhesion, during TPA-induced cohort migration.

Key words Cell motility · TPA · Tumour invasion · Fibronectin · Carcinoma cells

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Introduction

Migration of cells is a basic biological phenomenon in development, inflammatory reactions, immune responses, wound healing, and tumourigenesis. Cell motility includes stationary motility, such as membrane ruffling and pseudopodal extensions, and translocative motility which is defined as net translocation of the whole cell and is a synonym for locomotion [10]. Translocation of cells occurs not only as a single-cell event but also en masse. In developing embryos, for example, epithelial cells migrate or rearrange collectively during branching morphogenesis and mesodermal cells move as sheets of cells during gastrulation and heart formation [45]. These movements are termed "collective cell migration" or "cell sheet migration". Neuronal precursor cells have also been shown to move while maintaining cell–cell contact with one another via zonula adherens-like membrane specializations, and this is referred as "chain migration" [24]. Even in vitro, the normal epidermal and amniotic epithelial cells show cell sheet migration [44].

In histopathological tumour sections, invasion by contiguous sheets or cords of tumour cells is more frequently seen than infiltration by single tumour cells [39]. Carcinoma cells proliferate and form coherent cell nests, maintaining cell–cell contact with one another, and this mode of growth is also seen even at the invasion front. In well to moderately differentiated colon adenocarcinomas, for example, the invasion front consists of well-circumscribed, compact tumour glands or partially resolved

tumour glands with dissociated small clusters of carcinoma cells [11, 17]. The latter feature has been called tumour budding and is associated with a worse prognosis [11]. These observations suggest that groups or sheets of carcinoma cells at the advancing edge might be able to move as a unit or dissociate from the primary site and migrate within the host tissue as coherent aggregates; in both cases cells move together. We have called this type of movement cohort migration [32, 33]. The demonstration of migration of isolated V2 carcinoma cell aggregates as units in vitro [7] and in vivo [39] has provided support for the concept of cohort migration. Moreover, recently a time-lapse videomicroscopy and computer-assisted cell tracking study of primary neoplastic tissue explants placed in three-dimensional collagen gels showed that groups of clustered cells comprising 5 to more than 100 cells could detach from the primary tumour lesion and migrate within the adjacent collagen matrix [9]. Thus, we consider cohort migration to be a mode of carcinoma cell movement, as well as single cell locomotion. However, it might be also possible in vivo that carcinoma cells invade as single cells and then form aggregates or glands secondarily. In any consideration of the mechanisms involved in cohort migration, cell–substrate interactions are important as well as cell–cell interactions, since both are closely linked to the modification of the cytoskeleton, which leads to cell migration.

Fibronectin (FN) is known to play an important part in cohort-type migration during development [4, 20] and to induce or enhance single cell locomotion of various tumour cells in vitro [33, 46]. FNs from different sources are known to be slightly different with respect to subunit sizes, and the heterogeneity arises mainly from alternative splicing of a primary transcript at three distinct regions termed EDA, EDB, and IIICS [36]. FN content is increased in various tumour tissues, and FN expressed in fetal and tumour tissues contains a greater percentage of EDA and EDB segments than that expressed in normal adult tissues [6, 12, 18, 23, 35, 42]. The enhancing activity of the EDA segment in cell adhesion and motiltiy has recently been clearly demonstrated [26].

Previously we reported that an enhanced ability to invade Matrigel upon stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was one of the major properties of a highly metastatic variant (L-10) of the human colon adenocarcinoma cell line RCM-1 [22]. This TPA-enhanced invasion of Matrigel was associated with augmentation of cell motility but not metalloproteinase activity in the conditioned medium [29]. To elucidate the mechanism of this enhanced L-10 cell motility we used a simple Lab-Tek chamber motility assay [30], in which TPA-stimulated L-10 cells showed characteristic morphology: the cells moved outwards from the cell islands mainly as a localized coherent sheet of cells. The cells at the edges of the migrating cell sheets showed fan-shaped leading lamellae, and the following cells had cell contact with one another. Thus, TPA treatment clearly induced cohort migration of L-10 cells in vitro. These morphological characteristics of L-10 cell movement are similar to those previously reported for normal epithelial cell movement [44]. By electron microscopic study of the migrating cell sheets we previously demonstrated that localized release from cell–cell adhesion occurred only at the lower portion of the cells, which allowed the cells to extend leading lamellae, while close cell–cell contacts remained at the upper portion of the cells [31]. On immunoelectron microscopic analysis E-cadherin immunoreactivity was seen to be confined to the site of cell–cell adhesion at the upper portion of migrating cells and lost at the lower portion, and this was associated with increased tyrosine phosphorylation of the E-cadherin–catenin complex, including β-catenin [32].

In this study, we intended to determine: what type of cell–ECM adhesion, for example RGD-dependent or not, is involved in TPA-induced L-10 cell cohort migration, which ECM protein(s) is (are) produced by cells as substrate for cell migration, and whether alterations in cell–ECM adhesion can modulate cell–cell interaction by altering the phosphorylation levels of the E-cadherin–catenin complex. Cell motility assays in the presence of synthetic peptides of known cell-binding motifs indicated that this L-10 cell cohort migration was RGD-dependent, and this RGD-dependency was also observed during migration on surfaces coated by FN but not other ECM proteins examined. FN production by L-10 tumour cell themselves was demonstrated by immunocytochemical techniques and RT-PCR. Analysis of alternative splicing of FN gene pretranscripts showed that FN expressed by L-10 cells was mainly EDA+ and EDB–. Treatment of cells with a protein synthesis inhibitor, cycloheximide, or anti-FN antibody effectively inhibited L-10 cell cohort migration, which strongly supported predominant involvement of FN in the migration. Stimulation of migration by type I collagen coating and inhibition by RGD treatment did not affect the tyrosine phosphorylation of the E-cadherin–catenin complex induced by TPA, indicating that cell–cell interactions were adjusted to suit cell migration, irrespective of the condition of cell–ECM adhesion, during TPA-induced cohort migration.

Materials and methods

Synthetic peptides, Gly-Arg-Gly-Asp-Ser (GRGDS), Gly-Arg-Gly-Glu-Ser-Pro (GRGESP), and Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP), were purchased from Telios (San Diego, Calif.), and laminin pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR) from Peptide Institute (Osaka, Japan). Human FN and EHS mouse tumour laminin were obtained from Becton Dickinson Labware (Bedford, Mass.), and type I and IV collagens from Koken (Tokyo, Japan) and Nitta Gelatin (Osaka, Japan), respectively. TPA, cycloheximide, L-azetidine-2-carboxylic acid (LACA), and tunicamycin were purchased from Sigma (St. Louis, Mo.).

TPA was dissolved in absolute ethanol, and tunicamycin was dissolved in dimethyl sulfoxide (DMSO). Other reagents were dissolved in water or saline. The final concentrations of ethanol and DMSO in the culture medium were 0.001–0.005% and 0.0001–0.003%, respectively, and at these concentrations the solvents alone had no injurious effects on the cells or any detectable effects on cell motility.

A highly metastatic subline of human colon adenocarcinoma metastatic to the liver (L-10) was obtained by in vivo selection in nude mice as previously described [19, 27]. The cells were maintained as monolayer culture in growth medium (GM), a 1:1 mixture of RPMI 1640 and Ham's F-12 (Nissui Seiyaku, Tokyo, Japan) supplemented with 5% FCS, L-glutamine (746 μg/ml), 25 mM *N*-2-hydroxyethyl piperazine-*n'*-2-ethane sulfonic acid (HEPES), streptomycin (90 μg/ml) and penicillin G (90 μg/ml), pH 7.35. The cultures were passaged following incubation with 0.125% trypsin and 0.5 mM EDTA at a split ratio of 1:2.

Assays of cell motility were done as previously described [30] with some modifications. L-10 cells (1.5×10^5 cells / 0.4 ml GM) were seeded into compartments of a Lab-Tek tissue culture chamber slide (Nunc, Naperville, Ill.) and allowed to attach for 24 h at standard culture conditions (37°C, 5% CO_2 in air, 100% humidity). L-10 cells formed interlinked and piled-up cell islands on the tissue culture glass substrate of the Lab-Tek chamber slide. Then, the cells were rinsed with serum-free (SF) media three times and exposed to test medium (SFGM/0.1% BSA with or without TPA, 10 ng/ml) for various times up to 24 h. TPA stimulated the cells to migrate outwards from the cell islands into interisland spaces. Since the migrated cells did not overlap one another but formed coherent cell sheets one cell thick, quantitation was done by counting their number in 10 randomly selected high-power fields (×400) after the cells were fixed with 3.7% formaldehyde in PBS and stained with haematoxylin. All experiments were performed in duplicate and repeated three times. Means and standard errors of the mean were calculated and statistical differences were analysed using Student's *t*-test for nonpaired samples.

In experiments with ECM protein-derived peptides, cells were preincubated with the peptides for 30 min at 37°C, and migration was induced for 5 h in the presence of both TPA and the peptides.

In experiments with reagents inhibiting protein synthesis or glycosylation, cells were preincubated with the reagents for 2 h at 37°C prior to TPA addition. Twenty-four-hour incubation was then performed in the presence of both TPA and the reagents.

In experiments with anti-FN (Oncogene Science, Uniondale, N.Y.) or anti-vitronectin (VN) (Boehringer Mannheim, Germany) mouse monoclonal antibodies (mAb), cells were added to Lab-Tek chambers in SF-ITS medium: serum-free growth medium (SFGM) supplemented with insulin–transferrin–selenium (ITS): (5 ng/ml Na_2SeO_3, 5 μg/ml insulin, 5 μg/ml transferrin) premix (Collaborative Research, Bedford, Mass.), triiodothyronine (5×10-10 M; Sigma) and retinoic acid (10-9 M; Sigma). The attached cells were preincubated with the antibodies for 5 h at 37°C prior to TPA addition. Sixteen-hour incubation was performed in the presence of both TPA and the antibodies.

To assess the effect of ECM proteins on TPA-induced L-10 cell cohort migration, the glass substrate of each compartment of 8-well Lab-Tek chamber slides was coated with 200 μl of 1 μg/ml type I collagen, type IV collagen, laminin or FN at 4°C overnight (16 h). Diffuse coating was confirmed by protein staining. Using these coated chamber slides, L-10 cell migration was determined as described above, but migration was induced for only 1 h because ECM proteins stimulated the migration very effectively.

After cell motility assays were done in SF-ITS medium for 16 h as described above, the cells were immunostained after fixation with 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min at 4°C. They were then washed twice in cold phosphate-buffered saline (PBS), treated with 0.1% Triton X-100 in PBS for another 30 min at 4°C, and incubated with 10% normal goat serum (Cedarlane Laboratories, Horuby, Canada), 1% BSA (Sigma) and 0.025% Thimerosal (Sigma) in PBS for 1 h at room temperature (RT) to block nonspecific binding sites. They were subsequently incubated with mAb against human type I and V collagens (Fuji Chemical Industries, Takaoka, Japan), human pan-FN (reactive with both plasma and cellular FN; BioMakor, Kiryat Weitzmann, Israel), human laminin (BioMakor), and vitronectin (Boehringer Mannheim), which were diluted with the above blocking solution, for 1 h at RT. The cells were then washed in PBS, and incubated with anti-mouse IgG goat Fab' conjugated FITC (Tago, Burlingame, Calif.) for 1 h at RT. After rinsing in PBS, the cells were immediately viewed and photographed.

For the reverse-transcription / polymerase chain reaction (RT-PCR), total RNA was extracted from cultured L-10 tumour cells and fibroblasts using Trizol (Gibco BRL, Grand Island, N.Y.) and reverse-transcribed using random hexamer primers. The enzymatic reaction was performed in 20 μl reaction mixture containing 1 μg total RNA, 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 1 mM each dNTP, 200 pmol of hexamers, 200 U of M-MLV reverse transcriptase (Gibco), 1 U/μl of ribonuclease inhibitor (Takara, Tokyo, Japan), and 1 mM dithiothreitol, at 37°C for 1 h and heated at 95°C for 10 min.

PCR and analysis of alternative splicing of FN pre-mRNA at EDA and EDB regions were performed as described elsewhere [15, 25] with some modifications. RT product (3 μl) was diluted with PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001 (W/V) gelatin) to give a final volume of 15 μl, which contained 15 pmol of primer pairs and 0.375 unit of Ampli Taq DNA polymerase (Takara). PCR was run in a thermocycler (Perkin Elmer Cetus, Norwalk, Conn.) using cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min.

For semi-quantitative RT-PCR, since the amounts of PCR products for FN linearly increased between 20 and 30 cycles, the duplicate products of each of 25 and 30 cycles were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The stained bands were subjected to densitometric analysis. For internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by using the same RNA samples and analysed in the same manner. The relative abundance of mRNA for FN was expressed as a ratio of the integrated absorbance of the test band to that of the corresponding GAPDH band.

A primer pair, HA1 (antisense, 5′-AGAGCATAGACACTCACTTCATATTT-3′) and HA2 (sense, 5′-AAACAGAAATGACTATTGAAGGCTTG-3′), was used to amplify the EDA region of human FN [25]. HA1 and HA2 are present in exon III_{12} and III_{11} of the *FN* gene, respectively. Another pair, HB1 (antisense, 5′-TAATATCAGAAAAGTCAATGCCAGTTG-3′) and HB2 (sense, 5′-ATTACTGGT-TATAGAATTACCACAACC-3′) was used to amplify the EDB region of human FN [25]. HB1 and HB2 are present in exons III^9 and III^7 of the *FN* gene, respectively. Amplification of the correct sequence was confirmed by the size of PCR products and also by nested PCR using internal primer pairs. The sequences of forward and reverse primers for GAPDH were 5′-GTGAAGGTCGGAGTCAACG-3′ and 5′-GGTGAAGACGCCAGTGGACTC-3′. This PCR product of GAPDH was 300 bp.

Detection of phosphorylation was performed according to Behrens et al. [3] with some modifications. After cell motility was induced for varying times with TPA treatment, cells were lysed in nonionic detergent buffer (1% Triton X-100, 2 mM $CaCl_2$, 2 mM PMSF, 20 μg/ml leupeptin, and a phosphatase inhibitor 1 mM Na_3VO_4 (Sigma) in PBS). The protein concentrations of the cell lysates were determined by the Bradford assay (Bio-Rad). Aliquots of 200 μg of total cell proteins in 300 μl were incubated with 6 μg HECD-1 overnight at 4°C, followed by the addition of 2 μg rabbit anti-mouse IgG (MBL) and protein A-Sepharose. The pellet was collected by centrifugation, washed three times with detergent buffer, dissolved in SDS sample buffer, and immediately subjected to electrophoresis. Immunoblot analysis with anti-phosphotyrosine mAb (BioMakor) was done as described [32]. Chemiluminescence reagent (DuPont NEN, Boston, Mass.) was used to visualize the labelled protein bands according to the manufacturer's instructions. The same membrane was re-probed with anti-human E-cadherin mAb (HECD-1, Takara, Tokyo, Japan).

Results

To investigate the cell-ECM interaction involved in TPA-induced L-10 cell cohort migration, assays were done in the presence of synthetic peptides of known cell-binding motifs. With TPA treatment, L-10 cells migrated out as localized flat cell sheets at intervals along the margin of

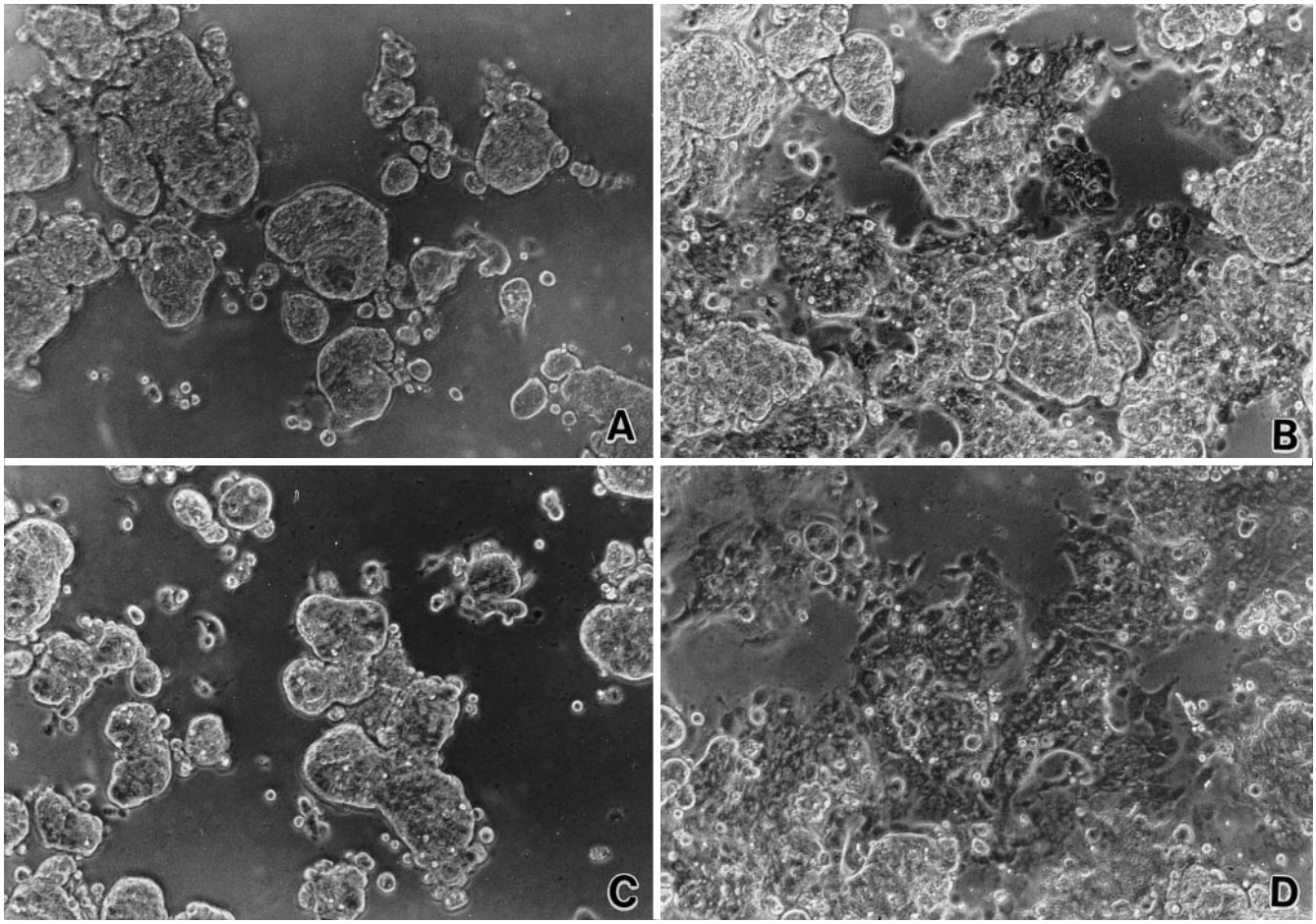

Fig. 1A–D Phase-contrast microscopy of TPA-induced cohort migration of L-10 cells with or without treatment with RGD-containing peptides. **A** L-10 cells mainly form piled-up cell islands without TPA treatment, while **B** the cells migrate outwards as localized coherent cell sheets at intervals along the margins of the cell islands. **C** In the presence of GRGDS (500 μg/ml) this TPA-induced cohort migration is almost completely inhibited, but **D** the control GRGESP peptide (500 μg/ml) does not show the inhibitory effect. Cells were photographed at a magnification of ×25

piled-up cell islands (Fig. 1B), while the cells remained piled up within the islands without TPA (Fig. 1A). This cell locomotion as coherent cell sheets (cohort migration) was inhibited by co-treatment with RGD-containing peptides (Fig. 1C) but not the control RGE-containing peptide (Fig. 1D). Dose responses of this inhibition of migration by peptides are shown in Fig. 2. RGD-containing peptides, GRGDS and GRGDTP, inhibited cohort migration by 65–85% at 50 μg/ml, 85–92% at 250 μg/ml and 92–94% at 500 μg/ml, while YIGSR and GRGESP caused almost no inhibition.

Modulation of cohort migration by ECM proteins was assessed by running assays on the glass substrate coated with type I and IV collagens, laminin and FN (Fig. 3). In these assays, since the cells formed less piled-up, flatter islands on the coated substrate and migration was stimulated very effectively and quickly after TPA addition, the migration was performed for only 1 h. Even without TPA treatment there was slight migration. With TPA treatment, however, migration was markedly stimulated compared with noncoated controls: type I and IV collagens stimulated by about 4.5 times, laminin by 3.6 times and FN by 2.5 times.

Next, effects of RGD-containing peptides on cohort migration on ECM protein-coated substrates were examined. GRGDS and GRGDTP inhibited cohort migration on FN effectively by more than 90%, while only slight inhibition, around 10–15%, was obtained on type I and IV collagens and laminin (Fig. 4). YIGSR did not show effective inhibition even on laminin.

Since RGD-containing peptides effectively inhibited cohort migration on both noncoated and FN-coated substrates, involvement of FN in cohort migration even on the noncoated substrate was considered. To explore this possibility, ECM protein production was examined by immunofluorescent staining. Weak FN staining was seen in L-10 cell cytoplasm, with accentuation along the cell borders in nontreated piled-up cell islands (Fig. 5A). With TPA treatment, FN was demonstrated preferentially in the migrating cells, diffusely in their cytoplasm or as dot-like staining at the perinuclear region and leading edge-like expanded cytoplasm (Fig. 5B, C). Laminin was seen along the cell borders even after TPA treatment (Fig. 5D). Collagen type V was observed very faintly in a similar pattern to laminin (data not shown). Type I collagen and VN could not be detected with this technique (data not shown).

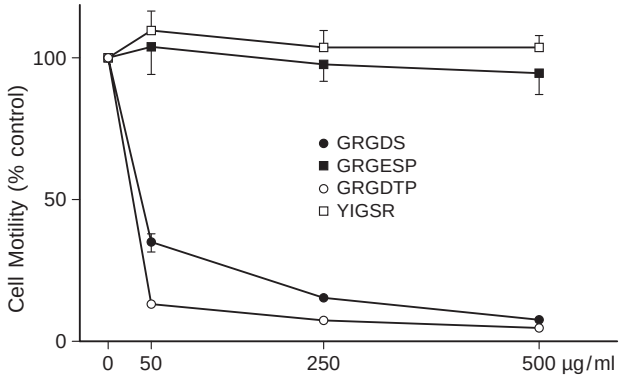

Fig. 2 Inhibition of TPA-induced cohort migration by RGD-containing peptides. After piled-up cell islands of L-10 cells were formed for 24 h at 37°C, cells were preincubated with GRGDS, GRGDTP, GRGESP and YIGSR peptides for 30 min followed by induction of migration for 5 h in the presence of both TPA (10 ng/ml) and the peptides. The control value was 114.7±6.6. Values are means and standard errors of the mean

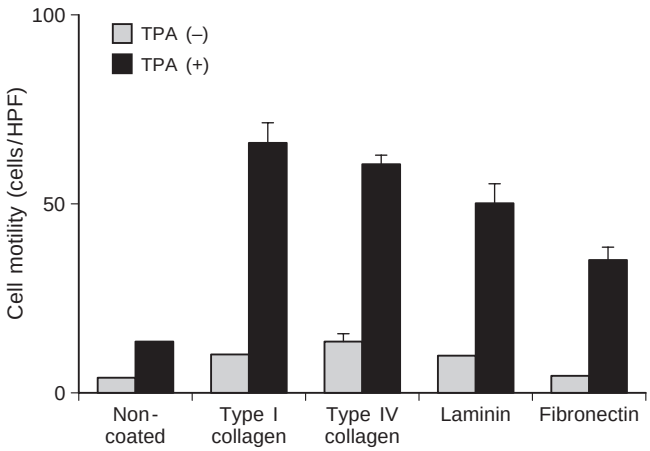

Fig. 3 Enhancement of TPA-induced cohort migration by coated ECM proteins. The glass substrate of Lab-Tek chamber slides was coated with 1 μg/ml type I and IV collagens, laminin and FN as described in Materials and methods. Migration was assessed in the presence and absence of TPA for 1 h

To confirm the production of FN by L-10 cells, RT-PCR was performed. In addition, alternative splicing at the EDA and EDB regions was examined, since both EDA and EDB regions of FN were reported to be increased in tumours, wound-healing sites and embryonal tissue, where cell migration frequently occurs [6, 8, 12, 18, 23, 35, 42]. At the EDA region, in both the presence and the absence of TPA, it was predominantly the 604-bp product of EDA-containing FN (EDA+ FN) that was expressed, and the 334-bp product of FN that lacks EDA (EDA– FN) was barely seen (Fig. 6A, lanes 2, 3). However, at the EDB region, the 502-bp product of EDB– FN was produced preferentially, and the 775-bp product of EDB+ FN was barely seen (Fig. 6B, lanes 2, 3). Thus, FN was expressed by L-10 cells, and this FN was mainly EDA+ and EDB–. In addition, semi-quantification using relative abundance of EDA+ FN and EDB– FN bands compared to corresponding GAPDH bands (data not shown; see Materials and methods) showed that TPA treatment increased expressions of EDA+ FN and EDB– FN about 1.3- and 1.6-fold, respectively.

Since an involvement of FN produced by L-10 cells in cohort migration was inferred from these results, the effect of reagents inhibiting protein synthesis or glycosylation on cohort migration was examined. A protein synthesis inhibitor, cycloheximide, inhibited cohort migration by 76.6%, and *N*-glycosylation inhibitor, tunicamycin, inhibited the migration by 66.9% (Table 1). Since it is reported that continual synthesis of collagen is necessary for epidermal cell movement and this movement is inhibited by proline analogue LACA [38], the effect of LACA on L-10 cell migration was also examined. However, no inhibition was observed with LACA treatment.

To investigate the possibility that FN produced by L-10 cells is required for cohort migration further the assays were run in the presence of anti-FN mAb, which recognizes the central domain of FN and thus inhibits cell adhesion to FN [28]. In these experiments, cells were added to Lab-Tek chambers in SF-ITS medium to avoid the contribution of serum-derived FN as well as

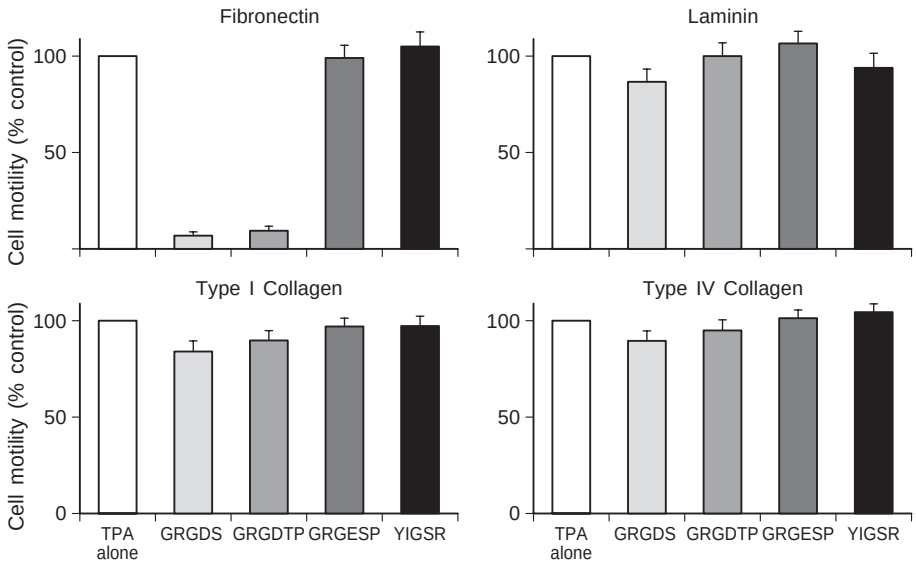

Fig. 4 Inhibition of cohort migration on ECM protein-coated substrates by RGD-containing peptides. The assays were run on ECM protein-coated substrates as described in Fig. 3, but cells were treated with 500 μg/ml peptides as in Fig. 2. The control values were 56.5±2.5, 68.8±2.5, 74.5±3.0, 70.2±2.1 for FN, laminin, type I and IV collagens, respectively. Values are means and standard errors of the mean

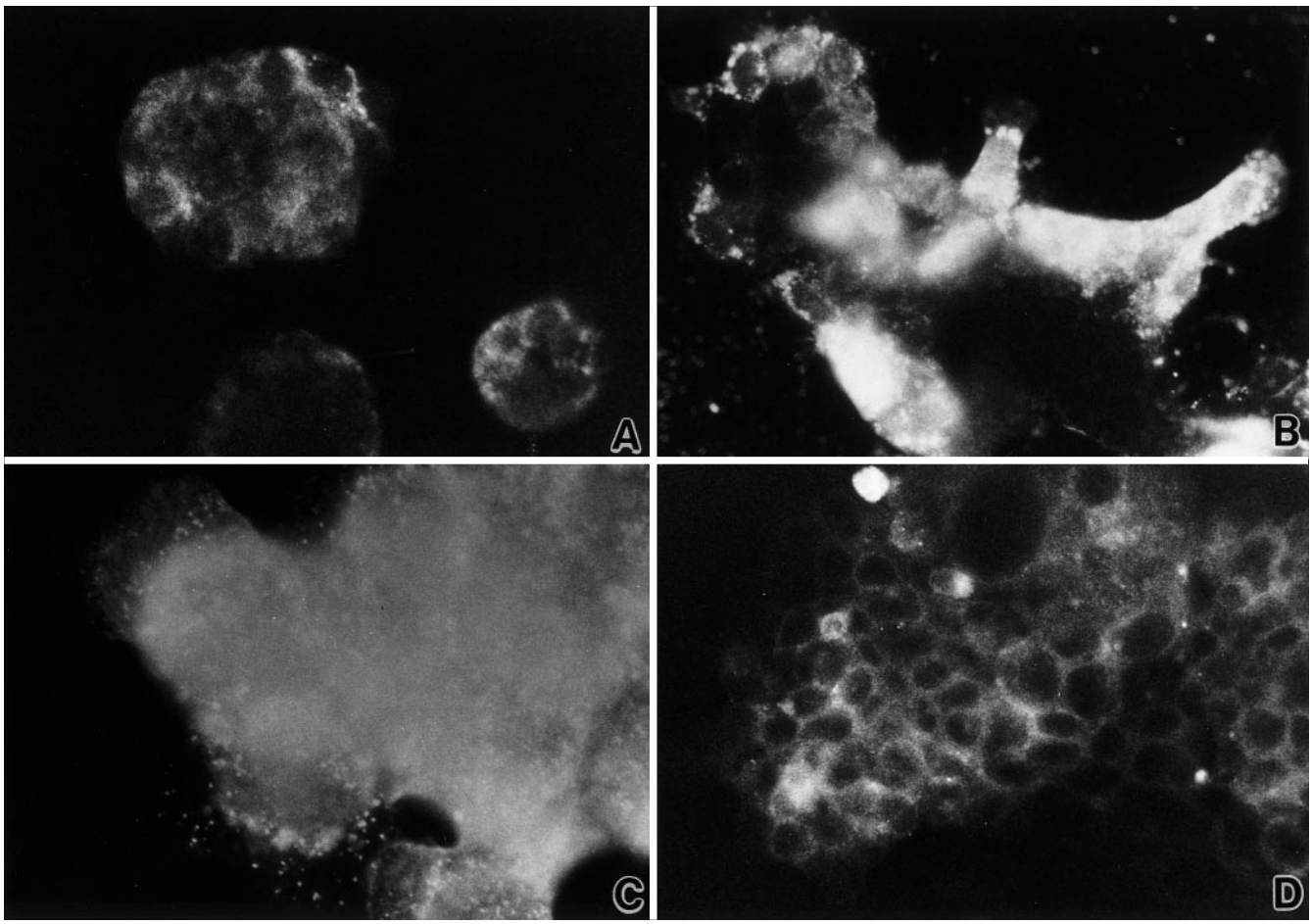

Fig. 5A–D Immunofluorescent microscopy of L-10 cells during cohort migration. Cells in SF-ITS medium were added to each well of Lab-Tek chamber slides and incubated for 24 h, followed by induction of migration for 16 h and immunofluorescent staining. **A** Without TPA treatment, piled-up cell islands show weak cytoplasmic staining of FN with some accentuation along the cell borders. **B** With TPA treatment, FN is demonstrated more intensely predominantly in migrating cells. **C** In a higher magnification, dot-like staining of FN is seen in the perinuclear region or expanded leading edges. **D** Laminin is demonstrated along the cell borders even in migrating cell sheets. Original magnifications **A**, **B**, **D** ×100, **C** ×250, exposure time **B** 4 s **A**, **C**, **D** 8 s

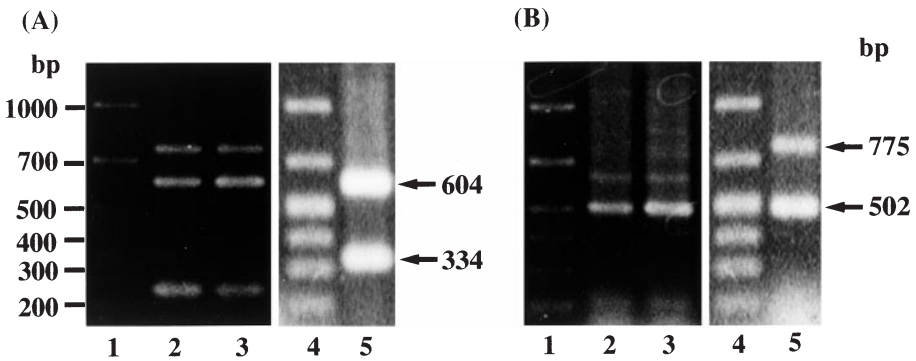

Fig. 6A, B Demonstration of FN expression in L-10 cells by RT-PCR. Alternative splicing at the EDA (**A**) and EDB (**B**) regions was determined as decribed in Materials and methods. Lane 5 s in **A** and **B** demonstrate the products of alternative splicing expressed in fibroblasts as positive controls: bands at 604, 334, 775 and 502 bp correspond to EDA+FN, EDA-FN, EDB+FN and EDB–FN, respectively. In both presence (*lane 3*) and absence (*lane 2*) of TPA, L-10 cells expressed mainly EDA+FN (604 bp) and EDB-FN (502 bp) products. *Lanes 1* and *4*, molecular weight markers

Table 1 Effect of protein synthesis or glycosylation inhibitors *LACA* L-azetidine-2-carboxylic acid

Agent	Cell motility (% control)
None (TPA alone)	100.0
Cycloheximide (1 μg/ml)	23.4 ± 5.8
LACA (10 μg/ml)	115.0 ± 9.7
Tunicamycin (1 μg/ml)	33.1 ± 6.9

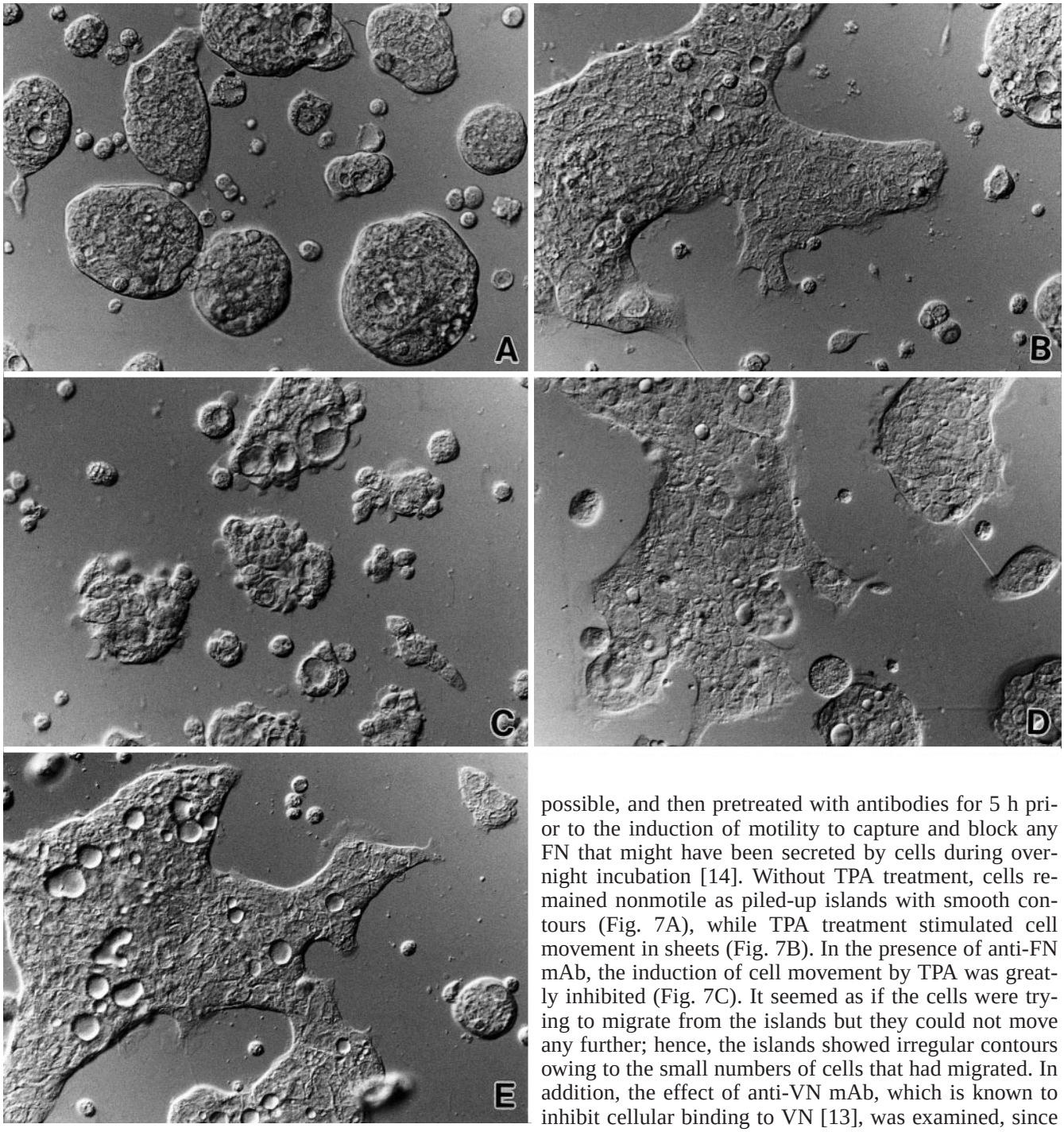

Fig. 7A–E Inhibition of cohort migration by anti-FN but not anti-VN antibodies. L-10 cells forming piled-up cell islands were pretreated with anti-FN mAb (**C**), anti-VN mAb (**D**) and control IgG_1 (**E**) for 5 h and then migration was induced for 16 h with TPA treatment in the presence of the above antibodies. Without any treatment, cells remain piled-up with few migrating cells (**A**), while treatment with TPA alone induced cell spreading and migration in sheets (**B**). This TPA-induced cohort migration is also seen even in the presence of anti-VN mAb (**D**) or IgG_1 (**E**). However, in the presence of anti-FN mAb the migrating cell sheet formation is almost completely inhibited (**C**). Only a few migrated cells are seen at the periphery of the piled-up cell islands. Original magnifications, ×100

possible, and then pretreated with antibodies for 5 h prior to the induction of motility to capture and block any FN that might have been secreted by cells during overnight incubation [14]. Without TPA treatment, cells remained nonmotile as piled-up islands with smooth contours (Fig. 7A), while TPA treatment stimulated cell movement in sheets (Fig. 7B). In the presence of anti-FN mAb, the induction of cell movement by TPA was greatly inhibited (Fig. 7C). It seemed as if the cells were trying to migrate from the islands but they could not move any further; hence, the islands showed irregular contours owing to the small numbers of cells that had migrated. In addition, the effect of anti-VN mAb, which is known to inhibit cellular binding to VN [13], was examined, since VN is included in serum and promotes cell adhesion and motility in an RGD-dependent manner in vitro [1]. Anti-VN mAb did not inhibit L-10 cell cohort migration at all (Fig. 7D). Treatment with normal IgG_1 also showed no inhibition (Fig. 7E). Quantitative analysis of these experiments is shown in Table 2. Anti-FN mAb treatment inhibited cell movement by approximately 95%, while the effect of anti-VN mAb was negligible.

We proposed earlier that in this TPA-induced cohort migration cells were released from cell–cell adhesion only at the lower portion of the cells, via phosphorylation of the E-cadherin–catenin complex. This change allowed

Table 2 Effect of anti-FN and VN antibodies on TPA-induced L-10 cell cohort migration

Agent added	Cell motility (cells / HPF)
None	1.0 ± 0.1
TPA alone	44.5 ± 4.4
TPA + IgG_1	51.4 ± 1.3
TPA + anti-FN Ab	2.8 ± 0.3
TPA + anti-VN Ab	49.5 ± 2.8

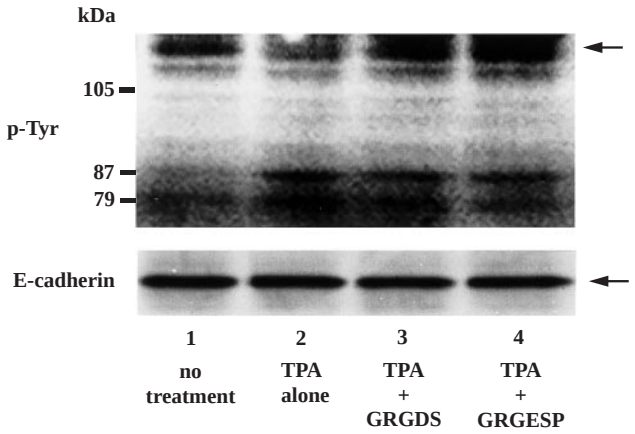

Fig. 8 Effect of RGD/RGE peptides on tyrosine phosphorylation of the E-cadherin-catenin complex. After the motility assay was run without any treatment (*lane 1*) or in the presence of TPA alone (*lane 2*), TPA and the GRGDS peptide (*lane 3*) or TPA and the GRGESP peptide (*lane 4*), the cells were lysed and subjected to immunoprecipitation with HECD-1. The immunoprecipitates were subjected to immunoblotting with an antiphosphotyrosine mAb as described. An *arrow* indicates the bands corresponding to E-cadherin (124 kDa). The bands at 105, 87 and 79 KDa correspond to the mobility of α-, β- and γ-catenins, respectively. The same blot as used for phosphotyrosine detection was reprobed with HECD-1 (*bottom*, indicated by E-cadherin), shown as control for applied protein contents

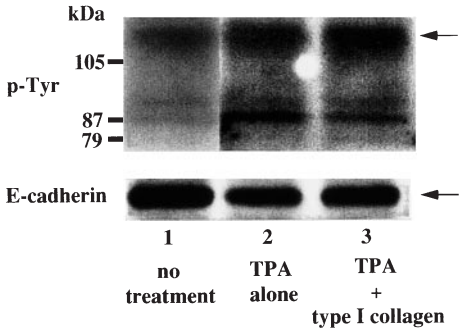

Fig. 9 Effect of type I collagen coating on tyrosine phosphorylation of the E-cadherin-catenin complex. After cell motility assay was run without (*lane 1*) or with TPA treatment on the glass substrate (*lane 2*) or type I collagen-coated substrate (*lane 3*), cells were treated as described in Fig. 8. An *arrow* indicates the bands corresponding to E-cadherin (124 kDa). The bands at 87 and 79 kDa correspond to the mobility of β- and γ-catenins, respectively. The same blot as used for phosphotyrosine detection was reprobed with HECD-1 (*bottom*, indicated by E-cadherin), shown as control for applied protein contents

the cells to extend leading lamellae and thus move together as coherent sheets [32]. In this study, this cohort migration was inhibited by RGD peptides and enhanced in the presence of coated ECM proteins. Thus, we investigated whether these modulations of cell–ECM interactions could cause alterations of the tyrosine phosphorylation levels of the E-cadherin–catenin complex: for example, whether the phosphorylation levels were decreased by RGD peptide treatment and whether they were increased during enhanced migration on ECM protein-coated substrates. After motility assays were run, cells were subjected to immunoprecipitation using an anti-E-cadherin antibody followed by immunoblotting with an anti-phosphotyrosine antibody (Figs. 8, 9). The band at 124 kDa (arrows) was E-cadherin, which was confirmed by reprobing with HECD-1. The bands at 105, 87 and 79 kDa correspond to α-, β- and γ-catenin, respectively, as described [5, 28]. With TPA treatment, irrespective of peptide treatment, the band at 87 kDa (β-catenin) showed increased tyrosine phosphorylation compared to negative control (Fig. 8). Treatment with GRGDS, which inhibited TPA-induced migration almost completely, did not noticeably decrease the levels of tyrosine phosphorylation of β-catenin compared with those of cells treated with TPA alone or with TPA and GRGESP (Fig. 8, lanes 2–4). For experiments with ECM protein coating type I collagen was used, since it enhanced TPA-induced cohort migration most effectively (Fig. 3). Tyrosine phosphorylation of β-catenin was induced by TPA treatment, on both noncoated and type I collagen-coated substrates compared with negative control (Fig. 9). However, the phosphorylation level on the type I collagen-coated substrate was not increased compared with that on the noncoated substrate (Fig. 9, lanes 2 and 3).

Discussion

We have investigated the cell–substrate interactions involved in TPA-induced cohort migration of carcinoma cells and the possibility of cross-talk between cell–cell and cell–substrate interactions. The results suggest that L-10 cells produce FN and deposit it into pericellular spaces and that this enables cells to move in a RGD-dependent manner when stimulated. The presence of exogenously added ECM proteins, such as type I and IV collagens, laminin, and FN, also stimulates TPA-induced cohort migration. However, stimulation of migration by type I collagen-coating and inhibition by RGD treatment did not affect the tyrosine phosphorylation of the E-cadherin–catenin complex induced by TPA, indicating that cell–cell interactions were adjusted to suit cell migration, irrespective of the condition of cell–ECM adhesion, during TPA-induced cohort migration.

In the cohort-type migration that occurs during development, FN also has an important role. During sea urchin and amphibian gastrulation, the primary mesodermal cells, once inside the blastocoele, appear to move collectively along the path lined with FN [4, 20]. An in-

jection of RGD-containing peptides completely blocks this migration. It has been speculated that this directed movement may result from a haptotactic response of each cell to the FN gradient, a model derived from and supported by experiments with individual cells in culture. However, another attractive model has been proposed recently [45]. During heart formation, since FN is arrayed as a localized patch rather than a gradient only front cells of collectively migrating precardiac mesodermal cells attach effectively to FN. Thus adhered, their cytoskeletal contractile activity generates force, which propagates throughout the migrating cell layer and efficiently pulls them in the proper direction. In this model, cell–cell adhesion plays a part in directional movement. In our cohort migration model in vitro, electron microscopic study suggests that not only the front cells but also the following cells are actively moving [31]. Since cell–cell adhesion is also maintained during this L-10 cell cohort migration, however, the above mechanism proposed by Wienst [45] could be in force when cells come across a localized patch of FN.

Increased FN content in various tumour tissues has been reported, and FN expressed in fetal and tumour tissues contains a greater percentage of EDA and EDB segments than that expressed in normal adult tissues [6, 12, 18, 23, 35, 42]. Very recently Manabe et al. [26] clearly showed the enhancing activity of the EDA segment in cell adhesion and motility: recombinant FNs containing the EDA segment were approximately twice as potent as those lacking EDA in their abilities to promote cell adhesion and migration, irrespective of the presence or absence of another domain, EDB. Since this activity was abolished by antibodies against integrin-α5 and -β1 subunits and by RGD-containing peptides, the EDA segment seemed to enhance the cell-adhesive activity of FN by potentiating the interaction of FN with integrin-α5β1. In the present study, FN expressed by L-10 cells was mainly EDA+ and EDB–. This seems suitable for carcinoma cell migration from the above report. Since L-10 cell cohort migration was RGD-dependent and integrin-α5β1was expressed in L-10 cells (data not shown), interaction of L-10 cells with FN via integrin-α5β1 during cohort migration is assumed. The enhancement of migration by precoated FN was relatively low; this might be due to the use of EDA– and EDB– plasma FN. Use of EDA+ FN may enhance migration more, as described above [26]. As for the cellular source of increased FN in tumour tissues, apart from tumour cells [35] there might be participation of host fibroblasts somehow stimulated by carcinoma cells [15]. In vivo, EDA+ FN produced by carcinoma cells and/or carcinoma-associated fibroblasts might be deposited on pre-existing ECM components and modulate their adhesiveness to make them suitable for cell migration. This process is very interesting, but could not be assessed in our in vitro assays because of the very short assay time (1 h) on precoated substrates.

ECM proteins other than FN, such as type I and IV collagens and laminin, also enhanced TPA-induced cohort migration, indicating that several different combinations of integrins were available on L-10 cells besides α5β1 since these migrations were not RGD dependent. This enhancement of migration by coated substrates may be partly due to shortening of the lag time that is otherwise necessary for L-10 cells to synthesize and deposit FN prior to migration. Requirement of type V collagen synthesis for epidermal outgrowth at the wounded edge, which include cell movement as continuous sheets, is reported in organ cultures [38]. This was not the case in our model: type V collagen production was barely seen by immunofluorescent microscopy and migration was not inhibited by proline analogue LACA. More recently it has been shown that at the wounded edge of monolayers of pancreatic carcinoma cells they deposit laminin-5 and migrate on the laminin-5-containing newly deposited basement membrane [41]. In our study, only the effect of EHS laminin (laminin-1) was examined. Since cohort migration on noncoated substrates was RGD-dependent while that on the EHS laminin-coated substrate was not effectively inhibited by RGD peptides, EHS laminin was considered not to be the major matrix protein working in L-10 cell cohort migration, in spite of its production by migrating cells. Modulation of FN-supported L-10 cell cohort migration by laminins cannot be excluded completely, and this is now under investigation in our laboratory.

The E-cadherin–catenin complex has an important role in cell–cell adhesion. The catenins [including α-, β-, γ- (plakoglobin) and $p120^{cas}$ catenins] complex with E-cadherin via its specific cytoplasmic binding domain, and this complex formation mediates the linkage of E-cadherin to the actin cytoskeleton and is thus essential for E-cadherin to express its full adhesive function [43]. This E-cadherin–catenin complex-based cell–cell adhesion is possibly regulated or altered via transient down-regulation of the expression, proteolysis, mutations in the genes, inappropriate expression of a nonepithelial cadherin (N-cadherin) instead of E-cadherin, binding to E-cadherin of $p120^{cas}$ which cannot bind to α-catenin and thus actin filaments, and tyrosine phosphorylation [2, 16, 21, 37, 40]. The tyrosine phosphorylation mechanism has been studied in most detail. Specific proto-oncogenic tyrosine kinases of the *src*-family (c-*yes* and c-*src*) are enriched to work as signal mediators in the adherens junction [43], and the elevation of tyrosine phosphorylation at the adherens junction induces its disassembly. The cadherin-associated proteins, especially β-catenin, are candidates for the specific substrates of the above tyrosine kinases. We previously showed the association of localized release from cell–cell adhesion at the lower portion of cells and increased tyrosine phosphorylation of the E-cadherin–catenin complex, including β-catenin, during TPA-induced L-10 cell cohort migration [32]. In the present study, this phosphorylation level of β-catenin was modulated by none of type I collagen coating, which promoted migration and treatment with RGD-containing peptides, which inhibited migration. This may suggest that the phosphorylation level induced by TPA was enough even for cell dissociation at the low-

er portion of cells during cohort migration enhanced by type I collagen coating. Nevertheless, the phosphorylation of β-catenin alone did not lead to cell movement without successful cell–ECM interactions. The E-cadherin–catenin complex was set ready to dissociate by TPA treatment, irrespective of the condition of cell-ECM interaction. Thus, cell–cell interaction seems to be adjusted independently in a manner permissive to cell migration during TPA-induced cohort migration.

We believe that our cohort migration model has some relevance to the epithelial or carcinoma cell movement in vivo, because of morphological similarities [32, 33, 44]. Recently it was shown that not only TPA but also hepatocyte growth factor/scatter factor (HGF/SF) induced cohort migration of L-10 cells and HGF/SF also caused this cohort type migration in several other human colon carcinoma cell lines [34]. These lines of evidence also support the relevance of our model.

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