The role of syndecan-2 in regulation of actin-cytoskeletal organization of Lewis lung carcinoma-derived metastatic clones

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Syndecans, a family of transmembrane heparan sulphate proteoglycans, contribute to various biological processes, including adhesion, motility, proliferation, differentiation and morphogenesis. We document here the involvement of syndecan-2 acting alone or co-operatively with integrin $\alpha 5 \beta 1$, for regulation of actin-cytoskeletal organization on cell adhesion to fibronectin, using fibronectin-recombinant polypeptides containing the ligands for either or both of these receptors as substrata. Lewis lung carcinoma-derived low-metastatic P29 cells binding to the substrata by both receptors formed actin stress fibres, whereas those binding by syndecan-2 or integrin $\alpha 5 \beta 1$ alone formed filopodia or cortex actin. In contrast, higher metastatic LM66-H11 cells formed cortex actin even on substrata containing both ligands. Northern-blot and flow-cytometric analyses revealed that syndecan-2 expression in LM66-H11 cells was significantly lower (1/4.5 in mRNA and 1/8 in cell-surface expression) than in P29 cells, whereas expression levels of integrin $\alpha 5 \beta 1$ and other

syndecans were similar in both cell types. These results suggest that the failure of LM66-H11 to form stress fibres is due to a lower expression of syndecan-2 than that due to a threshold for its function. This was confirmed by the finding that over-expression of syndecan-2 by transfection of its cDNA into LM66-H11 cells caused the formation of stress fibres on the fibronectin substratum. These *in vitro* cellular responses of the two clones might reflect their *in vivo* situation in primary tumours in which P29 cells with a stroma-inducing capacity were immediately surrounded by fibronectin-rich matrix formed by the induced stromal cells, whereas LM66-H11 cells without such capacity were not surrounded by a similar matrix.

Key words: actin stress fibres, cell adhesion, fibronectin-recombinant polypeptides, heparan sulphate proteoglycans, integrin $\alpha 5 \beta 1$.

INTRODUCTION

The extracellular matrix (ECM) is an extremely complex supra-molecular structure. Each of its components comprises several domains to allow them to interact with each other or with other ECM constituents and with cells. Cells generally interact with the ECM formed by themselves; epithelial cells interact with basement membranes and mesenchymal and stromal cells with interstitial-type matrix, but occasionally with allogenic ECM. Epithelial–mesenchymal or epithelial–stromal interactions play an important role in both physiological and pathological situations such as embryonic morphogenesis [1,2], wound healing [3] and tumorigenesis [4], and are accompanied by dynamic changes of ECM [5,6] to generate new cell–matrix interactions. In a metastatic process, tumour cells are always forced to interact with allogenic ECMs and these interactions are presumed to be important for the completion of metastasis.

The information encoded in an ECM is revealed by the interaction between individual ECM components and their respective receptors on the cell surface. It is well established that cell contact with a single ECM component initiates multiple signals affecting both cell behaviour and gene expression [7,8]. The interactions between fibronectin and integrin $\alpha 5 \beta 1$ have been studied extensively [9–11]. Their binding triggers the assembly of many cytoplasmic signalling proteins to form focal adhesions and re-organize actin cytoskeletons [12,13]. Recently,

it has become clear that syndecans, a family of transmembrane heparan sulphate proteoglycans whose external glycosaminoglycan chains can bind various extracellular soluble and insoluble ligands and whose core protein cytoplasmic domain can transduce signals into intracellular events, can modify signals generated by integrin-mediated cell adhesion to fibronectin [14]. Syndecan-4 signals co-operatively with integrins for the assembly of focal adhesions in fibroblasts inoculated on a fibronectin substratum [15,16] in a Rho-dependent manner [17]. Although details of the signalling processes remain to be determined, several components, which appear to be involved in the signalling, have been identified. Couchman and co-workers [18–20] have demonstrated that the variable region in the cytoplasmic domain of syndecan-4 binds phosphatidylinositol 4,5-bisphosphate and protein kinase C α (PKC α), and this interaction mediates its own multimerization, with resultant regulation of the intracellular distribution and activity of PKC α . Screening of a yeast two-hybrid library with a syndecan cytoplasmic domain identified several PDZ-containing proteins, syntenin [21], CASK/LIN-2 [22], synectin [23] and synbindin [24], which bind to the COOH-terminal EFYA sequence of syndecans, and syndesmos, which binds to both the membrane proximal and the variable regions [25].

Recently, we demonstrated that Lewis lung carcinoma-derived low-metastatic P29 cells induced actin stress-fibre formation on adhesion to a fibronectin substratum [26]. The formation of actin

Abbreviations used: ECM, extracellular matrix; PKC α , protein kinase C α ; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; RT, reverse transcription.

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stress fibres was completely inhibited by the addition of GRGDS peptide, heparin, anti-heparan sulphate monoclonal antibody or heparan sulphate oligomer [IdoA(2-OSO₃)-GlcNSO₃(6-OSO₃)₆], or treatment of the cells with heparitinase-I, suggesting that the signal to form actin stress fibres was transduced by a dual-receptor system with integrin $\alpha 5\beta 1$ and heparan sulphate proteoglycans. Moreover, the fact that the selective suppression of the expression of syndecan-2 with an antisense oligonucleotide for the core protein of this proteoglycan resulted in the failure to form actin stress fibres, indicated that the syndecan-2 is an essential component for the induction of this cytoskeletal organization. These results are consistent with our previous findings that more than 85% of the cell-surface heparan sulphate proteoglycans purified from P29 cells by fibronectin affinity is syndecan-2 [27] and that among these proteoglycans only syndecan-2 has a serine residue(s) in the cytoplasmic portion of the core protein, which is phosphorylated [28].

The highly metastatic LM66-H11 clone derived from the same carcinoma from which the P29 clone was isolated, was established after 66 episodes of an *in vivo* selection on the basis of spontaneous metastatic potential, exhibiting an ECM-dependent tumorigenesis distinctly different from the P29 clone [27]. Thus P29 cells with a capacity to elicit a host-stromal response, exhibit tumorigenesis dependent on a fibronectin-rich interstitial-type matrix generated by the induced stromal cells, whereas the highly metastatic cells, without any detectable capacity to elicit such a stromal response, show tumorigenesis dependent on the well-organized basement membranes formed by tumour cells themselves [29–31]. Therefore in the LM66-H11 tumour, the tumour cells encounter fibronectin only when they are confronted with the basement membrane of tumour blood vessels, whereas P29 cells are constantly interacting with fibronectin in the immediate surroundings. In the present study, we demonstrated that cellular responses of the two clones to adhere on a fibronectin substratum *in vitro* reflected the difference in their ECM dependence in tumorigenesis and the difference of the responses was attributable to the expression level of syndecan-2 on the cells to modify the integrin $\alpha 5\beta 1$ -mediating signal. To determine the ligands which initiate signals and their respective receptors, we used as substrata the fibronectin-relating recombinant polypeptides, RGD-containing cell-binding domain (designated as C-274), C-terminal heparin-binding domain (H-271) and a fusion polypeptide of both (CH-271). Moreover, we demonstrated that overexpression of syndecan-2 in LM66-H11 cells resulted in the formation of stress fibres on fibronectin as did the P29 cells. These clones of epithelial origin do not produce fibronectin [32], thus providing a useful system for this type of analysis without any complications due to synthesis of endogenous fibronectin.

MATERIALS AND METHODS

Materials

Human-plasma fibronectin was purchased from Iwaki Glass (Tokyo, Japan). Three species of fibronectin-recombinant polypeptides (Figure 1), i.e. C-274 (Pro¹²³⁹-Ser¹⁵¹⁵) with the RGD-containing Cell-I domain, H-271 (Ala¹⁶⁹⁰-Thr¹⁹⁶⁰) with the C-terminal heparin-binding Hep-II domain and CH-271, a fusion polypeptide of C-274 and H-271 with a methionine residue were generously supplied by TaKaRa Biomedicals (Otsu, Japan). Amino-acid sequences used here are numbered according to the system of Kornblihtt et al. [33]. Goat anti-human integrin $\alpha 5\beta 1$ antibody (AB1950) was purchased from Chemicon International (Temecula, CA, U.S.A.). Anti-heparan sulphate monoclonal

antibody, F58-10E4, was a gift from Dr K. Yoshida of Seikagaku Corp., Tokyo, Japan. The rabbit anti-mouse syndecan-2 antibody (SN2Ab), specific to the ectodomain of syndecan-2, was prepared as described previously [26]. [*Me*-³H]thymidine (3.1 TBq/mmol), [³⁵S]sulphate (carrier-free) and [α -³²P]dCTP (111 TBq/mmol) were purchased from DuPont-NEN Research Product (Boston, MA, U.S.A.).

Cell culture

The low-metastatic P29 and highly metastatic LM66-H11 cells cloned from a murine Lewis lung carcinoma 3LL [27] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) (Gibco, NY, U.S.A.), streptomycin (100 µg/ml) and penicillin (100 units/ml) at 37 °C in a humidified 5% CO₂ atmosphere. The syndecan-2 transfectants used in the present study were cultured in DMEM supplemented with 10% FCS G418 (800 µg/ml), streptomycin (100 µg/ml) and penicillin (100 units/ml) at 37 °C in a humidified 5% CO₂ atmosphere. The cells were harvested after incubation with 2 mM EDTA in PBS (EDTA/PBS) for 10 min at 37 °C, followed by gentle flushing with a pipette and subcultured twice a week.

Metastasis assay and immunostaining of primary tumour tissues

The metastatic potentials of the clones were measured by injecting single-cell suspensions of 1.0×10^5 or 2.0×10^5 cells in 200 µl of DMEM into the tail vein intravenously or subcutaneously to the right abdominal flank of syngenic C57BL/6 6-week-old male mice, respectively. After 4 weeks, the animals were killed and the number of visible parietal nodules in the lung, fixed in Bouin's solution, were counted.

The subcutaneous primary tumours were excised. Fresh frozen sections of 7 µm thickness were blocked with 1% BSA/PBS and treated with anti-fibronectin or anti-laminin antibodies at room temperature for 1 h. After washing with PBS, the sections were exposed to FITC-conjugated anti-rabbit immunoglobulin and 1.5 µM propidium iodide (Molecular Probes, OR, U.S.A.), and used for nuclear counterstaining. The sections were examined under an OLYMPUS Bx-50 microscope and images were captured using a High Sensitivity Cooled CCD Camera SenSys 0400 (RS-Photometrics, AZ, U.S.A.) and processed using Adobe PhotoShop 4.0.1J software.

Actin-cytoskeleton staining

Cells (5×10^3 cells in 50 µl of 0.2% BSA/DMEM) were inoculated on coverslips that had been coated with fibronectin (50 µg/ml), fibronectin-recombinant polypeptides (100 µg/ml) or combinations of C-274 (500 µg/ml) and antibodies F58-10E4 (15 µg/ml) or SN2Ab (0.7 µg/ml) overnight at 4 °C and blocked with 0.2% BSA/PBS for 30 min at room temperature. The cells were incubated for 1 h and then fixed in 3.7% paraformaldehyde containing 0.1% (v/v) Tween 20 for 5 min at room temperature. Actin filaments were stained with Rhodamine-conjugated phalloidin (Molecular Probes) for 30 min at room temperature and specimens were observed under a fluorescent microscope.

Cell-adhesion assay

Cells growing in a logarithmic phase were incubated with [*Me*-³H]thymidine (74 kBq/ml) for 12 h. After washing thrice with PBS, the cells were harvested with EDTA/PBS. The radio-labelled cells (1×10^4 cells) were suspended in 100 µl of 0.2%

Figure 1 Schematic models of fibronectin and the recombinant polypeptides used as substrata

Homology domain structures in fibronectin are represented by I, II, III and IIICS for type-I, -II, and -III homology units, and type-III homology connecting segment, respectively. Functional heparin-, fibrinogen-, collagen- and cell-binding domains are abbreviated as Hep, Fib, Col and Cell, respectively. The open arrowhead shows the position of the GRGDS sequence. The recombinant polypeptide, C-274, was designed with 8th to 10th type-III homology and H-271 with 12th to 14th. CH-271 is a fusion form of C-274 and H-271 with a methionine residue (M). ED-A and ED-B indicate that extra domains arise from alternative splicing.

BSA/DMEM and inoculated in 96-well plates coated with fibronectin (50 µg/ml) or its recombinant polypeptides (100 µg/ml). After incubation for 1 h at 37 °C, the plates were agitated on a microtube mixer at 1000 rev./min for 2 min to suspend unattached and loosely attached cells. The attached and unattached cells were solubilized separately in 1% SDS in 0.5 M NaOH. The radioactivity of an aliquot of each sample was measured in a liquid-scintillation counter (Beckman LS-9000).

Flow-cytometric assay

Cells (3×10^5 cells suspended in 50 µl of 0.2% BSA/DMEM) were incubated with antibodies or respective non-immune sera for 1 h at 4 °C with gentle agitation. After washing thrice with PBS, they were exposed to an FITC-conjugated second antibody for 30 min. The labelled cells were washed and the intensity of fluorescence was measured in a flow cytometer, Ortho Cytoron (Ortho Diagnostic Systems K.K.).

RT (reverse transcription)-PCR and Northern-blot analyses

Poly(A)⁺ RNAs were isolated from 1×10^7 cells of each clone, using a QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech) and analysed by RT-PCR using a TaKaRa RNA LA PCR Kit (TaKaRa Biomedicals) in a thermal cycler, Gene Amp PCR System 9600-R (PerkinElmer). The amounts of poly(A)⁺ RNA templates (50 ng) and cycle numbers (28 cycles) for amplification were chosen in quantitative ranges where the reactions proceeded linearly. Temperatures and time periods employed for melting, annealing and extension were 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 1.5 min, respectively. Oligodeoxyribonucleotide primers employed were as follows: 5'-GAGAATTCCACGGCAGACACCTTCCACTTG-3' (nucleotides 170–191 plus *Eco*RI adapter) and 5'-GACTGCAGGCACCTGTGGCTCCTTCGTC-3' (complement of nucleotides 709–728 plus *Pst*I adapter) for detection of mouse syndecan-1 mRNA (accession no. Z22532); 5'-GAGGATCCGAGACGA-GAACAGAGCTGAC-3' (nucleotides 487–506 plus *Kpn*I adapter) and 5'-GAGAATTCTTATTCTGTCCGTTTAAACAGATT-3' (complement of nucleotides 1151–1175 plus *Eco*RI adapter) for mouse syndecan-2 (U00674); 5'-GAGAATTCAC-CCACAACCGTTATCCAGCC-3' (nucleotides 370–390 plus *Eco*RI adapter) and 5'-GACTGCAGCAGAAGGGACTCTGAGTTGGG-3' (complement of nucleotides 910–931 plus *Pst*I

adapter) for mouse syndecan-3 (U52826); 5'-GAGAATTCGC-GAGTCGATTTCGAGAGACTGAGG-3' (nucleotides 69–23 plus *Eco*RI adapter) and 5'-GACTGCAGGTACACCAGCA-GCAGGATCAGG-3' (complement of nucleotides 498–520 plus *Pst*I adapter) for mouse syndecan-4 (W51352); and 5'-GTGGG-GCGCCCCAGGCACCA-3' (nucleotides 144–163) and 5'-CTC-CTTAATGTACGCACGATTTC-3' (complement of nucleotides 683–660) for β -actin [34]. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (5 µg/ml) and visualized under UV light. The RT-PCR products were sequence-verified.

Northern-blot analysis was performed as described previously [35]. In brief, 2 µg each of poly(A)⁺ RNAs from both clones were electrophoresed on a 1.5% agarose gel containing 1.1 M formaldehyde, transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech), and hybridized to a cDNA fragment of syndecan-1 (nucleotides 409–967), syndecan-2 (487–1175), syndecan-3 (370–931), or syndecan-4 (92–534), which had been labelled with [α -³²P]dCTP by the random labelling method. After hybridization for 14 h at 42 °C, the membranes were exposed to X-ray films and the films were scanned by CanoScan 600 (Canon, Tokyo). The quantification of the bands was performed with the public-domain NIH Image program in 256-grey-scale mode.

Transfection of syndecan-2 cDNA into LM66-H11 cells

The mouse syndecan-2 cDNA containing the entire coding region was amplified by RT-PCR with poly(A)⁺ RNA from P29 cells as template using 5'-GAGGTACCTCCTTTATCCGGG-TAGGAG-3' and 5'-GAGAATTCAAAGTTCAGTGCTCT-CCTAAATAG-3', which corresponded to nucleotides 487–506 and 1151–1174 (GenBank® accession no. U00674) respectively as primers. The 5'- and 3'-primers contained *Kpn*I and *Eco*RI sites respectively as indicated with underlines. The amplified cDNA was inserted into the *Kpn*I and *Eco*RI sites of a mammalian expression plasmid vector, pcDNA3 (Promega) at a site downstream of the cytomegalovirus promoter. The nucleotide sequence of the cloned syndecan-2 cDNA was verified with a Hitachi SQ-5500 sequencer (Hitachi, Tokyo, Japan). Recombinant plasmid DNA was purified with a Qiagen plasmid kit (Qiagen, Valencia, CA, U.S.A.) and used for transfection of LM66-H11 cells. After transfection using Tfx-50 reagent (Promega), cells were cultured in the presence of G418, and the

G418-resistant colonies were picked 2 weeks later. Cell clones were expanded individually and clones expressing high levels of syndecan-2 on their surface were selected by flow-cytometric analysis with SN2Ab. A clone (designated as H11-SN2) that expressed syndecan-2 on the cell surface at a similar level to that of P29 cells was used in the present study. LM66-H11 cells transfected only with vector (H11-Vec) were used as control cells.

Affinity chromatography of purified syndecan-2

[³⁵S]Sulphate-labelled syndecan-2 was isolated from both the clones as described below. P29 and LM66-H11 cells were labelled with [³⁵S]sulphate (7.4 MBq/ml) in 10% FCS/Ham's F-12 for 24 h at 37 °C in a humidified 5% CO₂ atmosphere. The radio-labelled macromolecules were extracted from the cell layer with 7 M urea/0.5% (v/v) Triton X-100/0.15 M NaCl/50 mM Tris/HCl, pH 7.3, containing protease inhibitors, 10 mM EDTA/10 mM *N*-ethylmaleimide/1 mM PMSF/0.036 mM pepstatin A, for 12 h on ice. After removal of insoluble materials by centrifugation at 65000 *g* for 30 min at 4 °C, the proteoglycan fraction was obtained on DEAE-Sephacel column chromatography and then applied to an Octyl-Sepharose 4B column. The bound hydrophobic proteoglycans were eluted with a linear-gradient concentration of 0–0.5% (v/v) Triton X-100 in 4 M guanidinium chloride/50 mM Tris/HCl, pH 7.3. Syndecan-2 was precipitated from this hydrophobic proteoglycan fraction with SN2Ab, as described previously [36]. Briefly, the sample was mixed with SN2Ab in 0.15 M NaCl/0.1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, for 1 h at room temperature, and 50% Protein A-Sepharose was added and mixed. One hour later, the beads were precipitated by a centrifugation and washed with 1 M NaCl/0.1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3. Syndecan-2 was eluted by 0.1% (v/v) Triton X-100/0.1 M glycine/HCl, pH 2.0, followed by neutralization with 1.0 M Tris/HCl, pH 9.0. The [³⁵S]-labelled syndecan-2 samples thus obtained were applied to a fibronectin-linked Sepharose 4B column. Elution was with a linear gradient concentration of 0.1–0.5 M NaCl in 0.5% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3.

RESULTS

Metastatic potential and tumorigenesis of P29 and LM66-H11 clones

The metastatic potentials of the two clones used in the present study are shown in Table 1. Irrespective of the sites of injection, a significant difference in the number of lung foci was observed between the clones. These clones also exhibited strikingly different patterns in the localization of fibronectin in primary tumours (Figure 2). The low-metastatic P29 cells having a strong ability to induce host-stromal response [31] were immediately surrounded

by a fibronectin-rich interstitial-type matrix formed by the induced host-stromal cells (Figure 2A). On the contrary, in the tumour formed by the highly metastatic LM66-H11 cells lacking the capacity to induce the host-stromal response, fibronectin was hardly observed in the extracellular compartment of tumour parenchyma except in the endothelial basement membrane of tumour blood vessels (Figure 2B). The results indicate that each clone grew in quite different microenvironments *in vivo*, especially in the interaction with fibronectin, which was an allogenic ECM component for them.

Cellular responses of P29 and LM66-H11 cells to substrata composed of fibronectin and fibronectin-recombinant polypeptides

When inoculated on a fibronectin substratum, the two cloned cells showed distinctly different responses in terms of actin-cytoskeletal organization (Figure 3). P29 cells spread extensively in association with actin stress-fibre formation (Figure 3A), whereas LM66-H11 cells spread to a slightly lesser extent in association with the formation of cortex actin (Figure 3B). We have previously demonstrated that the stress-fibre formation in P29 cells required a dual-receptor system with integrin $\alpha 5 \beta 1$ and syndecan-2 and thus selective suppression of syndecan-2 in P29 cells by *in vitro* treatment with its antisense oligonucleotides resulted in the failure to form actin stress fibres; the cells formed cortex actin instead [26], which was indistinguishable from the actin cytoskeleton induced in LM66-H11 cells. Therefore to clarify signalling by individual or co-operative action of the two receptors, we examined the responses of the cells on adhesion to substrata of fibronectin-recombinant polypeptides containing the respective ligands for the two receptors.

On CH-271, the actin cytoskeletons of the two cloned cells on fibronectin were reproduced (Figures 3C and 3D), suggesting that the other portions, except Cell-I and Hep-II domains of fibronectin, were not involved in these responses. Adhesion of both P29 and LM66-H11 cells to C-274 resulted in the formation of a cortex-actin structure (Figure 3E), indicating that cells adhering to fibronectin only by the interaction between integrin $\alpha 5 \beta 1$ and the Cell-I domain form cortex actin. The finding that the cortex-actin structure was induced in LM66-H11 cells not only on C-274 (Figure 3F) but also on both fibronectin and CH-271, despite the fact that the latter two substrata contain the ligand (Hep-II domain) for syndecan-2, suggested strongly that syndecan-2 expression on LM66-H11 cells was not sufficient in quality or quantity for co-operation with integrin $\alpha 5 \beta 1$. Adhesion of P29 cells to H-271 caused the formation of filopodia (Figure 3G), suggesting that the action of syndecan-2 resulted in the induction of filopodium formation, since the expression levels of other syndecans were similar between the two clones. In contrast, LM66-H11 cells hardly spread on an H-271 substratum and showed rounded shapes with small numbers of filopodia (Figure 3H), suggesting its insufficient expression on cells for their spreading and formation of filopodia.

The cell-adhesion assay demonstrated that, for the P29 clone, more than 90% of the cells adhering to a fibronectin substratum adhered not only to CH-271 but also to C-274 and H-271, indicating that the cells could adhere to the substratum by either of the receptors (Table 2). In contrast, LM66-H11 cells adhering to H-271 accounted for 68% of the cells adhering to fibronectin and the significantly lower percentage compared with those adhering to CH-271 or C-274 suggested again an insufficiency of probably syndecan-2 expressed on LM66-H11 cells in quality or quantity. Therefore we examined next the expression level of the two receptors on each of the cloned cell lines.

Table 1 Lung metastatic potentials of P29 and LM66-H11 clones

Clone	Route of injection	No. of cells injected ($\times 10^{-5}$)	Incidence	No. of lung foci	
				Mean	Range
P29	i.v.*	1.0	3/7	0.4	0–1
LM66-H11	i.v.	1.0	7/7	516.2	467–744
P29	s.c.†	2.0	0/7	0	–
LM66-H11	s.c.	2.0	7/7	35.2	23–56

* i.v., intravenous.

† s.c., subcutaneous.

Figure 2 Localization of fibronectin in primary tumours

Primary tumours formed by subcutaneous injection of P29 (A) and LM66-H11 (B) cells were treated with antibody against fibronectin. The reactive components were visualized by an FITC-conjugated second antibody (green) and the nuclei were counterstained with propidium iodide (red). Arrowheads show basement membranes of tumour blood vessels. Bar, 50 μ m.

Figure 3 Actin-cytoskeletal organization of P29 and LM66-H11 cells on substrata of fibronectin and fibronectin-recombinant polypeptides

P29 (A, C, E, G) and LM66-H11 (B, D, F, H) cells suspended in 0.2% BSA/DMEM were inoculated on coverslips coated with fibronectin (A, B), CH-271 (C, D), C-274 (E, F) and H-271 (G, H), incubated for 1 h at 37 °C, fixed and stained with Rhodamine-conjugated phalloidin to visualize actin fibres. Bar, 20 μ m.

Expression and fibronectin-binding affinity of syndecan-2 produced by P29 and LM66-H11 cells

The cell-surface expression of the two receptors was compared using flow cytometry (Figure 4). As expected, the expression levels of integrin $\alpha 5 \beta 1$ were almost the same in the cloned cell lines (Figures 4A and 4B), whereas that of syndecan-2 on P29 cells was approx. eight times higher in fluorescence intensity than that of LM66-H11 cells (Figures 4C and 4D). To compare the

Table 2 Adhesion of cells to substrata of fibronectin and fibronectin-recombinant polypeptides

[3 H]Thymidine-labelled cells were inoculated on 96-well plates coated with fibronectin or the recombinant polypeptides and incubated for 1 h at 37 °C. Cells attached and unattached were separated and dissolved in 0.5 M NaOH. Radioactivity of each fraction was measured. The results show values obtained from two different experiments done in triplicate.

Substratum	P29		LM66-H11	
	% of inoculated cells	Ratio	% of inoculated cells	Ratio
Fibronectin	83.8 $\pm$ 1.88	1	81.3 $\pm$ 4.38	1
CH-271	81.3 $\pm$ 4.38	0.97	72.5 $\pm$ 3.75	0.89
C-274	76.3 $\pm$ 3.50	0.91	68.8 $\pm$ 1.88	0.85
H-271	76.3 $\pm$ 3.63	0.91	55.0 $\pm$ 5.00	0.68

binding ability of the syndecan-2 proteoglycan which was isolated from the two cloned cell lines with fibronectin, [35 S]sulphate-labelled syndecan-2 precipitated with SN2Ab from the hydrophobic proteoglycan fractions was subjected to fibronectin-affinity chromatography (Figures 4E and 4F). The syndecan-2 proteoglycan from each of the clones was eluted with 0.22 M NaCl, indicating that the binding abilities of the two samples to fibronectin were the same. These results suggest that the formation of stress fibres in P29 cells, which was induced in co-operation of syndecan-2 and integrin $\alpha 5 \beta 1$, requires a certain threshold of syndecan-2 below which the formation of cortex-actin structure ensues, as observed in LM66-H11 cells in this study and in the antisense-treated P29 cells in the previous study [26].

Participation of syndecan-2 in actin-cytoskeletal organization

Most cells express more than one species of syndecans [37] and our previous study also suggested that P29 cells synthesized cell-surface heparan sulphate proteoglycans other than syndecan-2 [27]. Hence, the expression of mRNAs of all members of the syndecan family was examined in the two clones (Figure 5). The RT-PCR analysis showed that the two clones expressed all members of the syndecan family although the levels of syndecan-3 were extremely low (Figure 5A). Among them, only syndecan-2 exhibited different expression levels between the two clones, i.e. the expression in P29 cells was significantly higher

Figure 4 Expression of integrin $\alpha 5 \beta 1$ and syndecan-2 on P29 and LM66-H11 cells

P29 (A, C) and LM66-H11 (B, D) cells suspended in 0.2% BSA/DMEM were treated with anti-integrin $\alpha 5 \beta 1$ antibody, AB1950 (A, B) or anti-syndecan-2 core protein antibody, SN2Ab (C, D) for 1 h at 4 °C and then FITC-conjugated second antibodies for 30 min. Intensity of fluorescence was measured with a flow cytometer. Peaks shown with dotted lines are for control samples reacted with non-immune sera as the first antibodies. [35 S]Sulphate-labelled syndecan-2 purified from P29 (E) and LM66-H11 (F) cells was applied to a fibronectin-linked Sepharose 4B column and eluted with a linear-gradient concentration of NaCl to compare the binding affinity of the two samples with fibronectin.

Figure 5 Expression of mRNAs of a syndecan family in P29 and LM66-H11 cells

Expressions of mRNAs of syndecan-1, -2, -3, -4 and β -actin in P29 and LM66-H11 cells were analysed by RT-PCR (A) and by Northern blot (B). Their levels were normalized by the expression of β -actin.

than that in LM66-H11 cells. By Northern-blot analysis (Figure 5B), mRNA of syndecan-3 was not detected but that of other syndecans, syndecan-1 (2.4 kb), syndecan-2 (1.2, 2.1 and 3.6 kb) and syndecan-4 (2.3 kb) were detected in both clones. Three syndecan-2 mRNA species of different sizes have been reported in human foetal lung fibroblasts [38] and shown to result from different use of alternative polyadenylation signals. The densities of the bands were quantified using the NIH Image program and normalized with the amount of β -actin. The ratios of the individual densities of the P29 and LM66-H11 cell-derived syndecan-1, -2, and -4 bands were 1.00, 4.47 (ratio of total densities of three bands) and 1.47 respectively demonstrating a significant difference in the expression of syndecan-2 mRNAs.

To confirm the participation of syndecan-2 in actin-cytoskeletal organization, responses of the cells to mixed substrata

Figure 6 Actin cytoskeletons formed in P29 and LM66-H11 cells on adhesion to substrata comprising a mixture of C-274 and antibody against heparan sulphate (F58-10E4) or syndecan-2 core protein (SN2Ab)

P29 (A, B) and LM66-H11 (C, D) cells suspended in 0.2% BSA/DMEM were inoculated on coverslips coated with a mixture of C-274 (500 μ g/ml) and F58-10E4 (15 μ g/ml) (A, C) or SN2Ab (0.7 μ g/ml) (B, D) and incubated for 1 h at 37 °C. Cells were fixed and stained with Rhodamine-conjugated phalloidin. Bar, 20 μ m.

comprising C-274 and antibodies against two portions of the syndecan-2 proteoglycan, heparan sulphate (F58-10E4) and the ectodomain of core protein (SN2Ab), were assessed (Figure 6). When P29 cells were inoculated on a mixed C-274 and F58-10E4 substratum, they spread extensively in association with the formation of stress fibres (Figure 6A), whereas those pretreated with heparitinase-I did not form stress fibres under the same conditions (results not shown), indicating a necessity for clustering of heparan sulphate proteoglycans, mediated by binding of their glycosaminoglycans to the ligand. Moreover, a mixed substratum of C-274 and SN2Ab exerted similar effects on the organization of the actin cytoskeleton in P29 cells (Figure 6B), whereas heparitinase-I digestion of the cells did not affect the formation of stress fibres (results not shown). These results lead us to speculate that clustering of syndecan-2 on the cell surface

Figure 7 Induction of stress-fibre formation by overexpression of syndecan-2 in LM66-H11

LM66-H11 cells were transfected with syndecan-2 cDNA and the vector only, and the transfectants were cloned and named H11-SN2 and H11-Vec, respectively. The cell-surface expression of syndecan-2 in H11-Vec (A) and H11-SN2 (B) was measured with a flow cytometer. Peaks shown with dotted lines are for control samples reacted with non-immune sera as the first antibodies. Open and closed arrows show the peak positions of fluorescent intensity due to syndecan-2 expressed on LM66-H11 and P29 cells, respectively. H11-Vec (C) and H11-SN2 (D) cells were inoculated on a fibronectin substratum and incubated for 1 h at 37 °C. Cells were fixed and stained with Rhodamine-conjugated phalloidin. Bar, 20 μ m.

is sufficient to work co-operatively with integrin $\alpha 5 \beta 1$ for signal transduction to induce stress fibre formation, and both glycosaminoglycan- and core protein-mediated clusterings are equivalent in terms of this signal transduction. On the contrary, LM66-H11 cells failed to form stress fibres, but exhibited the formation of cortex actin on these two substrata (Figures 6C and 6D), suggesting that the clustering of syndecan-2 expressed on their surface was not sufficient to form stress fibres under the conditions used, or that the cells lack some components to complete the stress-fibre formation. This possibility has been investigated below.

Induction of stress-fibre formation by overexpression of syndecan-2 in LM66-H11

To confirm that syndecan-2 is specifically involved in the induction of stress-fibre formation in the present system, we inserted a full-length cDNA of the mouse syndecan-2 into LM66-H11 cells with its low expression and obtained a stable clone, H11-SN2, expressing it at a level similar to that of P29 cells (Figure 7B). On the contrary, the clone transfected only with the vector, H11-Vec, expressed syndecan-2 at a level similar to that of the parental LM66-H11 cells (Figure 7A). Its overexpression did not affect the expression of other syndecans and integrin $\alpha 5 \beta 1$ (results not shown). When H11-Vec cells were allowed to adhere to the fibronectin substratum, they formed cortex actin (Figure 7C) as did the parental cells (Figure 3B). On the other hand, H11-SN2 cells spread extensively in association with the formation of stress fibres (Figure 7D). This actin-cytoskeletal organization was indistinguishable from that of P29 cells on the same substratum (Figure 3A). These results indicate that LM66-H11 cells have all the features required for the induction of

stress-fibre formation, except the expression of syndecan-2 beneath the threshold.

DISCUSSION

In the present study, using the fibronectin-recombinant polypeptides as substrata, we have clearly demonstrated that signal transduction by (1) integrin $\alpha 5 \beta 1$ alone results in the induction of cortex-actin structure in association with cell spreading; (2) syndecan-2 alone results in the formation of filopodia; and (3) both receptors cause the formation of stress fibres (Figure 8). Increasing evidence suggests that many heparin-binding growth factors are activated by binding to cell-surface heparan sulphate chains, acting as physiological co-receptors before binding to their intrinsic receptors [37,39–41]. Thus it is noteworthy that unlike co-receptors for soluble growth factors, syndecan-2 itself has the potential to induce the formation of filopodia by binding to an immobilized substratum, indicating that ECM-ligand-mediated clustering of syndecan-2 can trigger the cytoplasmic cascade for signalling to rearrange the actin cytoskeleton. Another new finding is that the signals binding to fibronectin by integrin $\alpha 5 \beta 1$ alone induced the formation of cortex actin in the cells used in the present study. In addition, syndecan-2 can act co-operatively with integrin $\alpha 5 \beta 1$ to generate a new signal for the formation of stress fibres, which differs from the respective signals by syndecan-2 or integrin $\alpha 5 \beta 1$ alone. Moreover, the fact that the stress fibre formation was induced in P29 cells on adhesion to a mixed substratum composed of C-274 and not only anti-core protein of syndecan-2 antibody but also anti-heparan sulphate antibody, further corroborates our previous finding that syndecan-2 participates specifically in the induction of stress-fibre formation [26]. However, it remains to be elucidated how syndecan-2 and integrin $\alpha 5 \beta 1$ cross-talk with each other to generate a new signal in terms of the molecular mechanism.

The responses of LM66-H11 cells with low syndecan-2 expression to adhesion substrata indicated that a certain threshold in the expression of syndecan-2 might be required for co-operation with integrin $\alpha 5 \beta 1$. The expression level of syndecan-2 on P29 cells is sufficient to work co-operatively with integrin $\alpha 5 \beta 1$ to induce the formation of stress fibres on fibronectin. Thus an elevated expression of syndecan-2 in LM66-H11 cells by transfection of its cDNA caused the formation of stress fibres in co-operation with integrin $\alpha 5 \beta 1$. This also suggests that LM66-H11 cells have all the features required for the induction of stress fibres, except for a low expression of syndecan-2.

The facts that the mixed substratum composed of C-274 and SN2Ab is sufficient to induce the formation of stress fibres in P29 cells and treatment of P29 cells with the antisense oligonucleotide for syndecan-2 prevents the formation of stress fibres [26], indicate that a certain level of syndecan-2 is not only essential but also sufficient to receive the ECM signals co-operatively with integrin $\alpha 5 \beta 1$ to induce stress-fibre formation. Although the clones used in this study expressed all members of the syndecan family, the molecular species exhibiting specific binding to fibronectin or H-271 was syndecan-2 [26–28]. On the other hand, it has been well established that syndecan-4 signals co-operatively with integrin $\alpha 5 \beta 1$ to result in the assembly of focal adhesions and actin stress fibres in fibroblasts inoculated on to a fibronectin substratum [16,17,42]. The cytoplasmic domain of syndecan-4 binds phosphatidylinositol 4,5-bisphosphate and PKC α at its variable region, and this interaction mediates its own multimerization, with resultant regulation of the intracellular distribution and activity of PKC α [18, 19]. We have recently demonstrated [43] that the cross-talk of syndecan-2 and -4 is

Figure 8 Fibronectin-induced signalling mediated by integrin $\alpha 5 \beta 1$ and/or syndecan-2

The results obtained in this study show that cells adhering to the Cell-I domain of fibronectin by integrin $\alpha 5 \beta 1$ spread and form cortex actin. In contrast, the cells adhering to the Hep-II domain by heparan sulphate side-chains of syndecan-2 spread to a lesser extent and develop filopodia. When they adhere to fibronectin by both receptors, stress-fibre formation is induced. It is also revealed that there is a threshold level for syndecan-2 signalling to form filopodia or regulating integrin $\alpha 5 \beta 1$ -mediated signalling to form stress fibres. However, this depends on the ability of a ligand to bind syndecan-2. Therefore, on a fibronectin substratum, P29 cells with high syndecan-2-expression form stress fibres and LM66-H11 cells with low expression form cortex actin.

required for the induction of those cytoskeletons in co-operation with integrin $\alpha 5 \beta 1$. This cross-talk may be explained by a substrate–enzyme interaction, i.e. a phosphorylation reaction at the serine residue(s) of the syndecan-2 cytoplasmic domain, which is catalysed by PKC α bound to the syndecan-4 cytoplasmic domain [20]. However, it should be noted that fibroblasts derived from syndecan-4-null mice have been recently demonstrated to form focal contacts and stress fibres on adhesion to fibronectin as observed in normal fibroblasts [44], indicating that syndecan-4 is not essential and can be compensated for by some other molecule(s). Thus all these results suggest that both syndecans appear to function co-operatively with integrin $\alpha 5 \beta 1$ for the assembly of focal adhesions and actin stress fibres on cell adhesion to the fibronectin substratum.

Finally, it is appropriate to discuss the correlation between the metastatic potential and the expression level of syndecan-2. An *in vivo* selection of different metastatic clones from Lewis lung carcinoma cells on the basis of spontaneous metastatic potential results in the selection of clones with different abilities to induce the host-stromal responses, and also different expressions of syndecan-2. Thus each of the cloned cell lines grow in quite different microenvironments *in vivo* and cells adapt to such a milieu by all the receptors that they express on the cell surface. In consequence, the two cloned cells behave differently. Indeed, the actin cytoskeleton formed in the highly metastatic LM66-H11 cells on adhesion to a fibronectin substratum is indistinguishable from that in the low-metastatic P29 cells whose expression of syndecan-2 is selectively suppressed by *in vitro* treatment with antisense oligonucleotides for its core protein [26]. Moreover, we have recently demonstrated that over-expression of syndecan-2 by transfection of its cDNA into LM66-H11 cells causes the formation of actin stress fibres in the cells on adhesion to fibronectin, which is indistinguishable from the actin cytoskeleton induced in P29 cells on fibronectin and, surprisingly, results in striking suppression of metastasis [43]. The results reported here consistently suggest that syndecan-2 plays a pivotal role in the determination of the degree of metastasis.

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