Effect of syndecan-1 overexpression on mesenchymal tumour cell proliferation with focus on different functional domains

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Abstract

Objectives: Syndecan-1 is a transmembrane proteoglycan involved in various biological processes. Its extracellular, transmembrane and cytoplasmic domains may all participate in signal transduction. The aim of this study was to investigate the biological roles of these domains of syndecan-1.

Materials and methods: We transfected cells of two mesenchymal tumour cell lines with a full-length syndecan-1 construct and three truncated variants, namely 78 construct lacking the EC domain with exception of DRKE sequence; 77 construct lacking extracellular the whole domain and RMKKK corresponding to a short cytoplasmic motif. Subcellular distribution was revealed using confocal laser microscopy. Overexpression of the constructs was verified using real-time RT-PCR and by FACS analysis and effects of syndecan-1 on cell behaviour were explored. Cell cycle analysis allowed for dissection of mechanisms regulating cell proliferation.

Results: Overexpression of syndecan-1 influenced expression profile of the other syndecan members, and decreased tumour cell proliferation significantly by two mechanisms, as follows: increased length of G0/G1 phase was the most evident change in RMKKK and 77 transfectants, whereas prolonged S phase was more obvious in full-length transfectants. Overexpression of syndecan-1 chan-

ged the tumour cell morphology in an epithelioid direction.

Conclusions: Both full-length and truncated syndecan-1 inhibited proliferation of the mesenchymal tumour cells, providing new insights into the importance for cancer growth of different functional domains of this proteoglycan.

Introduction

Mammalian syndecans represent a family of transmembrane proteoglycans (PGs), transcribed from four distinct genes (*syndecan-1*, -2, -3 and -4). They participate in a number of biological processes, such as cell–cell and cell–matrix adhesion, and cell differentiation, proliferation and migration (1), which also indicate an essential role of the syndecans in malignant properties of a tumour. These functions are mediated through interactions of the core protein and/or *via* binding of the glycosaminoglycan (GAG) chains to various regulatory proteins.

The generic syndecan core protein can be broadly divided into three domains as follows: an extracellular (EC) domain or ectodomain, a transmembrane (TM) domain and a cytoplasmic domain (CD) (1). The EC domain is specific for each syndecan, but they share conserved sequences for GAG attachment, cell interaction, proteolytic cleavage and oligomerization. Thus, the ectodomain carries heparan sulphate (HS) chains to facilitate interactions with matrix proteins (2,3), growth factors (4–6) and growth factor receptors (7). On the cell membrane, the syndecans are turned over within a few hours through proteolytic cleavage and shedding of most of the ectodomain as an intact fragment.

The conserved TM domain is believed to be important for localizing syndecans to distinct membrane

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compartments (8). This domain is essential for activation of the CD for downstream signalling (9). Choi *et al.* (10) showed that TM domains of syndecan-2 and syndecan-4 are sufficient for inducing oligomerization, which is crucial to the functions of these PGs. In the case of syndecan-3, this oligomerization needs the last four amino acids (ERKE) of the EC domain in addition to the TM domain (11). It has not been shown whether the corresponding sequence in syndecan-1, DRKE, has an equivalent function.

The CD contains two conserved regions: one proximal, close to the cell membrane (C1) and the other distal (C2) at the C-terminus of the molecule. Between C1 and C2, there is a variable region (V) unique to each syndecan member (12). The CD is postulated to play a role in binding cytoskeletal elements and possibly in formation of cytoplasmic signalling complexes (8).

Syndecan-1 is the prototype representative of the syndecan family, expressed in epithelial cells in a temporo-spatial manner (13). Syndecan-1 is overexpressed in many tumour types but downregulated in others, which correlates with the cells' malignant behaviour (14). There is only a limited number of reports regarding the structure–function relationship of syndecan-1. Targeting of syndecan-1 to uropods of myeloma cells requires the presence of HS chains on the syndecan-1 core protein and also the ectodomain, but not CD and TM domains (15). An invasion regulatory domain within the ectodomain of syndecan-1 was identified and proved to inhibit myeloma cell invasion (16). CD Peterfia *et al.* (17) have reported that and TM domains of syndecan-1 are sufficient to promote metastasis formation in HT-1080 fibrosarcoma. A very recent study has reported different effects of membrane-bound or soluble syndecan-1 on breast cancer progression (18).

Although syndecans play major roles on the cell surface, a substantial proportion of them at intracellular locations has previously been reported by our group. In particular, syndecan-1 was found to accumulate in the cell nucleus in a time-dependent manner. This nuclear translocation was seen in different cell types and was shown to depend on the presence of functional tubulin (19). The role of nuclear syndecan so far is not well understood, although indirect evidence indicates its association with cell proliferation (20). How and to what extent the different syndecan domains regulate proliferation of tumour cells is still poorly comprehended. Thus, our aim was to study functional effects of different on domains of syndecan-1 human mesenchymal tumour cells, by transfecting two different malignant tumour cell lines with human full-length syndecan-1 construct or truncated variants.

Materials and methods

Cell lines and cell culture conditions

STAV-AB human cells (21) were grown in RPMI 1640 medium malignant mesothelioma (MM) containing 25 mM HEPES (42401; Gibco, Grand Island, NY, USA) and 2 mM L-glutamine, supplemented with 10% human AB serum. B6FS human fibrosarcoma cells (22) were grown in RPMI 1640+ GlutaMAX™-I (72400; Gibco) supplemented with 10% foetal bovine serum (FBS) and 50 µg/mL gentamicin (Gibco). Cells were cultured in 75 cm² tissue culture flasks (Sarstedt, Newton, NC, USA) and incubated in humidified 5% (v/v) CO₂ atmosphere at 37 °C, and culture medium was changed twice a week. Both cell lines used in this study have mesenchymal origin and were selected based on their low endogenous syndecan-1 expression level on the cell surface.

Plasmid constructs and DNA transfections

Human full-length syndecan-1/enhanced green fluorescence protein (EGFP) construct (FL) and truncated variants (Fig. 2), namely 78 lacking the EC domain with exception of DRKE sequence, 77 lacking the whole EC domain and RMKKK, which we hypothesized to be a nuclear localization signal (NLS), were all obtained from Szilák Labour Ltd (Szeged, Hungary). The short RMKKK sequence CD on the of syndecan-1 is mostly composed of basic amino acids. Similar basic amino acids are commonly found in classical NLS motifs (23,24). pEGFP-N1 vector, used as negative control, was purchased from BD Biosciences (Clontech, Palo Alto, CA, USA) and full-length and RMKKK constructs were cloned in-frame with the N-terminus of EGFP, whereas 77 and 78 constructs were cloned in-frame with the C-terminus of EGFP. Plasmids were amplified in *Escherichia coli* and purified using EndoFree Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany). Their purity was determined using spectrophotometry and agarose gel electrophoresis.

MM and fibrosarcoma cells were transfected with the constructs above, using Effectene Transfection Reagent (Qiagen GmbH). Optimization of transfection was carried out according to the manufacturer's guidelines. Briefly, around 2×10^5 cells were seeded in six-well plates and incubated for 24 h to reach 40–80% confluence at the time of transfection. The recommended amount of DNA (0.4 µg for six-well plate) and DNA/Effectene ratio of 1:25 were chosen for transient transfections, of no longer than 72 h.

To obtain stable transfectants, EGFP-positive cells were selected by Geneticin incubation (G418; Roche Diagnostics GmbH, Mannheim, Germany). Mock transfected cells were used as a reference for selection.

Transfection efficiency and subcellular localization of syndecan-1

Appearance of EGFP fusion proteins was monitored over time using a fluorescence time-lapse microscope (Leica DM IRBE; Openlab 3.0.4 software Improvision, Coventry, UK) to follow the EGFP-positive cells. Transfection efficiency was determined by calculating proportion of EGFP-positive cells to total number of cells in the same visual field. For each cell line, three randomly selected visual fields were evaluated.

Distribution of syndecan-1/EGFP fusion protein was further examined using immunocytochemical analysis and subsequent confocal laser microscopy. Stably transfected cells were seeded on to POLYSINE[®] microscope slides (Art. no. 041400; Menzel-Gläser, Braunschweig, Germany) which were allowed to adhere for 6–48 h before being fixed in 3% paraformaldehyde for 10 min at 37 °C. The cells were then permeabilized with 0.1% Triton X-100 (Sigma, Steinheim, Germany) for 10 min at room temperature. Non-specific binding was blocked with 3% goat serum (Dako A/S, Glostrup, Denmark) for 30 min, and then primary antibody against EGFP (Living Colors[®] A.v. Monoclonal Antibody (JL-8) Mouse IgG2a, Catalogue no. 632381; Clontech) was added. Slides were incubated overnight in a humidified chamber at 4 °C, followed by 30 min incubation with secondary antibody (Alexa 488 goat anti-mouse F(ab')₂ fragment of IgG (H + L), Molecular Probes, Leiden, The Netherlands, A11017) in the dark at room temperature. Samples were then counterstained with 1 mg/L bisbenzimidazole H33342 (Fluka, Steinheim, Germany) and were mounted using Dako fluorescent mounting medium (Dako, CA, Carpinteria, USA).

Detailed visualization was performed using a Leica TCS NT confocal laser scanning microscope, equipped with an ArKr laser, and detecting emission of signals at 568 nm. Scanning was performed using a 63 × 1.2 NA objective lens. Images were obtained by scanning in the

XY direction with focal depth of 0.3 µm. Images were processed using Adobe Photoshop software. For each experiment, excitation and sensitivity of the detector were normalized to corresponding negative controls.

Expression of syndecans

Total RNA was isolated from subconfluent cell cultures using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) with optional on-column DNase 1 digestion, according to the supplied protocol. Yield and purity of RNA preparations were estimated spectrophotometrically by measuring absorbance at 260 nm and A260/A280 ratio respectively.

Synthesis of cDNA was performed through reverse transcription of 2 µg RNA, using Omniscript reverse transcription kit (Qiagen) with random primers (final concentration 12 µM, Catalogue no. C1181; Promega, Madison, WI, USA).

Quantitative real-time RT-PCR was performed using Bio-Rad ICycler (Bio-Rad, Hercules, CA, USA) with 20–50 ng of cDNA/reaction, in triplicate, in 96-well plates using Platinum SYBR Green qPCR super mix (Invitrogen, Carlsbad, CA, USA). Final volume for each reaction was 25 µL with primer concentration of 500 nM. All syndecan primers were designed to correspond to the EC domain of respective syndecans (Table 1). Each primer pair provided only one amplicon, as judged from the melting curves. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. mRNA level was determined as an arbitrary unit by calculating the ratio between starting quantity for each syndecan and GAPDH. Three independent experiments were performed. Samples were analysed in triplicate for each independent experiment.

Synthesized syndecans were detected using fluorescence-activated cell sorting (FACS). Sub-confluent cells were detached using cell dissociation buffer (Gibco, Catalogue no. 13151-014), or 5 mM EDTA in phosphate-buffered saline (PBS) and were fixed in 1% formaldehyde.

Table 1. Syndecan primers sequences (5' to 3' orientation)

Analyte	Primers (5' to 3' orientation)	Ref
Syndecan-1	TCT GAC AAC TTC TCC GGC TC CCA CTT CTG GCA GGA CTA CA	Ridley RC (1993), Blood 81(3), 767-774
Syndecan-2	GGG AGC TGA TGA GGA TGT AG CAC TGG ATG GTT TGC GTT CT	Marynea P. (1989). J.Biol.Chem. 264, 7017-7024
Syndecan-3	GGT GAC AGA AGT CCC GGA AGA CTT GTG GCA GCA GTG GTA GC	Own design, sequence information from GeneBank
Syndecan-4	GAT GTG TCC AAC AAG GTG TCA ATG TGA GGA AGA CGG CAA GAG GAT	Own design, sequence information from GeneBank
Ref.gene: GAPDH	GGA CCT GAC CTG CCG TCT AG TGT AGC CCA GGA TGC TTT GA	Own design, sequence information from GeneBank

Blocking non-specific binding was performed using 0.5–1% bovine serum albumin (BSA) in PBS for 10 min. To detect syndecans, the cells were directly incubated with PE-conjugated specific antibody against syndecan-1 (CD138, Clone B-A38, Ref no. IQP-153R, IQ[®] Products; Groningen, The Netherlands) or, alternatively, with non-conjugated syndecan-1 (sc-12765), syndecan-2 (sc-9492), syndecan-3 (sc-9495) and syndecan-4 (sc-12766) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The latter non-conjugated antibodies were then visualized using donkey anti-mouse IgG Alexa Fluor 488 (Catalogue no. A21202) or donkey anti-goat IgG Alexa Fluor 488 (Catalogue no. A11055) antibodies from Molecular Probes. To detect intracellular syndecan-1, fixed cells were permeabilized with 0.1% saponin/PBS at room temperature for 15 min, prior to incubation with antibodies as above, but with all buffers containing 0.1% saponin. Flow cytometry was performed using Becton Dickinson Flow Cytometry (Mountain View, CA, USA) and analysing results using Cell Quest software according to the manufacturer's specifications. Syndecan-1 protein expression data were obtained from three independent experiments. For each sample, at least 10 000 cells were analysed. Obtained profiles were compared to those of the corresponding isotype controls.

Cell proliferation

Each stably transfected cell line was seeded in 96-well plates at density of 5000 cells/well; WST-1 colorimetric reagent (Roche, Catalogue no. 1 664 807) was used for quantification of metabolically active cells. Cell proliferation rate was calculated from their growth curve, as measured by WST-1 analysis at 4, 12, 24, 48 and 72 h after seeding, WST-1 reagent having been added 4 h before each respective time point. Samples were analysed using a Spectramax spectrophotometer, measuring absorbance at 450 nm and subtracting that at 650 nm as background control.

In parallel, the same cell culture set-ups were used for BrdU labelling, which was carried out by means of cell proliferation ELISA, BrdU Kit (Roche, Catalogue no. 11 647 229 001). This assay was used to monitor rate of DNA synthesis in proliferating cells; BrdU was added 4 h before each time point and incorporated BrdU was quantified according to the manufacturer's instructions, by measuring absorbance at 370 nm using the Spectramax spectrophotometer with absorbance at 492 nm as background control. Amount of incorporated nucleotide was calculated relative to total cell content, as determined by WST-1 reaction described earlier, normalizing rate of incorporation to that of EGFP transfectants. WST-1 assay and BrdU ELISA were performed in at least three independent experiments with six replicates in each.

Cell cycle analysis

Cell cycle analysis was performed by single parameter DNA flow cytometry. For DNA histograms, cells were harvested and analysed as described previously (25). Briefly, cells were fixed in 4% buffered formaldehyde for 18 h at room temperature. Formaldehyde was then removed using 95% ethanol for 1 h followed by rehydration in distilled water for 1 h. After treatment with subtilisin Carlsberg solution [0.1% Sigma protease XXIV, 0.1 M Tris-HCl and 0.07 M NaCl (pH 7.5)] and staining with DAPI-sulphorhodamine solution [8 mM DAPI, 50 mM sulphorhodamine 101, 0.1 M Tris-HCl and 0.07 M NaCl (pH 7.5)], samples were analysed using a PAS II flow cytometer (Partec, Münster, Germany) equipped with a 100 W mercury arc lamp HBO 100. DAPI fluorescence was measured above 435 nm. A multicycle program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA, USA) was used for histogram investigation; number of nuclei/histogram was 40 000; S-phase was fitted to a broadened trapezoidal model. For background correction, the sliced-nuclei model was applied. Average times required for the different cell cycle phases were calculated by combining these FACS data with the cell doubling times.

Cell morphology

Systematically randomized micrographs ($\times 500$) were taken from phase contrast microscopy from cells at 50–80% confluence. Cell shape was monitored as ratio between longest diameter and perpendicular diameter at the centre of the nucleus. The ratio was calculated in all cells where the cell borders could be identified (26). For each phenotype – stable transfectants and EGFP control cells – at least 220 cells were measured.

Statistical analysis

Differences between means were evaluated using Student's *t*-test. Null hypothesis of no difference was rejected at $\alpha = 0.05$.

Results

Generation of stable transfectants and subcellular localization of synthesized syndecan-1/EGFP fusion proteins

Initially, we examined expression of syndecan family members in B6FS fibrosarcoma cells compared to STAV-AB malignant mesothelioma (MM) cells. Endogenous expression of syndecan-1 was low in the latter (26) and

Figure 1. Overall syndecan profile of non-transfected B6FS fibrosarcoma cells. Protein expression levels were quantified using FACS analysis with specific antibodies (black histogram) and their respective isotype controls (white histogram). B6FS cells did not express syndecan-1 (a) and syndecan-3 (c), whereas they expressed a low level of syndecan-2 (b) and a high level of syndecan-4 (d).

virtually absent in the former (Fig. 1). B6FS fibrosarcoma cells also expressed some syndecan-2, but predominantly syndecan-4 (Fig. 1), similar to MM cells (26). The amount of syndecan-3 was too low to be detected in both cell lines. The two mesenchymal tumour cell lines thus have very similar syndecan profiles and thus are suitable to study effects of syndecan-1 overexpression. We transfected these two tumour cell lines with human full-length syndecan-1/EGFP construct a number of and truncated variants (Fig. 2), and generated stable transfectants.

Synthesized syndecan-1/EGFP fusion proteins were detected by confocal laser microscopy 24 h after transfection (Fig. 3). Overall transfection efficiency of the four and constructs EGFP control ranged from 15% in STAV-AB cells to 70% in B6FS cells. Control cells transfected with EGFP empty vector exhibited only faint cytoplasmic reactivity, whereas RMKKK/EGFP-transfected cells had strong fluorescence with distinct nuclear localization. Mainly 77/EGFP transfectants revealed cytoplasmic reactivity, whereas the slightly longer 78/EGFP construct showed both nuclear and faint cell membrane localization. Full-length syndecan-1/EGFP fusion protein was mainly seen in the cytoplasm with focally faint cell membrane reactivity. Interestingly, transfection with the 77 construct, lacking the whole EC domain of syndecan-1, initially caused the B6FS cells to round up and detach. A small

proportion of the 77 transfected cells had unchanged adhesive capacity and developed into a stable cell line.

After transfection, the cells were cultured under genetic selection thereby obtaining stable transfectants of both cell lines. After 2 months selection, STAV-AB cells formed colonies that could grow even under high selection pressure (800 µg G418/mL), whereas reference cells died after 2 weeks selection of only 200–400 µg/mL. The drug-resistant colonies were taken for further expansion,

Figure 2. Schematic representation of the syndecan-1 constructs. FL denotes the human full-length syndecan-1/EGFP construct. The truncated syndecan-1 variants include the 78 construct lacking the extracellular domain with the exception of the DRKE sequence, the 77 construct lacking the whole extracellular domain and the RMKKK construct corresponding to a hypothesized nuclear localization signal. The short red bar corresponds to the export signal peptide; EC, the extracellular domain (blue); TM, the transmembrane domain (grey); CD, the cytoplasmic domain (orange); and EGFP, the enhanced green fluorescence protein (green).

Figure 3. The subcellular localization of syndecan-1/EGFP fusion proteins detected by confocal laser microscopy 24 h after transfection of B6FS fibrosarcoma cells. Nontransfected cells were used as a background control (upper left column); EGFP transfected control cells revealed only cytoplasmic reactivity (upper middle column); Distinct nuclear localization was seen in the RMKKK/EGFP transfected cells (upper right column); 77/EGFP transfected cells showed strong reactivity in the peri-nuclear area (lower right column); 78/EGFP transfected cells showed weak nuclear localization and faint cell membrane reactivity (lower middle column); and the full-length syndecan-1/EGFP fusion protein revealed cytoplasmic and focally faint cell membrane reactivity (lower right column).

finally generating a stably transfected cell line. B6FS cells were further selected by repeated FACS sorting for EGFP-positive cells. Strongly positive cells were then grown out as a stable cell line. Attempts to sort STAV-AB cells in the same way failed as sorted cells could not be grown out in the subsequent culture. Overexpression of syndecan-1 mRNA by all stable transfectants was first evaluated using quantitative real-time RT-PCR. Transfection with full-length syndecan-1 construct caused a 5- to 10-fold increase in mRNA levels compared to corresponding EGFP vector controls (Fig. 4). Interestingly, truncated 77 and 78 constructs, which do not contain the ectodomain and were thereby not able to hybridize with the PCR primers used, also caused significant increases in syndecan-1 expression. Short RMKKK construct stimulated 2-fold increased syndecan-1 transcription in B6FS cells, whereas STAV-AB cells were unaffected.

Overexpression of syndecan-1 was also verified at the protein level by FACS analysis (Fig. 5), which showed 2- to 3-fold increase after transfection with the FL construct. In accordance with the mRNA results (Fig. 4), degree of overexpression was greater in STAV-AB cells than in B6FS cells. Likewise, increased amounts of syndecan-1 after transfection with the 77 construct were shown in both cell lines, which correlated well with the mRNA data (Fig. 4).

Overexpression of syndecan-1 influenced expression profile of other syndecan family members

In parallel with syndecan-1 mRNA expression in stable transfectants, we also measured the expression of the other

syndecan family members, syndecan-2, -3 and -4. Interestingly, we found that overexpression of syndecan-1 profoundly influenced the expression of the other syndecans. In MM cells, syndecan-1 was upregulated by all constructs except for the short RMKKK. There was, however, simultaneous downregulation of syndecan-2 in a compensatory way (Fig. 4). None of these constructs influenced the expression of syndecan-3 significantly.

The truncated domains had different effects on syndecan expression profile in MM cells (Fig. 4). Similar to transfection with full-length syndecan-1, the 78 construct decreased the expression of syndecan-2. The slightly shorter 77 construct had a less pronounced effect on syndecan-2 expression, but increased that of syndecan-4 significantly, whereas the 78 construct did not. There were no significant effects on syndecan-3 by any of the truncated constructs. The B6FS cells reacted slightly differently upon these transfections. Thus the 78 construct increased both syndecan-2 and -4 expressions, whereas the 77 slightly increased only syndecan-2 expression. The RMKKK construct, mainly found in cell nuclei, increased both syndecan-2 and -4 expression in B6FS cells, although it only increased syndecan-4 expression in MM cells.

Effects on cell proliferation and cell cycle distribution

Cell proliferation rates were calculated as doubling times based on the growth curves (Fig. 6). Most transfectants clearly showed longer doubling times compared to EGFP vector control, which showed the highest growth rate in both cell lines. This growth inhibition was observed in

Figure 4. The mRNA expression profile of syndecans in stable transfectants of STAV-AB MM (left column) and B6FS fibrosarcoma (right column) cells. The expression pattern was examined using quantitative real-time RT-PCR with syndecan-1, -2, -3, and -4 primer pairs (abbreviated as Syn-1, 2, 3 and 4). The mRNA level was given as an arbitrary unit (AU) calculated by the Starting Quantity ratio of each syndecan to GAPDH (as an endogenous control). Values are mean $\pm$ SEM ($n = 9$), obtained from three independent experiments with triplicate in each experiment. Asterisks denote a statistically significant difference from the EGFP control ($P < 0.05$).

both cell lines with both full-length syndecan-1 and the truncated constructs. The most profound effect was around a 30% decrease in cell proliferation rate. To investigate further the underlying reason for this decrease in cell proliferation, cell cycle analysis was performed, based on cell DNA content as determined using FACS analysis (Fig. 7a). While the time for G2/M phase progression was similar for different transfectants, cell lines with the most retarded proliferation had significantly prolonged G0/G1 and S phases. Thus, increased G0/G1 phase was the most evident change after transfection with RMKKK and 77 constructs, whereas prolonged S phase was more obvious in the FL transfectants. When normalized to the EGFP control, DNA incorporation rate (Fig. 7b) decreased after transfection of the FL construct. In these, significant decrease paralleled prolonged S phase, whereas with the truncated constructs, an increase in G0/G1 phase progression was most apparent and possibility of increased number of cells in G0 must be considered.

Effects on cell morphology

Previously, we have shown that STAV-AB cells vary their morphology depending on their overall syndecan content; they have lower length/width ratio with high syndecan content (26). When the fibrosarcoma cells were transfected to overexpress syndecan-1, this resulted in a similar significant decrease in length/width ratio from 2.7 to 2.2–2.3 for all constructs (Fig. 8). These cells, however, remained still more fibroblast-like than the epithelioid STAV-AB cells, but changed their morphology in an epithelioid direction. Interestingly, this decrease in length/width ratio seemed to vary, becoming greater with increasing length of the syndecan-1 constructs.

Discussion

Syndecan-1 is the main syndecan of epithelial tumours, whereas in sarcomas, expression levels of syndecan-1 are

Figure 5. The protein expression levels of syndecan-1 in stable transfectants of STAV-AB MM (a,c) and B6FS fibrosarcoma (b,d) cells. Cell surface syndecan-1 was detected with non-permeabilized cells (a,b). Total amount of syndecan-1 including both the cell surface and the intracellular protein was detected with permeabilized cells (c,d). The expression pattern was quantified using a PE-conjugated antibody against syndecan-1 (CD 138), with background subtraction of the corresponding IgG1 isotype control. Fold change was calculated based on the Mean Intensity value normalized to the EGFP control. Values are mean $\pm$ SEM ($n = 3$), obtained from three independent experiments. Asterisks denote a statistically significant difference from the EGFP control ($P < 0.05$).

Figure 6. Cell proliferation decreased in most syndecan-1 stable transfectants. The cell doubling time (left column) was calculated from the logarithmic phase of the growth curve (right column) obtained from the WST-1 reaction. Values are mean $\pm$ SEM ($n = 3$), obtained from three independent experiments with six replicates in each. Asterisks denote a statistically significant difference from the EGFP-transfected control cells ($P < 0.05$).

generally low (in accordance with their mesenchymal phenotype) and highly malignant behaviour (27). Very few previous studies have addressed the issue of syndecan-1 regulation in mesenchymal tumour cells, or importance of the respective functional domains of this PG. Our results reveal that overexpression of syndecan-1, in both MM and fibrosarcoma cells, significantly inhibited their proliferation. This effect seemed to correlate with both level of syndecan-1 expression and size of the PG construct. Thus,

prominent effects were seen with both full-length syndecan-1 and the highly expressed, short RMKKK sequence (Fig. 6).

For both cell lines, transfection of full-length construct resulted in a 2-fold increase in syndecan-1 protein levels. There is a substantial change in MM cells, and they simultaneously decreased their expression of syndecan-2. Therefore, transfection mainly changed syndecan profile, rather than total quantity of syndecans. The fibrosarcoma

Figure 7. Cell cycle analysis (a) was performed using DNA flow cytometry. DNA synthesis (b) was measured as incorporated BrdU using Cell Proliferation ELISA. The average times (a) required for the different cell cycle phases were calculated by combining cell populations from FACS data with the cell doubling times. The amount of incorporated nucleotides (b) was calculated by combining the total cell content determined in parallel by the WST-1 reaction and normalized to EGFP control. Three independent experiments were performed with all stable transfectants of B6FS fibrosarcoma cells. Asterisks denote a statistically significant difference from the EGFP-transfected control cells ($P < 0.05$).

cells have much lower endogenous expression of syndecan-1. Therefore, a 2-fold increase in protein would probably represent only a minor increase in absolute amount of this PG, corresponding to less compensatory changes of the other syndecans. Overexpression of syndecan-1 profoundly influenced expression of other syndecan family members, which are also important for regulating cell behaviour in mesenchymal tumours, and thus influence of other syndecans must be taken into consideration.

Many studies have suggested that syndecan-1 overexpression influences tumour cell proliferation in a cell type-dependent manner. In mouse squamous cell carcinoma (28), human endometrial cancer cells (29) and hepatocytes (30), overexpression of full-length syndecan-1 has been reported to increase cell proliferation, both *in vitro* and *in vivo*. Increased proliferation has also been seen another in fibrosarcoma cell line HT1080 when transfected with the same full-length syndecan-1 and 78 constructs as we used (17).

On the other hand, similar to the present findings in mesenchymal cell lines, decrease in cell growth has

Figure 8. Morphological changes in syndecan-1-overexpressing cells. Phase contrast micrographs ($\times 500$) of stably transfected B6FS cells were taken at 50–80% confluence. The length/width ratio of cells was measured and calculated as described in 'Materials and methods'. The length/width ratio is shown as the mean $\pm$ SEM for $n > 220$ cells. For overexpressing cell lines, these are $2.21\text{--}2.32 \pm 0.05\text{--}0.06$, and for EGFP-transfected control cells, they are 2.74 ± 0.08 . Asterisks denote a statistically significant difference from the EGFP-transfected control cells ($P \leq 0.001$).

been witnessed when full-length syndecan-1 is overexpressed in mouse mammary epithelial tumour cells (31); this latter effect has also been seen in benign fibroblasts (32). Interestingly, overexpression of syndecan-1 in benign renal cells gave a proliferative advantage to them when the cells were grown in serum-rich medium, whereas in low serum-containing medium, it was disadvantageous (33). This duality in the role of syndecan-1 in cell proliferation indicates a complex regulatory mechanism, which is tissue- and/or tumour type-specific, and at least partly dependent upon serum conditions (33).

Furthermore, membrane-bound and soluble syndecan-1 may have differential effects in the same tumour as shown by Nikolova *et al.*. Overexpression of wild-type syndecan-1 increased cell proliferation, whereas overexpression of constitutively shed syndecan-1 decreased cell proliferation of human breast cancer cells (18). This effect can partly be explained by co-receptor function of syndecan-1 for growth factors. Shedding of syndecan-1 converts membrane-bound co-receptor into soluble effectors, which, in turn, may act as agonists or antagonists that regulate cognate growth factor receptors (1). So variously engineered syndecan-1 ectodomain can mimic shedding and compete with intact syndecan-1 to regulate cell proliferation.

When cell proliferation rates (Fig. 6) are correlated with cell cycle distribution (Fig. 7a), it becomes apparent that growth delay in mesenchymal cells was mainly caused by prolonged S-phase, increasing from 7.2 ± 0.5 h to 10.1 ± 0.5 h. This was also seen in the BrdU experiment as decreased rate of DNA synthesis (Fig. 7b). Simultaneously with the decreased rate of proliferation, the

phenotype of transfected B6FS cells became even more epithelioid (Fig. 8). This phenotypic change is consistent with our previous finding that downregulation of endogenous syndecan-1 in epithelial MM cells by antisense targeting changes cell shape from polygonal to spindle-like (26).

Limited numbers of publications have so far dissected the role of distinct syndecan-1 domains in tumour cell proliferation, mainly focusing on the ectodomain. Overexpression of truncated syndecan-1 core protein lacking the C-terminal cytoplasmic tail induces hepatocyte proliferation (30) and growth of myeloma tumours *in vivo* (34). However, inhibitory effects have been described as well. For example, exogenously added entire mouse syndecan-1 ectodomain (as opposed to overexpressed full-length syndecan-1) suppressed the growth of mouse mammary tumour cells (35). A similar effect was obtained by transfecting these cells with minican, a shorter segment of syndecan-1 ectodomain, containing distal GAG attachment sites (36).

Little information is so far available regarding the possible role of TM and CDs domains of syndecan-1. The effect on syndecan expression profile was, however, also dependent on truncated constructs. Interestingly, transfections with the 77 and 78 constructs (Fig. 2), differing only in absence or presence respectively, of the DRKE motif corresponding to the four amino acids of the EC domain proximal to the TM domain, yielded somewhat variable results, with differences both between the two constructs and between the two cell lines. Both the 77 and 78 constructs upregulated syndecan-1 expression in both cell lines and affected expression of other syndecans. In the MM cells, both constructs downregulated syndecan-2 expression similar to that of the full-length construct, whereas the main effect of the 77 construct was upregulation of syndecan-4. In B6FS cells, the 78 construct increased expression of both syndecan-2 and syndecan-4, whereas the 77 construct only increased syndecan-2 expression. This may indicate that these effects on syndecan expression include oligomerization of the transcripts, and the DRKE motif in syndecan-1 may also be important for its polymerization.

Both the 77 and 78 constructs hampered cell proliferation, the mechanisms, however, seem to be different. Removal of the entire extracellular portion with the GAG chains normalized length of S-phase progression, whereas the 77, but not the 78 construct, prolonged the G0/G1 phase. Nevertheless, reduced cell proliferation also correlated with a more epithelioid morphology in these cells.

The TM domain is essential for oligomerization of syndecans in the cell membrane, and such self-association of core proteins within the membrane could result in formation of complexes that are able to regulate down-stream

signalling, including cell proliferation. The TM domains are highly conserved across family members, suggesting that this region may have overlapping functions (37,38); specifically, the TM domain is sufficient for inducing oligomerization of syndecan-2 and -4 (10). In the case of syndecan-3, on the other hand, the TM domain was found to be required, but not sufficient, for formation of multimeric complexes. Addition of the four flanking extracellular amino acids (ERKE) was necessary to confer this property, with conservation of the two basic residues being essential in this. There is only one amino acid difference between syndecan-1 and syndecan-3 within this tetrapeptide, glutamic acid (E) being replaced with aspartic acid (D). It has, however, not been shown that syndecan-1 can oligomerize in the same way as syndecan-3 (11), in fact, it has been suggested that cytoskeletal coupling may also contribute to syndecan-1 self-association (39,40). The more recent study by Dews and Mackenzie showed that TM domains of the syndecan family display a hierarchy of homotypic and heterotypic interactions. Syndecan-1 TM domain exhibits selectivity in binding syndecan-2 and syndecan-3, but not itself and syndecan-4. Weak self-association of syndecan-1 TM domain suggests that homodimerization of the full-length protein would require additional interaction partners. Selectivity of syndecan-1 heteromeric interactions shows that specific residues outside the conserved GxxxG dimerization motif must contribute to specificity (37).

We found that the short RMKKK motif was localized almost entirely in the nucleus, while the EGFP control cells showed only cytoplasmic fluorescence. This supported our hypothesis that the RMKKK motif serves as an NLS in syndecan-1. We also observed that FL transfectants in which EGFP was fused with the C-terminus of syndecan-1 had no nuclear localization, whereas the 78 transfectants fused with N-terminus had obvious nuclear localization. EGFP located at the CD of syndecan-1 in the FL construct may influence binding interactions with scaffolding proteins such as syntenin or CASK, and may thus also influence interactions with the cytoskeleton. Similar to our findings, data from Gaudin *et al.* and Davidson *et al.* indicate that specific residues near the carboxyl terminus of galectin-3 are required for nuclear translocation (41,42). The effect of RMKKK on cell proliferation was similar to that of the 77 construct, that is, simultaneously prolonging the G0/G1 phase. The mechanism for this remains unclear, but blocking nuclear translocation of syndecan-1 has been previously associated with hampered cell proliferation (20).

The present study shows that syndecan-1 influences cell proliferation through at least two different mechanisms. For the first mechanism of syndecan-1 action, participation of its EC domain is obligatory, and mainly

prolongs S-phase progression. The shorter constructs reduced proliferation by delaying the G0/G1 phase. This would then indicate a second mechanism, which could be related to capacity of the cell to translocate its syndecan-1 to the nucleus. Furthermore, cells overexpressing syndecan-1 showed a significant decrease in cell length/width ratio, suggesting that syndecan-1 may initiate cytoskeletal changes, which in turn influence cell division. This correlates well with our previous finding that antisense treatment against syndecan-1 leads to more elongated cells (26). Loss of syndecan-1 can also transform epithelial cells into a more mesenchymal morphology (43). Correlated with the decreased cell growth, this finding provides more evidence that malignant behaviour is associated with fibroblast-like morphology here.

Taken together, our study is the first report on overexpression of syndecan-1 in malignant mesothelioma cells. We demonstrate that this upregulation of syndecan-1 influences the expression profile of the other syndecan members and hampers tumour cell proliferation. Both full length and truncated syndecan-1 inhibit proliferation of the two malignant tumour cell lines studied, with two different mechanisms related to the size of the constructs. All constructs lead to a less fibroblast-like morphology. Our results provide new insights into importance of the different functional domains of syndecan-1 in mesenchymal tumours.

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