Steroid-induced epithelial-fibroblastic conversion associated with syndecan suppression in S115 mouse mammary tumor cells

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Cell-matrix interactions play an important role in the maintenance of cell shape, supposed to be mediated by the anchorage of cellular cytoskeleton to extracellular matrix via matrix receptors. In this work the expression of one of the known matrix receptors, syndecan, was studied during the hormone-induced change in the phenotype of Shionogi 115 (S115) mouse mammary tumor cells. In the presence of testosterone, when S115 cells express fibroblastic phenotype, they increased their growth rate and became gradually anchorage independent. These cells, however, revealed strong RGDS-dependent binding to fibronectin (FN) but no binding to the heparin-binding domain of FN. Instead, S115 cells grown without testosterone showed epithelial morphology and binding to the heparin-binding domain of FN, suggesting an alteration of syndecan expression in hormone-treated S115 cells. As quantitated by radioimmunoassay and by Western blot, the amounts of both matrix-binding ectodomain of syndecan and syndecan mRNA (2.6 kb) declined in hormone-treated S115 cells. The addition of antiandrogen cyproterone acetate to culture medium opposed the effect of testosterone on syndecan mRNA. We thus propose that the inactivation of syndecan gene and the consequent suppression of syndecan expression is related to the altered adhesion properties, the disappearance of epithelial phenotype, and, on the other hand, to the appearance of transformed-like phenotype in hormone-treated S115 cells.

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Introduction

Cell adhesion, spreading, proliferation, and differentiation are all based on close contacts between cell surface and surrounding extracellular matrix (ECM).¹ Cell surface molecules that mediate cell anchorage to ECM are called matrix receptors (Buck and Horwitz, 1987). The selective usage of ligand-receptor interaction in vivo may create the diversity of specific cell-matrix interactions needed for the development of organs (Ekblom *et al.*, 1986). Transformation is known to alter the response of cells to ECM (Liotta, 1986), suggesting that changes can occur in the expression of matrix receptors by malignant cells (Cheresh *et al.*, 1989; Plantefaber and Hynes, 1989). These changes are largely unknown, but they may have a fundamental role in the outcome of malignant behavior of various cell types.

One recently described matrix receptor is a cell-surface proteoglycan called syndecan (Saunders *et al.*, 1989). Syndecan consists of a matrix-binding ectodomain to which the glycosaminoglycan chains (heparan sulfate and/or chondroitin sulfate) are attached (Rapraeger *et al.*, 1985), and a membrane domain (Rapraeger and Bernfield, 1985), stretching 34 residues intracellularly (Saunders *et al.*, 1989; Mali *et al.*, 1990). Known matrix ligands for the ectodomain are type I, III, and V collagen fibrils (Koda *et al.*, 1985); C-terminal heparin-binding domain of fibronectin (Saunders and Bernfield, 1988); and trombospondin (Sun *et al.*, 1989). Ligand binding promotes syndecan to the close interaction with

¹ Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate-dehydrogenase; HBD, heparin-binding domain; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; i-FCS, heat-inactivated fetal calf serum; mAb, monoclonal antibody; NMuMG, mouse mammary epithelial cell line; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; S115, Shionogi S115 mouse mammary tumor cell line; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

actin-rich cytoskeleton (Rapraeger *et al.*, 1986), but cells can detach themselves from this interaction by proteolytic cleavage of the core protein that results in the shedding of the matrix-binding ectodomain from the cell surfaces (Jalkanen *et al.*, 1987). By these kinds of associations, syndecan can participate in the regulation of cell shape essential to the maintenance of cell morphology and phenotype.

The expression of syndecan during development and organ formation follows morphogenetic rather than histological boundaries (Thesleff *et al.*, 1988), a feature that also supports an active role for syndecan as a matrix receptor during development. Syndecan has been localized mainly on various epithelial cells (Hayashi *et al.*, 1987) but can also be found on the surfaces of plasma cells (Hayashi *et al.*, 1988). It localizes also to condensating mesenchyme next to budding epithelium during organ formation (Thesleff *et al.*, 1988). Very interestingly, its expression in the mesenchyme has been shown to be regulated by epithelial contact both in tooth (Vainio *et al.*, 1989a) and in metanephric kidney (Vainio *et al.*, 1989b), and in both tissues its expression can be correlated both spatially and temporally to the morphogenesis of these organs.

In this paper we have analyzed syndecan expression in relation to the induction of malignant phenotype in Shionogi 115 (S115) mouse mammary tumor cell line. These cells provide an excellent model for the studies of the transformed phenotype (Darbre and King, 1988) because they respond to androgens by increasing growth rate (King *et al.*, 1976) and by changing from epithelial to fibroblastic morphology (Yates and King, 1981). In the presence of androgen, the cells adhere loosely to substratum and acquire an ability to grow in suspension without anchorage to matrix, whereas in the absence of androgen they adhere tightly to the matrix and do not grow at all in suspension (Yates and King, 1981). The basis for the morphological change and for anchorage independency is largely unknown, although several mechanisms—including changes in cytoskeletal organization (Couchman *et al.*, 1981) and in paracrine mechanisms (Darbre and King, 1988)—have been proposed. We show in this paper that addition of androgen to S115 cultures results in the suppression of syndecan expression because of the inactivation of syndecan gene. This suppression closely correlates to the acquisition of anchorage independency and to the loss of epithelial phenotype and thus can be one of the

reasons to the outcome of transformed-like behavior of S115 cells in the presence of androgen.

Results

Growth rate and morphology of S115 cells is regulated by testosterone

The cloned cell line S115 from mouse mammary carcinoma exhibited a positive proliferative response to androgen (Figure 1), and the removal of hormone from the culture medium resulted in a total change of cells from fibroblastic to epithelial morphology (Figure 2, A–D), as shown previously by King *et al.* (1976) and Yates and King (1981). Besides stimulating the growth of cells anchored to tissue culture plastic (Figure 1), testosterone also caused some of the cells to detach and grow in suspension (see round cells in Figure 2, B and D). This behavior was profoundly apparent during the extension of testosterone exposure (compare Figure 2, panels A and C, with B and D). However, the shift from epithelial to fibroblastic morphology provided us with a model to analyze possible changes in the expression of syndecan and to relate these changes to the maintenance of the epithelial phenotype of S115 cells. By the use of the monoclonal antibody 281-2, which recognizes the core protein of the extracellular part (ectodomain) of syndecan (Jalkanen *et al.*, 1985), we observed S115 cells to express this molecule on their surfaces (Figure 2E), suggesting that syndecan could be one of the adhesive mole-

Figure 1. Growth of S115 monolayers. The S115 monolayers were cultured in the presence (■) or absence (□) of 10 nM testosterone as described in the text. Monolayers were harvested for cell counts on days 1, 3, 5, and 7 after plating (day 0), as indicated in the figure. The cells showed a positive proliferative response to testosterone. Each point represents the mean of triplicated counts of four dishes. Three separate experiments gave similar results.

Figure 2. Testosterone-induced conversion of S115 cell morphology. Panels A–D represent phase contrast pictures of the S115 cells grown in the absence (A and C) and presence (B and D) of testosterone to subconfluent (A and B) and early confluent (C and D) cultures. In panels E and F, intact cells were stained both with 281-2 (E) and MEL-14 (F; negative control), indicating the presence of syndecan at the cell surface of S115 cells.

cles on the surface of S115 cells, that its expression could be altered at the cell surfaces of hormone-treated cells, and that the possible lack of syndecan from hormone-treated cells may be one of the reasons for poor formation of adhesion plaques observed in these cells (Couchman *et al.*, 1981).

Testosterone-exposed S115 cells fail to bind to the heparin-binding domain of fibronectin

To study the possible functional alteration in S115 cell-matrix interactions, we analyzed their ability to bind fibronectin (FN), which is known to contain binding sites both for integrins (RGD-dependent binding) and for syndecan (heparin-

Figure 3. The effect of testosterone treatment on the binding of S115 to FN. After 3 d culture of S115 cells with or without testosterone, monolayers were metabolically labeled with ^{35}S -methionine and detached; suspended cells were used in cell-binding assay as described in the text. Total binding of hormone-treated (left panel) and nontreated (right panel) S115 cells to FN was 56 and 62%, respectively. For comparison, maximal binding of 100% was given for these values. Each bar represents a mean $\pm$ SD of four individual wells. In the competition assay, RGDS-peptide and heparin were used with concentrations of 500 and 100 $\mu\text{g}/\text{ml}$, respectively.

binding domain; HBD). For this purpose ^{35}S -methionine prelabeled hormone-treated and nontreated cells were tested for their binding to intact FN and HBD. Both cell types showed strong binding to FN (Figure 3). RGDS-peptide almost completely inhibited the binding of hormone-treated cells to FN, but these cells revealed low binding to HBD (Figure 3). In the case of (non-treated) epithelial S115 cells, RGDS only partially inhibited binding to FN. Furthermore, these cells showed good binding to HBD and were inhibitable with heparin (Figure 3). These binding results indicated that hormone treatment of S115 cells reduces binding of these cells to FN by decreasing heparan sulfate-mediated binding but not integrin-mediated binding. This finding suggested that testosterone treatment of S115 cells could influence syndecan expression, which was therefore studied further.

Loss of syndecan expression follows testosterone-regulated phenotype shift in S115 cells

Initial stainings with the monoclonal antibody (mAb) 281-2 against the core protein of syndecan ectodomain (Jalkanen *et al.*, 1985) demonstrated less intense mAb 281-2 stain on S115 cells grown in the presence of testosterone, when their phenotype was fibroblastic, than on

cells without testosterone, when S115 cells express epithelial phenotype. We analyzed possible differences in syndecan expression of S115 cells by established quantitative assay for the ectodomain (Jalkanen *et al.*, 1987). In this assay the trypsin-released ectodomain was loaded onto cationic nylon membrane, which subsequently was probed with radioiodinated 281-2. The assay was standardized with the ectodomain from mouse mammary epithelial (NMuMG) cells (Jalkanen *et al.*, 1985; Rapraeger and Bernfield, 1985).

Stock S115 cells are routinely grown with full serum in the presence of testosterone. These cultures clearly contained syndecan (Figure 4, d0). However, as soon as these cultures were maintained in the dextran charcoal-treated serum (to remove endogenous hormones of serum) with or without androgen, the differential expression of syndecan was evident. Without testosterone the epithelial morphology was prominent and the expression of syndecan was doubled after 3 d compared with cultures at the time of plating (Figure 4, d3). When the S115 cells were grown for 3 d without testosterone, maximal syndecan expression was induced and

Figure 4. Quantitation of cell-surface syndecan in S115 cultures. For quantitation of cell-surface syndecan content, ectodomains from 10^5 cells were trypsin released at various time points of testosterone treatment (day 0, 3, 5, or 7) and loaded onto cationic nylon membrane as described in the text. The filter was probed with radioiodinated 281-2 and visualized autoradiographically. Standardization of each experiment was carried out with the 0.1 μg of ectodomain from NMuMG cells. Day 0 stands for the stock cells grown in full serum (5%) and 10 nM testosterone and plated for the experiment. Each column shows the value (mean $\pm$ SD) of densitometric scanning of four independent experiments. ■, cultures grown without testosterone; □, cultures with testosterone. The standard amount was blotted as a reference in each experiment, and the relative amounts of syndecan ectodomain from S115 cells after various treatments were calculated (scale on the left).

it was maintained high for the rest of the 7-d period of culture (Figure 4). The introduction of dextran charcoal-treated serum supplemented with hormone, however, resulted in a significant decrease in syndecan expression, which was only one-ninth of that observed in cultures without any hormone on day 3 of the culture (Figure 4, d3). The 5- or 7-d treatment with testosterone reduced syndecan expression even further, and the smallest amounts were obtained on day 7 of the cultures (Figure 4, d7).

Syndecan expression was also studied by Western blotting the ectodomain from various time points of cultures. Again the well-characterized syndecan from NMuMG cells was analyzed simultaneously as a comparison (Figure 5, lane S on the right: the average modal molecular mass of this preparation is 200–250 kDa; see Jalkanen *et al.*, 1985, 1987). All S115 cell cultures grown in the absence of testosterone expressed syndecan, which was similar to that of NMuMG cells (Figure 5, lanes d3–, d5–, d7–, and S). Reduced amounts of syndecan were also observed in testosterone-treated cultures with this technique (Figure 5, lanes d3+, d5+, and d7+). However, when ectodomains were extracted from a larger number (2.5-fold) of cells, faint stainings for syndecan were also found in testosterone-treated cells (Figure 5, lanes d3+, d5+, and d7+). It was notable that, when syndecan level decreased after testosterone treatment, total protein labeling with ^{35}S -methionine concomitantly increased (51 360 and 21 730 cpm/ 10^4 cells when labeled with ^{35}S -methionine for 24 h after 4 d in culture, with or without testosterone, respectively; similar stimulation by testosterone was also observed on day 7).

It was interesting to see that hormone-treated cells were capable of synthesizing syndecan that has similar size to that of syndecan from NMuMG cells or from S115 cells without hormone treatment (Figure 5, lanes d3+ and d7+). This supports the idea that cells under hormone influence are capable of synthesizing fully glucosylated syndecan even though the total expression of syndecan was reduced.

Syndecan gene is suppressed in hormone-induced transformed phenotype of the S115 cells

One way to down-regulate syndecan expression is to suppress transcription of the syndecan gene. We isolated total RNA preparations from cultures grown in the presence and absence of testosterone to see the levels of syndecan

Figure 5. Analysis of the size of syndecan ectodomain by Western blotting. Ectodomains from 2.5×10^6 S115 cells grown with testosterone (d+) or from 10^6 cells grown without testosterone (d-) were released with trypsin and precipitated with ethanol. The whole yield was solubilized in sample buffer, fractionated on SDS-PAGE gradient (4–10%), and transferred onto a cationic nylon membrane for ^{125}I 281-2 probing. Ectodomain (0.5 μg) from NMuMG cells was used as a positive control and for the molecular weight comparison.

mRNA in these cultures. As shown in Figure 6, testosterone-maintained stock cells expressed hardly any detectable mRNA for syndecan (Figure 6, lane 1), and levels of syndecan mRNA observed in cultures after extended testosterone treatment were also low (Figure 6, lanes 3, 5, and 7). However, removal of testosterone for 3 d permitted the expression of syndecan, which was maximal 5 d after hormone withdrawal (Figure 6, lanes 2, 4, and 6). The levels of two other mRNAs—glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) and alpha-actin (Fort *et al.*, 1985 and Minty *et al.*, 1981, respectively)—commonly used for comparison did not show any change in expression at the same time points (Figure 6B). Syndecan reexpression and specificity were further studied by quantitation against ribosomal RNA. By this way a 5- to 7-fold stimulation of syndecan mRNA content was observed on days 3, 5, and 7 of the culture (Figure 7) compared with the sample collected from the same time point but grown in the presence of testosterone (Figure 7).

In the original NMuMG cultures and in several tissues in vivo, the ratio of 2.6-kb mRNA versus 3.4-kb mRNA has been described to be 3:1 (Saunders *et al.*, 1989). S115 cells, however, especially in the absence of testosterone, expressed these two messages in the ratio of 8:

Figure 6. Northern analysis of syndecan mRNA levels. Total RNA was isolated from androgen-maintained stock cells used for plating on day 0 (lane 1) and thereafter from the cultures grown with (lanes 3, 5, and 7) and without (lanes 2, 4, and 6) testosterone for 3 d (lanes 2 and 3), for 5 d (lanes 4 and 5) and for 7 d (lanes 6 and 7). From each sample, 15 μ g of total RNA was fractionated on 1% agarose gel, transferred to a filter, and hybridized with syndecan (A) or GAPDH and alpha-actin (B) cDNA probes as described in the text. The migration of ribosomal RNA in A is indicated. Sizes for syndecan mRNAs are 2.6 and 3.4 kb (Saunders *et al.*, 1989) and for GAPDH and alpha-actin are 1.3 and 2.1 kb, respectively.

1 (Figure 6), indicating that the activation of syndecan gene expression resulted mainly in the production of smaller mRNA (2.6 kb), and that whatever the mechanism for the production of two mRNAs, it favors the expression of the smaller 2.6-kb mRNA during the epithelial-fibroblastic phenotype shift of S115 cells. These results clearly indicate that syndecan gene is suppressed as long as the hormone is present in S115 cultures but is activated as soon as hor-

mone has been removed, suggesting that this gene is sensitive to hormone exposure directly or indirectly and that its activation closely correlates to the appearance of epithelial phenotype in S115 cells.

Finally, we tested whether antiandrogen cyproterone acetate, which is specifically known to block androgen effects (Poyet and Labrie, 1985), would also prevent syndecan gene suppression. As seen in Figure 8, testosterone-induced reduction of syndecan mRNA content (Figure 8, lane 3) was not observed in S115 cells treated both with testosterone and antiandrogen cyproterone acetate for 3 d (Figure 8, lane 5), indicating that, whether the testosterone effect on syndecan expression is direct or indirect, the initial events must involve the activation of androgen receptor complex.

Discussion

Transformation alters cell anchorage to substratum (Buck and Horwitz, 1987). As this anchorage is dependent on the expression of adhesive molecules at the cell surface, it is a natural conclusion to study the expression of individual adhesive molecules in biological models of malignant transformation (Cheresh *et al.*, 1989; Plantefaber and Hynes, 1989). The cell surface proteoglycan-syndecan has several features of a matrix receptor: it is an integral membrane component (Rapraeger and Bernfield, 1985; Saunders *et al.*, 1989; Mali *et al.*, 1990), it binds several ECM components with high specificity (Koda *et al.*, 1985; Saunders and

Figure 7. Quantitation of syndecan mRNA levels. For accurate quantitation of syndecan mRNA levels, autoradiographs of syndecan Northern blots were scanned and corrected against the ribosomal RNA loading as described. Columns show the levels of syndecan mRNA from the cultures grown with (□) or without (■) testosterone.

Figure 8. Reversal of testosterone-induced syndecan suppression by anti-androgen. Total RNA was isolated and 15 μ g loaded for Northern analysis from S115 cells grown without hormone for 0 (lane 1) or 3 d (lane 2), with 10 nM testosterone for 3 d (lane 3), with 1 μ M cyproterone acetate for 3 d (lane 4) and with 10 nM testosterone and 1 μ M cyproterone acetate for 3 d (lane 5). Observe the reduced syndecan mRNA content in the presence of testosterone in lane 3, which was reversed with anti-androgen in lane 5.

Bernfield, 1988; Sun *et al.*, 1989), it can connect and/or disconnect epithelial cytoskeleton to ECM (Rapraeger *et al.*, 1986; Jalkanen *et al.*, 1987), and its expression is strongly regulated during development (Thesleff *et al.*, 1988; Vainio *et al.*, 1989a,b). Our present work presents additional evidence for this role of syndecan. Using S115 cell line, which changes its phenotype from epithelial to fibroblastic according to the hormonal status in the growth medium (Yates and King, 1981), we were able to correlate the maintenance of the epithelial morphology to the expression of syndecan in these cells. Furthermore, the loss of syndecan expression also coincided with the shift to a malignant phenotype in these cultures.

Expression of syndecan correlates to the appearance of epithelial phenotype in S115 cells

The change in morphology from epithelial cell type to a more fibroblast-like cell type can be induced in S115 cell cultures by exposing the cells to androgen. Both our radioimmunoassay and Western blotting results indicated that S115 cells grown in the presence of testosterone sig-

nificantly reduced their syndecan expression. This can be explained by reduced expression of syndecan gene, because testosterone-exposed cells revealed very low amounts of syndecan mRNA, thus possibly preventing the expression of syndecan core protein. This finding is supported by the fact that, as soon as the hormone was removed, S115 cells reexpressed the syndecan mRNA and also cell-surface syndecan. Transformed cells could also have higher turnover for syndecan mRNA, which would result in lower syndecan content, and we are currently analyzing this possibility, although it appears unlikely to be the main regulatory mechanism to maintain syndecan mRNA levels in S115 cells. The suppression of syndecan in testosterone-treated S115 cells is rather specific. Namely, testosterone generally enhances rather than suppresses the protein synthesis: we measured a 2.5-fold increase in protein synthesis of S115 cells after 4- or 7-d culture with testosterone. Furthermore, hormone-treated cells did not show any significant changes in another FN receptor, α -5/ β -1 receptor of the integrin family, at either protein or mRNA levels (our unpublished results). This also explains the good binding of hormone-treated S115 cells to FN by an RGDS-dependent mechanism (Figure 3).

S115 cells cultured without hormone adhered tightly to the underlying substrate (Figure 2). One explanation could be the increased binding of S115 cells to HBD of FN because of increased expression of syndecan (see Figure 3). Heparan sulfate proteoglycans have been found in focal adhesion sites (Woods *et al.*, 1984), and cells failing to express heparan sulfate do not form focal adhesion sites on FN substrate (LeBaron *et al.*, 1988). Further evidence for the involvement of heparan sulfate proteoglycans in the formation of focal adhesion sites comes from the work by Woods *et al.* (1986), who showed that fibroblasts cannot form focal adhesions without HBD of FN adsorbed to the substrate (Woods *et al.*, 1986). Our work supports the idea that syndecan as a matrix receptor can be one of the integral membrane components, that connect S115 cells to substratum. This can also explain the poor attachment of S115 cells treated with testosterone (Figure 2). S115 cells provide an excellent and simple model to study these questions in the future. The analysis of S115 genetic variants for anchorage independence (Härkönen *et al.*, 1990) will further evaluate the role and regulation of syndecan in cell attachment.

How is syndecan expression regulated?

In this work we have demonstrated that androgen treatment of S115 cells decreased syndecan expression. Because an androgen receptor antagonist reversed this effect, syndecan expression is likely to be mediated by androgen receptor complex. Concomitant changes in syndecan expression were seen at both mRNA and protein levels. This would suggest that the hormone regulates syndecan gene at transcriptional and/or posttranscriptional levels. Androgens are supposed to influence responsive genes as other steroid hormones do, primarily by modulating transcription rates by the interaction of the hormone receptor with the regulatory enhancer elements (Beato *et al.*, 1989; Berger and Watson, 1989); but they can also affect posttranscriptional processes, such as mRNA processing and stabilization (Page and Parker, 1982). Androgens have a stimulatory effect on most of the known target genes (Page and Parker, 1983; Darbre *et al.*, 1986; Cato *et al.*, 1988), but some androgen-repressed genes have also been described (Chatterjee *et al.*, 1987; Leger *et al.*, 1987; Saltzman *et al.*, 1987). These are of unknown function, but they have been supposed to have an important role in androgen-regulated growth and differentiation of the target tissues. The enhancer elements of androgen-regulated MMTV-LTR (Cato *et al.*, 1988; Ham *et al.*, 1988) have been analyzed, but any molecular mechanisms of androgen repression have not yet been characterized. It is not known whether syndecan gene contains any steroid response elements. If it does, the results of this study could be explained by a direct suppression of syndecan gene by androgens and a consequent disappearance of syndecan from cell surfaces.

Syndecan expression has been shown to be regulated in other steroid-responsive tissues such as vagina, in which epithelial differentiation fluctuates during the estrous cycle. In vagina, changing levels of estrogen and progesterone result in dramatic spatial and quantitative changes in syndecan expression (Hayashi *et al.*, 1988). These results demonstrate another example of parallel changes in syndecan expression and morphological change under steroid influence, but no conclusions of the mutual consequences can be drawn before further analyses of the mechanisms of the regulation of syndecan gene by steroid hormones.

Another possibility is that syndecan expression is more related to the phenotype switch

and that its disappearance in S115 cells is just a result of the change from epithelial to fibroblastic morphology in the presence of androgens. In this case, syndecan expression would not be directly related to steroid-regulated pathways but to more distal events in the cascade(s) leading to the morphological change. It is of interest that, during the development of organs *in vivo*, syndecan expression again follows morphological rather than histological patterns (Thesleff *et al.*, 1988; Vainio *et al.*, 1989b), which supports the idea that syndecan expression could be linked to the regulation of morphology of the cells.

Transformation changes the expression of adhesive cell surface molecules

Transformed cells are known to synthesize poorly organized pericellular matrix, which, e.g., does not contain FN fibrils found in normal counterparts. The failure in the deposition of pericellular FN matrix is due not to reduced biosynthesis (Vaheri and Ruoslahti, 1975) but to reduced ability of transformed cells to adhere to FN (Wagner *et al.*, 1981), which subsequently prevents the formation of cell-matrix connections. The latter can also be facilitated by increased secretion of proteolytic enzymes by transformed cells (Chen and Chen, 1987). Syndecan is a matrix receptor for FN (Saunders and Bernfield, 1988), and its reduced expression suggests a putative molecular mechanism for reduced adherence of transformed cells to substratum. Our results with transformed S115 cells indicate that hormonal transformation results in the suppression of syndecan gene followed by the disappearance of syndecan from the cell surfaces. Thus we think that the loss of syndecan expression in these cells may be one of the reasons for reduced ECM recognition. This can be a common mechanism in carcinogenesis because spontaneously transformed mouse mammary epithelial cells also express reduced amounts of syndecan (Saunders *et al.*, 1987). Our preliminary data with epithelial cells transfected with inducible *ras*-oncogene construct indicate that, when these cells get transformed and become anchorage-independent, they express reduced amounts of syndecan (Kirjavainen, J., Hynes, N., and Jalkanen, M., submitted).

Syndecan is not the only matrix receptor that shows altered expression during transformation. Plantefaber and Hynes (1989) recently observed changes in the expression of integrin re-

ceptors (especially in those binding to FN) on oncogenically transformed cells. In addition, the cytoplasmic domain of integrins has been shown to be more phosphorylated in transformed cells (Hirst *et al.*, 1986) and this may reduce the ability of integrins to link ECM to cytoskeletal elements of cells (Buck and Horwitz, 1987). Obviously, S115 cells provide an excellent model to study also the regulation of integrins during the transformation of epithelial cells.

In conclusion, our study supports the view that matrix receptors may represent a new and important way of looking at the explanations for the altered behavior of malignant cells in response to ECM. This can be more complex than the present data suggest and it can be different depending on the tissue or cell type studied. The alterations in the expression of matrix receptors may also vary according to the triggering elements that transform the cells.

Methods

Cell culture

Mouse mammary tumor S115 stock cells (kindly provided by Dr. Philippa Darbe and Dr. R. King, The Imperial Cancer Research Fund, London) were routinely cultured in DMEM Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (i-FCS), pyruvate (1 mM), glutamine (1 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and testosterone (10 nM). For studies involving hormone treatment, growth medium-supplemented with 4% dextran charcoal-treated fetal calf serum was used with or without 10 nM testosterone and/or 1 µM cyproterone acetate. For experiments, cells were plated at density of 10 000 cells/cm² on Nunc tissue culture dishes. For cell count cells were lysed in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 1.5 mM MgCl₂ containing Zaboglobin (Coulter Electronics, Harpenden, UK); the nuclei released were suspended in Isoton (Coulter) and finally counted on a Coulter cell counter.

For metabolic labeling of proteins with ³⁵S-methionine, S115 cells were grown in 6-cm dishes for 3, 4, or 7 d with or without testosterone. Each dish was washed three times with saline and labeled in 2.5 ml of methionine-free DMEM supplemented with 1% dialyzed dextran charcoal-treated FCS, with or without testosterone, and with 50 µCi ³⁵S-methionine (New England Nuclear, Boston, MA) for 18 h. The cells were washed and harvested in phosphate-buffered saline (PBS). Aliquots were taken for cell count; the rest of the cells were lysed in 0.3 M NaOH, and proteins were precipitated with 20 volumes of 5% trichloroacetic acid (TCA). The precipitates were collected on Whatmann 3 M discs; air dried; washed in 5% TCA, ethanol, ethanol-ether (1:1), ether; and dried. The discs were counted by liquid scintillation counter.

Antibodies and immunostainings

S115 cells were stained on day 4 of culture, intact as described (Jalkanen *et al.*, 1987). The cell-surface proteoglycan, syndecan, was immunolocalized with a rat mAb 281-2

against the core protein of the proteoglycan (Jalkanen *et al.*, 1985); these stainings were controlled by the use of another IgG_{2a} mAb, Mel-14, specific for lymphocyte homing receptor (Gallatin *et al.*, 1983). Detection of immobilized rat antibodies was done with rabbit anti-rat fluorescein isothiocyanate conjugate (Janssen Biochimica, Beerse, Belgium).

Cell binding assay

Binding of S115 cells to FN and to its HBD was studied in microtiter plates (Nunc, Roskilde, Denmark) with the following protocol. Wells were first coated with FN or its HBD (Calbiochem, La Jolla, CA) at 50 µg/ml overnight at +4°C. Before use, the wells were saturated with bovine serum albumin (BSA; Sigma, St. Louis, MO) at 1 mg/ml for 1 h at room temperature and rinsed several times with PBS. Cells grown for 4 d with or without hormone were labeled with 40 µCi ³⁵S-methionine for 24 h in methionine-free DMEM supplemented with 1% dialyzed serum and with or without 10 µM testosterone. Labeling medium was removed and monolayers were rinsed several times with EDTA-PBS. Then cells were detached by brief EDTA-PBS incubation, rubber policeman detachment, and suspension as described by Saunders and Bernfield (1988). The detached and repelled cells were suspended into warm DMEM and 50 000 cells/well were pipetted into each well. Wells were incubated at 37°C for 45 min followed by several rinses with cold PBS. Finally, bound cells were lysed into 1% sodium dodecyl sulfate (SDS) and counted by liquid scintillation. If an inhibitor (RGDS-peptide or heparin) was included in the assay, cells were incubated with it for 15 min before plating them into wells. Wells coated with BSA (100 µg/ml) and saturated similar to FN or HBD wells were used to determine the background for the assay.

Quantitation of syndecan

For quantitation of syndecan on cell surfaces of S115 cells, monolayers were washed several times with cold PBS and the ectodomain of the molecule was released with bovine pancreatic trypsin (Sigma, Type III; 20 µg/ml) by 10-min incubation on ice as described by Rapraeger and Bernfield (1985). After trypsin inactivation by trypsin inhibitor (100 µg/ml), cells were centrifuged, supernatant was recovered for ectodomain quantitation, and cells were suspended into Isoton for cell count.

The mAb 281-2 was radioiodinated by the chloramine-T oxidation method (Stähli *et al.*, 1983) to a specific activity of 14.1 × 10⁶ cpm/µg. For the assay, supernatants from 2 × 10⁵ cells and a purified control syndecan from NMuMG cells were loaded onto cationic nylon membrane (Zeta-Probe, BioRad, Richmond, CA) in a minifold-slot apparatus (Schleicher and Schuell, Dassel, Germany) as described earlier (Jalkanen *et al.*, 1987). Membrane was detached from the slot apparatus and incubated for 1 h at room temperature in PBS supplemented with 10% FCS to block the membrane. Then it was incubated overnight at +4°C in PBS containing ¹²⁵I-labeled 281-2 (10 000 cpm/ml). After washing the filter five times with PBS, we exposed it to Kodak X-Omat film (Rochester, NY) to visualize the bound 281-2. For quantitation, each slot was analyzed by LKB Ultrascan XL enhanced laser densitometer and compared with known amount of syndecan from NMuMG cells.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Trypsin-released ectodomains from S115 cells were ethanol precipitated and fractionated on SDS-PAGE gradient (4–

10%) gel (O'Farrell, 1975). After electrophoresis, samples were transferred onto Zeta-Probe membrane using electroblotting 2005 Transphor apparatus (LKB, Bromma, Sweden) as described earlier (Jalkanen *et al.*, 1985; Rapraeger *et al.*, 1985), and the membrane was exposed to ¹²⁵I-labeled 281-2 as described above. Again, isolated syndecan ectodomain from NMuMG cells was used for comparison (Figure 5, lane S).

Northern blotting

Total RNA was isolated using 4 M guanidine isothiocyanate and CsCl pelleting as described by Chirgwin *et al.* (1979). RNA aliquots of 15 µg were fractionated on formaldehyde agarose gel (1%) and transferred to Gene Screen Plus membrane. Hybridization with multi-prime (Amersham, Arlington Heights, IL) labeled insert of PM-4 cDNA probe for syndecan (Saunders *et al.*, 1989) was performed under conditions suggested by the manufacturer of the membrane (New England Nuclear). Immobilized probe was visualized by exposing the membrane to Kodak X-Omat film at -70°C, and quantitation of 2.6 kb mRNA was done by densitometric analysis described above. The expression of syndecan mRNA was correlated to ribosomal RNA as described by Denis *et al.* (1988). The specificity of syndecan suppression was further studied by probing the same samples for rat GAPDH (Fort *et al.*, 1985) and mouse alpha-actin (Minty *et al.*, 1981).

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