Tumour regulation of fibroblast hyaluronan expression: a mechanism to facilitate tumour growth and invasion

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Hyaluronan, a high molecular weight glycosaminoglycan is associated with cellular proliferation and migration. In a number of different tumour types, there is a close correlation between tumour progression and hyaluronan production, either by the tumour cells or the surrounding stromal cells. We have examined the ability of an aggressive melanoma cell line (C8161) to stimulate the synthesis of fibroblast hyaluronan, and the association of cell-surface CD44 receptors and hyaluronan with invasion. Melanoma cell-conditioned medium (CM) prepared in low glucose medium (1 mg/ml) stimulated the synthesis of fibroblast glycosaminoglycan as measured by $[^3H]$ glucosamine incorporation, and the synthesis of hyaluronan as measured using a specific hyaluronan-binding plate assay, while tumour cell-CM prepared in high glucose medium (4.5 mg/ml) inhibited the synthesis of fibroblast glycosaminoglycan. High glucose tumour cell-CM contained large amounts of lactate that appeared to inhibit the tumour-derived factor stimulation of fibroblast glycosaminoglycan synthesis, as removal of the lactate restored the stimulating activity. Melanoma cells seeded on contracted collagen lattices and incubated at the air/liquid interface rapidly formed a multilayered cell mass on the surface, with significant invasion of the gel. Hyaluronan staining was apparent within the collagen gel, and strong staining was seen around the invading tumour cells, but not around those cell layers near the surface. CD44 expression on the tumour cells was confined to those invading cells and corresponded to cellular hyaluronan staining. Hyaluronan staining was also apparent around and between tumour cells invading fibroblast-free collagen lattices. Monolayer cultures of C8161 cells stained strongly for CD44, but few cells stained for hyaluronan, while no detectable hyaluronan was released into the medium. In summary, the C8161 melanoma cells stimulated the synthesis of fibroblast hyaluronan, and in collagen lattices, only the invasive tumour cells expressed CD44 and hyaluronan, either in the presence or absence of fibroblasts.

Introduction

Hyaluronan is a ubiquitous, very large, linear non-sulfated glycosaminoglycan composed of a repeating disaccharide unit of glucuronic acid and $N$-acetyl glucosamine. The polymer may contain in excess of 25,000 disaccharide units resulting in a molecular weight of up to $10^7$ Da. Hyaluronan directly affects tissue homeostasis owing to its biophysical properties, and as it is one of the most hygroscopic natural molecules known, it has a profound effect on tissue hydration. Hyaluronan also interacts with several extracellular and pericellular matrix molecules (hyaladherins) and thereby influences the deposition of matrix molecules and the structural integrity of tissues, while cell function is influenced by hyaluronan through interaction with cell surface receptors such as CD44 (1). Hyaluronan-rich stroma and pericellular matrices are associated with cellular proliferation and migration, as observed during morphogenesis (2), wound healing (3) and tumour growth and invasion (4,5).

Several tumour cell types exhibit elevated levels of hyaluronan expression compared with their normal counterparts. This is particularly apparent if the normal cells are non- or poor expressers of hyaluronan, such as some epithelia of breast, stomach and colon (6,7). Tumours arising from stratified epithelia frequently exhibit a decreased expression of hyaluronan that appears to contradict the proposed role of hyaluronan in facilitating tumour cell growth and invasion (8,9). However, there is probably still sufficient hyaluronan to fulfil this role, and the decreased expression may simply reflect the loss or reduction of the differentiated phenotype. While many tumours differ in their tumour cell-associated hyaluronan, most human epithelial tumours are surrounded by a hyaluronan-rich stroma. The extent of this elevated stromal hyaluronan correlates with a poor prognosis in many tumour types, including those of breast, prostate, ovary and lung (6,10–12). The elevated levels of hyaluronan in the stroma surrounding tumours is most likely predominantly synthesized by mesenchymal cells, such as fibroblasts in response to factors released by the tumour cells, or by direct cell–cell contact (13–16).

Hyaluronan is synthesized by hyaluronan synthases Has 1, Has 2 and Has 3 (17), integral membrane proteins whose activity increases during cell proliferation and peaks at mitosis during the cell cycle. Overexpression of Has in certain tumour cells promotes growth and metastasis (18), and even promotes anchorage-independent growth of fibrosarcoma cells in soft agar (19). Although macromolecular hyaluronan promotes tumour cell growth and invasion, hyaluronidase-generated hyaluronan oligosaccharides promote angiogenesis (20), and it has been shown that there is a correlation between hyaluronan levels and hyaluronidase activity with tumour angiogenesis and progression (21,22). CD44 is the major cell surface receptor for hyaluronan, and considerable experimental evidence confirms that the interaction of CD44 with hyaluronan promotes tumour cell growth, migration and metastasis in several tumour types (23,24).

Abbreviations: bHABP, biotinylated hyaluronic acid-binding protein; CM, conditioned medium; DMEM, Dulbecco’s modified Eagle medium; MEM, minimal essential medium; PBS, Dulbecco’s phosphate-buffered saline; SF, serum-free.
These observations support the role of hyaluronan and CD44 in facilitating tumour growth and spread. Studies with melanoma cells have demonstrated a role of hyaluronan and CD44 in facilitating growth and migration; however, Karjalainen et al. (9) have observed a correlation between reduced hyaluronan and CD44 in primary melanomas with an unfavourable prognosis. Here, we have examined the ability of the highly aggressive C8161 melanoma cell line to stimulate the synthesis of fibroblast hyaluronan, and determined the expression and distribution of CD44 and hyaluronan in a 3D collagenous invasion model.

Materials and methods

Cell culture

Human adult breast skin fibroblast cultures were initiated from explants obtained from the reduction mammoplasty skin of a 28-year-old Caucasian, and were used between passages 5 and 10. The C8161 melanoma cell line was established from an abdominal wall metastasis and has been characterized by Welch et al. (25). All cultures were routinely grown in Eagle’s minimal essential medium, supplemented with 10% foetal calf serum (Invitrogen Ltd, Paisley, UK). Conditioned medium (CM) was prepared by washing almost confluent cultures twice with serum-free minimal essential medium (SF-MEM), incubating for 2 h with SF-MEM, and finally changing to fresh SF-MEM, and incubating for 48 h. The CM was harvested and passed through a 0.45 μm filter, and stored at -30°C.

Rat tail tendon collagen solution was prepared by the extraction of tendons with 0.5 M acetic acid, and gels prepared essentially as described previously (26). The final collagen mixture contained ~2 mg/ml collagen, and with 0.5 M acetic acid, and gels prepared essentially as described previously (26). The final collagen mixture contained ~2 mg/ml collagen, and with 0.5 M acetic acid, and gels prepared essentially as described previously (26).

Ultraltrification

Conditioned media were passed through an Amicon ultrltrifiltration membrane with a molecular weight cut-off of 30 kDa (YM-30). The material retained by the membrane was concentrated 40-fold, and diluted with SF-MEM for fibroblast glycosaminoglycan stimulation assays.

Glucose assay

Glucose levels in CM were determined using a glucose assay kit (Sigma-Aldrich Co. Ltd, Poole, UK) that is based on the conversion of glucose to gluconic acid and hydrogen peroxide by glucose oxidase. The assay was performed according to the manufacturer’s instructions, and the levels of glucose in CM medium normalized by the addition of glucose from a standard solution of 100 mg/ml.

Glucose dose response

To determine the effect of different concentrations of media glucose on glycosaminoglycan-stimulating factor production, media containing glucose concentrations ranging from 1.0 to 4.5 mg/ml were prepared by mixing appropriate volumes of high and low glucose Dulbecco’s modified Eagle medium (DMEM).

Lactate assay

Lactate levels in CM were measured using a lactate assay reagent (Sigma-Aldrich Co. Ltd, Poole, UK) that involves the conversion of lactate to pyruvate and hydrogen peroxide by lactate oxidase. The assay was performed according to the manufacturer’s instructions.

Radiolabelled glycosaminoglycans

Twenty-four well multiwell plates containing confluent breast skin fibroblasts were washed with 1.0 ml SF-MEM/well, and 0.6 ml of SF-MEM or CM containing 3H glucosamine (5 μCi/ml) (Amersham Biosciences, Little Chalfont, UK) was added to triplicate wells. The cultures were incubated for 24 h at 37°C, after which the contents of each well, along with 2 × 100 μl Dulbecco’s phosphate-buffered saline (PBS) washes were transferred to 1.5 ml eppendorf tubes. The cells were detached with trypsin and counted in a Coulter counter. A sample of the harvested medium was diluted 1 in 15 with SF-MEM and retained for subsequent hyaluronan determination. One hundred micro-litres of carrier glycosaminoglycan solution (3.5 mg chondroitin sulfate and 3.0 mg hyaluronan/ml H2O) were added to the remaining radioactive medium, followed by boiling for 5 min. Pronase (100 μl of 3 mg/ml 50 mM Tris/HCl, pH 7.6) was added, and the samples were incubated for 24 h at 30°C followed by heat inactivation and centrifugation at 13 000 g for 5 min. One-fifth volume of 10% cetylpyridinium chloride (CPC) in 0.03 M NaCl was added to the supernatant, and incubated for 20 min at 40°C, followed by centrifugation at 13 000 g for 5 min. The resultant precipitate was washed twice with 0.1% CPC in 0.03 M NaCl, and the pellet was finally dissolved in 2 M NaCl. Samples were taken and suspended in Optiphase HiSafe 3 scintillant (Fisher Chemicals, Loughborough, UK), and radioactivity was determined using a Packard TriTec scintillation counter.

Hyaluronan plate assay

Hyaluronan present in various media was determined using the Corgenix hyaluronic acid test kit (Corgenix Ltd, Peterborough, UK) that is based on an enzyme-linked hyaluronic acid binding protein (HABP) assay. Briefly, diluted media samples and appropriate controls were incubated in HABP-coated microwells, allowing hyaluronan present in the samples to react with the immobilized binding protein. After the removal of unbound material by washing, HABP conjugated with horseradish peroxidase solution was added to form complexes with the bound hyaluronan. Following another washing step, the chromogenic substrate tetramethylbenzidine with hydrogen peroxide was added to develop a colour reaction. The intensity of colour was measured in an ELISA plate reader at 450 nm. Levels of hyaluronan were determined from a reference curve obtained using reference hyaluronan solutions provided with the kit. The minimum detectable hyaluronan level that the assay can accurately measure is 10 ng/ml.

Hyaluronan staining

The biotinylated complex of hyaluronan-binding region of the binding protein and link protein (bHABP) was prepared as described previously (27). Paraffin sections of collagen lattices were deparaffinized in xylene, rehydrated with graded alcohols, and washed in PBS. Endogenous peroxidase was blocked with 1% hydrogen peroxide for 5 min, and non-specific binding blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Control sections were preincubated with Streptomyces hyaluronidase (100 TRU/ml 0.1 M sodium acetate buffer, pH 5.0; Seikagaku Kogyo Corp., Tokyo, Japan) in the presence of protease inhibitors (28), and the biotinylated complex pre-treated with hyaluronan oligosaccharides acted as controls. Sections were incubated with bHABP (3 μg/ml diluted in 1% BSA) overnight at 4°C, washed twice with PBS, and treated with avidin–biotin peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) for 1 h at RT and washed with PBS. Colour was then developed with 3,3'-diaminobenzidine, and the sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted in DePex. Monolayer cultures of C8161 cells and fibroblasts were grown until they were almost confluent on coverslips, washed in PBS, and stained for 30 min in 2% paraformaldehyde, 0.5% glutaraldehyde 0.5% CPC in PBS, followed by washing in PBS. The cells were permeabilized with 0.3% Triton X-100 in 3% BSA (30 min, RT), washed with PBS, and probed with 3 μg/ml of bHABP in 3% BSA overnight at 4°C. Hyaluronidase-treated cells acted as controls. After washing with PBS, the cells were incubated with avidin–biotin peroxidase for 30 min and then washed. Colour was then developed using the VIP kit of Vector labs (Vector Laboratories, Peterborough, UK).

CD44 immunostaining

CD44 was detected using the Hermes 3 antibody. Sections (3 μm) from collagen lattices were deparaffinized and rehydrated using xylene and graded alcohols. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 5 min, followed by washing twice for 5 min with PBS and the sections incubated with 1% BSA for 30 min at RT. The Hermes 3 primary antibody (from Dr Sirpa Jalkanen, University of Turku, Finland) was diluted 1/100 with 1% BSA and incubated on the slides overnight at 4°C, followed by washing with PBS. The bound antibody was detected using a biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) with avidin–biotin peroxidase detection, and visualized by incubation with 3,3'-diaminobenzidine. The sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted with DePex. In controls, an isotype control antibody replaced the primary antibody.

Monolayer cultures of C8161 cells and fibroblasts were grown until they were almost confluent on coverslips, washed in PBS, and fixed for 30 min in 2% paraformaldehyde, 0.5% glutaraldehyde in PBS, followed by washing in PBS. The cells were permeabilized with 0.3% Triton X-100 in 3% BSA (30 min, RT), washed with PBS, and incubated with normal rabbit serum (1/5 dilution in PBS) for 30 min. After washing with PBS, the cells were incubated with the primary antibody (clone 2C5, R&D Systems, Abingdon, UK) at a concentration of 1 μg/ml. After washing with PBS, the bound antibody was detected using a biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) with avidin–biotin peroxidase detection, and visualized by incubation with 3,3'-diaminobenzidine. The sections were counterstained with Mayer’s haematoxylin, dehydrated, and mounted with DePex. In controls, an isotype control antibody replaced the primary antibody.
Statistical analysis
Comparisons were performed using one-way analysis of variance (ANOVA) and Dunnett’s post-test. The dose response data (Figure 4B) were subjected to regression analysis.

Results

CM from C8161 cells stimulates fibroblast glycosaminoglycan and hyaluronan synthesis

The CM from both C8161 melanoma and fibroblast cultures was assayed for glucose levels, as reduced glucose would affect the incorporation of [3H] glucosamine into glycosaminoglycans. Following conditioning for 48 h, the level of glucose in the fibroblast culture medium was reduced from 989 to 666 µg/ml, while the C8161 melanoma cultures depleted the medium of all glucose. Using a stock solution of 100 mg/ml, the CM was readjusted to the original level of glucose before any further analysis. The incorporation of [3H] glucosamine into fibroblast glycosaminoglycans and release into the culture medium was determined in response to CM from C8161 melanoma cells, with fibroblast-CM, and SF-medium acting as controls (Figure 1A). Complete C8161 melanoma cell-CM, and that concentrated using a 30 kDa cut-off ultrafiltration membrane stimulated the incorporation of [3H] glucosamine into fibroblast GAGs. The >30 kDa fraction that was concentrated 40-fold was diluted with SF-MEM to give fractions >30 kDa x1, x2, x3 and x4, which showed a gradual increase in stimulating activity, with the >30 kDa x4 fraction stimulating the synthesis of fibroblast glycosaminoglycan 2.8-fold compared with the SF-MEM control (P < 0.01). No increase in stimulating activity was observed with fibroblast-CM, while the C8161-CM passing through the 30 kDa cut-off membrane, when incubated with fibroblasts, inhibited the synthesis of glycosaminoglycan compared with the SF-MEM control. These results demonstrate that C8161 melanoma cells released factors >30 kDa into the culture medium that stimulated the synthesis of fibroblast glycosaminoglycan.

The majority of the glycosaminoglycan released into the culture medium by fibroblasts was hyaluronan (29), but to confirm that the synthesis of hyaluronan was stimulated by the tumour cell-derived factors, the amount of hyaluronan synthesized was assayed using a specific plate assay (Figure 1B). Levels of hyaluronan present in the CM were subtracted from the total hyaluronan present in the media taken from the fibroblast cultures exposed to the CM to determine newly synthesized hyaluronan in response to the different media. The tumour cell-CM stimulated the synthesis of hyaluronan by fibroblasts, with the >30 kDa x 4 fraction exhibiting the greatest activity (2.75-fold increase compared with SF-MEM control; P < 0.01), while the <30 kDa fraction showed no stimulating activity compared with the SF-MEM control. It was also found that the C8161 cells did not release any detectable hyaluronan into the culture medium themselves, with the hyaluronan assay being able to accurately detect hyaluronan down to 10 ng/ml.

Activity of tumour cell-derived fibroblast glycosaminoglycan-stimulating activity is dependent upon glucose levels in the medium during conditioning

Experiments using high glucose DMEM for conditioning resulted in no detectable glycosaminoglycan-stimulating activity. As tumour cells are known to rapidly metabolize glucose, it is possible that a depletion of medium glucose may stimulate the production of the tumour cell-derived fibroblast glycosaminoglycan-stimulating activity. Figure 2 shows that the tumour cells rapidly depleted low glucose (1.0 mg/ml) DMEM of glucose, and by 18 h it was completely utilized. In high glucose (4.5 mg/ml) DMEM, the glucose levels again rapidly decreased, and following incubation for 48 h, the levels were 1.35 mg/ml. Fibroblasts on the other hand utilized only small amounts of glucose, the level of glucose being reduced to 0.65 mg/ml in low glucose medium, and 3.77 mg/ml in high glucose medium following incubation for 48 h.

To determine the exact effect of glucose levels in the medium on the production of tumour cell-derived fibroblast glycosaminoglycan-stimulating activity, CM was prepared using DMEM containing a range of glucose concentrations ranging from 1.0 to 4.5 mg/ml. Figure 3 shows that the increasing levels of glucose in the tumour conditioning medium resulted in a decrease in glycosaminoglycan-stimulating activity, and at the highest levels of glucose, there was even inhibition of the synthesis of fibroblast glycosaminoglycan. The increasing
levels of medium glucose had little effect on the activity of fibroblast CM. Before assaying the activity of the CM, glucose was added so that all media contained 4.5 mg glucose/ml before addition to fibroblast cultures. It therefore appears that the tumour cell production of the fibroblast hyaluronan-stimulating activity was affected by glucose depletion.

Lactate produced by tumour cells suppresses fibroblast glycosaminoglycan synthesis in response to tumour cell-derived factors

It has been shown previously that lactate can stimulate the synthesis of hyaluronan (30,31); therefore, we examined the ability of both fibroblasts and C8161 melanoma cells to produce lactate in the different media, and the ability of lactate to stimulate fibroblast hyaluronan in this system. While lactate was released in increasing amounts into the tumour cell-CM over time (Figure 4A), the highest levels were detected in the high glucose DMEM tumour cell-CM; yet, this medium did not possess any stimulating activity. Using a range of lactate concentrations in SF-DMEM, we did not detect any lactate-mediated stimulation of hyaluronan synthesis (Figure 4B), but instead observed an inhibition of fibroblast glycosaminoglycan synthesis. It is therefore possible that the elevated levels of lactate present in the high glucose CM may be suppressing the tumour cell-derived factor stimulation of fibroblast glycosaminoglycan synthesis. Concentration of high glucose tumour cell-CM using a 30 kDa cut-off ultrafiltration membrane 40-fold, and then rediluting the >30 kDa fraction with SF-medium restored the stimulating activity (Figure 5A). Addition of lactate to low glucose tumour cell-CM to the levels present in the high glucose CM abolished the stimulating activity (Figure 5B).

Tumour cell-CM stimulates fibroblast glycosaminoglycan synthesis in contracted collagen lattices

The stimulation of fibroblast glycosaminoglycan synthesis was examined using fibroblasts embedded in contracted collagen lattices, as many factors exhibit different activities on fibroblasts in collagen gels compared with monolayer culture. Again, the tumour cell-CM stimulated fibroblast glycosaminoglycan synthesis, although the overall levels synthesized in
collagen gels was less than monolayer cultures (Figure 6). The stimulation of glycosaminoglycan synthesis was apparent in both the medium fraction, and that associated with the collagen lattice where the synthesis of glycosaminoglycan was stimulated 4.25-fold in the medium fraction \( (P < 0.01) \), and 2.75-fold in the collagen lattice-associated fraction \( (P < 0.01) \).

**Melanoma cells invading fibroblast-contracted collagen lattices and fibroblast-free collagen gels express cell surface CD44 receptors and hyaluronan**

It was necessary to determine if the tumour cell-CM was active on fibroblasts within collagen lattices as we wished to examine the expression and distribution of hyaluronan in fibroblast-containing collagen lattices on which melanoma cells were growing and invading. Figure 7A shows C8161 melanoma cells grown on fibroblast-contracted collagen lattices incubated at the air/liquid interface for up to 12 days. It is apparent that the tumour cells rapidly formed a multilayered tumour cell mass on the surface, with a large number of cells invading into the gel. Staining of these models using an anti-CD44 antibody showed that the invasive cells express high levels of CD44; yet, the tumour cell layers near the surface were negative for CD44 (Figure 7C). CD44 isotype control antibody staining was completely negative. When sections of these tumour models were stained for hyaluronan (Figure 7E), it was apparent that the C8161 cells on the surface did not stain, whereas many of the invading cells exhibited intense staining around their cell surface whereas the collagen matrix stained moderately intensely, presumably owing to hyaluronan synthesized by the fibroblasts. The staining pattern for tumour cell surface-associated CD44 and hyaluronan was therefore very similar. As the C8161 cells did not release detectable levels of hyaluronan into the culture medium, it is possible that they bind hyaluronan in the gels produced by fibroblasts, or are induced to produce hyaluronan themselves when within the lattices. Collagen lattices without fibroblasts were set up to examine the invasion of the C8161 melanoma cells, and to determine if the invasive tumour cells express hyaluronan in the absence of fibroblasts. Figure 7B shows that the C8161 cells formed a multilayered mass of cells when the fibroblast-free collagen gels were raised to the air/liquid interface, and readily invaded the collagen lattice. As with the fibroblast-containing lattices, the tumour cells in the fibroblast-free gels expressed CD44 strongly only on the invasive cells \( (P < 0.01) \). However, the C8161 cells also expressed hyaluronan in those cells invading the collagen lattice while those on the gel surface did not (Figure 7F). In addition to cell-associated hyaluronan, there appeared to be a substantial accumulation of hyaluronan in areas between the invasive tumour cells while the main fibroblast-free collagen matrix was essentially negative (Figure 7F). The C8161 cells grown as monolayers did not release detectable levels of hyaluronan into their culture medium, but may express hyaluronan retained on their cell surface. To examine this possibility, the monolayer cultures...
of C8161 cells were stained, along with fibroblast cultures for hyaluronan and also CD44. Figure 8A shows that the mono-layer cultures of C8161 cells stained strongly for CD44, but hyaluronan staining (Figure 8C) was very variable, with many cells expressing no hyaluronan. In comparison, fibroblast monolayer cultures stained intensely for both CD44 (Figure 8B) and hyaluronan (Figure 8D), while the CD44 isotype controls and hyaluronidase-treated cells were negative.

Discussion

As a solid tumour grows, a stage is reached when the blood supply is unable to provide sufficient oxygen and nutrients, and parts of the tumour become anoxic and starved of nutrients such as glucose. Our observations support the contention that tumours release a number of soluble factors that promote a modulation of the surrounding stroma, and in particular, increased hyaluronan, to facilitate growth, invasion and angiogenesis and thus, overcome restrictive conditions. We have also shown that the C8161 melanoma cells in organotypic cultures are able to enrich their immediate pericellular environment with hyaluronan, although their capacity to do so in monolayer culture is limited. Although levels of hyaluronan expression by different tumour cells do not necessarily correlate with tumour progression or patient prognosis, in most cases, the presence of a hyaluronan-rich stroma surrounding the tumour does correlate with aggressiveness and poor prognosis. Tumours that poorly express hyaluronan may therefore be potent stimulators of fibroblast hyaluronan synthesis as observed here with the C8161 cell line.
Stern et al. (30) have demonstrated the lactate stimulation of fibroblast hyaluronan synthesis, and suggest that tumour cell-derived lactate may promote the development of a hyaluronan-rich stroma surrounding the tumour. Tumour cells produce large amounts of lactate, as we have demonstrated here, but they also produce lactate in aerobic conditions, commonly termed the Warburg effect (32). However, we found no lactate stimulation of fibroblast hyaluronan synthesis, and indeed the tumour-CM prepared with high glucose DMEM contained the highest levels of lactate, and actually inhibited the synthesis of fibroblast glycosaminoglycan. Moreover, fibroblasts incubated with tumour cell-CM passing through a 30 kDa cut-off ultrafiltration membrane synthesized less hyaluronan than the controls. This apparent anomaly is probably owing to the CM being prepared in the absence of serum, as Stern et al. (30) found little or no lactate stimulation of fibroblast hyaluronan synthesis in serum-free DMEM. Lactate may therefore play a role in vivo in modulating the synthesis of fibroblast hyaluronan, but it is apparent here that the C8161 cells produce a potent fibroblast hyaluronan-stimulating factor that is >30 kDa, but whose effect on the synthesis of fibroblast glycosaminoglycan is modulated by the levels of lactate. In the organotypic cultures, it is unlikely that levels of lactate would be high enough to inhibit the tumour cell-derived factor stimulation of fibroblast hyaluronan as the medium was replaced daily. The elevated uptake and metabolism of glucose, characteristic of most tumours is primarily owing to hypoxia-inducible factor-1α (32), whose targets include the glucose transporters Glut-1 and Glut-3, and several glycolytic enzymes. As the culture conditions used here were normoxic, it is possible that the C8161 cells express HIF-1α constitutively as a result of mutations in genes encoding oncogenes and tumour suppressors. This will result in activation and stabilization of the hypoxia-inducible factor (33,34). The mechanism by which levels of lactate affect the synthesis of fibroblast hyaluronan is unclear, but in vivo, levels of available glucose and lactate produced will vary within a solid tumour and it is possible that glucose depletion will result in a decrease in lactate levels, and therefore enhance the activity of the tumour cell-derived glycosaminoglycan-stimulating activity.

The C8161 cells readily formed a multilayered cell mass and invaded fibroblast-contracted collagen lattices when cultured at the air/liquid interface. This system provides an ideal system in which to examine the expression and distribution of hyaluronan and CD44. As expected, the upper layers of the tumour cells did not express hyaluronan, or indeed CD44; yet, many of those cells invading the collagen lattice did express CD44 and stained for hyaluronan around their cell surface. The invading cells are either a population of CD44-positive cells in the original cell population and are the cells capable of invasion, or the invading cells are induced to express CD44. Presumably, these CD44-positive melanoma cells bind hyaluronan from the collagen lattices produced by the resident fibroblasts, in addition to possibly expressing hyaluronan themselves, and are also likely to stimulate the fibroblasts to upregulate their synthesis of hyaluronan. While it appeared that the melanoma cell CD44 binds hyaluronan, the exact role of this interaction in facilitating invasion is unclear. CD44 is considered to be primarily involved in mediating cell adhesion to hyaluronan, and to promote tumour cell growth and invasion (35); however, a number of studies suggest its role is more complicated. In certain cancers such as those of the ovary, a high expression
correlates with a good prognosis (36), while in melanoma, increased and decreased expression of CD44 has been reported to be associated with a poor prognosis (9,37). Other studies have demonstrated a number of spliced variants of CD44 that differ in their ability to bind hyaluronan (38,39), suggesting that the overall expression of CD44 may not reflect the hyaluronan-binding capacity of the cell.

While the C8161 melanoma cells did not release detectable levels of hyaluronan into the culture medium in monolayer culture, it was important to eliminate the possibility that the invading tumour cells were induced to express hyaluronan within the collagen lattice. Relatively dense collagen gels were prepared without fibroblasts, and in this case, the invading tumour cells did express hyaluronan. As monolayer cultures of C8161 cells showed variable hyaluronan staining, with many cells negative for hyaluronan, it is possible that only the hyaluronan-positive cells invade the collagen lattices. This would explain the surface layers of cells being negative for hyaluronan. However, the monolayer cultures of C8161 cells stained strongly for CD44; yet, only the collagen lattice-invasive cells expressed CD44. Exposure of the cultures to the air/liquid interface may therefore contribute to the observed pattern of expression of both CD44 and hyaluronan. These observations suggest that contact with the collagenous substrate is required for fibroblast detachment and mitosis.

The exact relevance of C8161 melanoma cell stimulation of fibroblast hyaluronan to its invasive capacity is unclear, as the tumour cells readily invade collagen lattices in the absence of fibroblasts, although this fibroblast-free collagen gel is less dense. It is, however, of interest to note that the invasive cells do express CD44 and hyaluronan, suggesting that this expression may be required for invasion.

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References


