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Inhibition of hyaluronan degradation by dextran sulphate facilitates characterisation of hyaluronan synthesis: An *in vitro* and *in vivo* study

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The concentration and molecular weight of hyaluronan often dictates its physiological function. Consequently full characterisation of the anabolic products and turnover rates of HA could facilitate understanding of the role that HA metabolism plays in disease processes. In order to achieve this it is necessary to interrupt the dynamic balance between concurrent HA synthesis and degradation, achievable through the inhibition of the hyaluronidases, a group of enzymes which degrade HA. The sulphated polysaccharide, dextran sulphate has been demonstrated to competitively inhibit testicular hyaluronidase in a non-biological system, but its application to *in vitro* biological systems had yet to be developed and evaluated. This study determined the inhibitory concentrations of dextran sulphate against both testicular and *Streptomyces* hyaluronidase in a cell-free and breast cancer model followed by characterisation of the effect that hyaluronidase inhibition exerted on HA synthesis and degradation. The IC_{100} of dextran sulphate for both hyaluronidases in a cell-free and biological system was determined to be $\geq$400 μg/ml. At concentrations up to 10 mg/ml the dextran sulphate did not effect breast cancer cell proliferation or morphology, while at 400 μg/ml HA degradation was totally inhibited, enabling an accurate quantitation of HA production as well as characterisation of the cell-associated and liberated HA. FACS quantitation of the HA receptor CD44, HA synthase and the hyaluronidases HYAL 1 and HYAL 2 demonstrated that dextran sulphate down-regulated CD44 and HA synthase while upregulating the hyaluronidases. These results suggest dynamic feedback signalling and complex mechanisms occur in the net deposition of HA *in vivo*.
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Keywords: **hyaluronidase, dextran sulphate, hyaluronan, CD44, HA synthase**

Abbreviations: **HA: hyaluronan; DxS: dextran sulphate; HAase: hyaluronidase; *HAS*: hyaluronan synthase genes; HAS: hyaluronan synthase proteins; HYAL: hyaluronidase protein; *HYAL*: hyaluronidase genes; MW: molecular weight; FACS: Fluorescence Activated Cell Sorter Analysis; ECM: extracellular matrix; Da: Daltons; GAG: glycosaminoglycan; FCS: foetal calf serum.**

Introduction

Hyaluronan (HA) is a non-sulphated glycosaminoglycan (GAG) that plays an important role in normal and pathogenic states. The function of HA is highly dependent on its molecular weight (MW). As a normal constituent of the extracellular matrix (ECM), and in its native form, HA exists as a high MW polysaccharide, typically in excess of 10^6 Da, where it is associated with the structural fluid dynamic, homeostasis and maintenance of tissue connective tissue integrity. In biological fluids such as blood or lymph, or at sites of inflammation or disease, the molecular weight of HA is often significantly lower [1]. Both intermediate (200–500 kDa) and smaller MW HA (<20 kDa) are involved in cellular processes, in particular as signalling molecules for angiogenesis [2], induction of inflammatory gene expression [3], wound repair [4], cell proliferation and migration [5]. These processes are important in inflammation and tumour invasion [6]. Cellular processes initiated by HA are mediated by its binding to high affinity HA receptors, particularly RHAMM [7] and CD44 [8]. The receptor-ligand interactions trigger cell signalling cascades that direct lymphocytes

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to inflammatory sites [9], lead to the assembly of a pericellular matrix during chondrogenesis [10], and initiate wound healing and tissue morphogenesis during development [11]. The dynamics involved in the synthesis of large MW HA and its depolymerisation into biologically active fragments appears to be an intricate, multi-factorial and ubiquitous balancing act.

Hyaluronan is synthesized by a multi-isoform family of transmembrane glycosyltransferases termed the HA synthases [12], while HA is depolymerised by a combination of enzymic and non-enzymic mechanisms [13]. HA polymerization occurs on the inner face of the plasma membrane where it is extruded onto the extracellular surface of the cell [14]. Three eukaryotic HAS isoforms have been identified, termed HAS1, HAS2 and HAS3. Sequence data of the HAS isoforms suggests they contain seven membrane-associated regions and a central cytoplasmic domain possessing several consensus sequences that are substrates for phosphorylation by protein kinase C [12,15]. The catalytic rate for each HAS isoform is different [16]. HAS1 is the least active and drives the synthesis of high MW HA (2000 kDa), suggesting low constitutive levels of HA synthesis. HAS2 is more catalytically active and is associated with synthesis of high MW HA (2000 kDa). HAS2 is implicated in developmental processes involving tissue expansion and growth. HAS3, the most active drives the synthesis of short (100 to 1000 kDa) HA chains. HAS3 expression may be activated to produce large amounts of low MW HA, to contribute to the pericellular matrix or may interact with cell surface HA receptors, triggering signaling cascades and profound changes in cell behavior [16].

Depolymerisation of HA is the result of two processes: hyaluronidase activity and exposure to reactive oxygen intermediates (ROI). The hyaluronidases are β-endoglucosaminidases distinguished by their pH requirement. HYAL 1 and HYAL 2 are widely distributed and in collaboration with CD44, degrade high MW HA [17]. It is hypothesised that high MW HA binds to CD44 and in co-operation with the GPI-anchored HYAL 2 [18] the HA is internalised and degraded in unique acid endocytic vesicles [19] to 20 kDa HA fragments. The fragments are transported intracellularly and further digested by HYAL 1 together with two β-exoglycosidases, β-glucuronidase and β-*N*-acetyl glucosaminidase resulting in very low-MW oligosaccharides [18]. The bone-marrow associated HYAL 3 has not been fully characterised. The sperm-associated HAase, PH-20 plays an important role in fertilisation and differs from the other HAases by exhibiting enzymic activity at neutral pH [20]. The ROI driven degradation of HA occurs by preferential random cleavage of internal glycoside linkages [21,22]. *In vivo* the respiratory burst of leukocytes are considered a major source of these free radicals [23].

When investigating the potential correlation between the native molecular weight of HA synthesised by human breast cancer cells, HAS isoform expression and their propensity to invade the ECM it became evident that both synthetic and degradative processes were simultaneously occurring subsequently complicating the quantitation and characterization of the HA produced by metastatic and non-metastatic cells. Analysis of similar retrospective studies clearly demonstrated that there were numerous discrepancies in the data where for example mesothelioma and lung cancer cells were found not to synthesise HA *in vitro* [24], while *in vivo* both tumour types secrete large quantities of HA [25], potentially suggesting that high degradative rates *in vitro* may have hindered the detection of any synthesised HA. Due to the differential effect that varying molecular weight HA exerts on physiological function it became evident that investigations which require an accurate quantitation of HA synthesis and characterisation of end product requires the inhibition of HA degradation. Inhibition of the enzymic degradation pathways could potentially allow the characterization of the role played by HA concentration and molecular weight during the malignant process, therefore highlighting the need for a potent, yet non-cytotoxic inhibitor for mammalian HAase.

Hyaluronidase activity can be inhibited by endogenous compounds found in body fluids or tissues, plant-derived molecules, anti-inflammatory drugs and sulphated GAGs [13]. The best characterised sulphated GAG inhibitor of HAase activity is heparin which acts in a non-competitive fashion. To selectively inhibit HAase activity *in vitro* it would be advantageous to utilise a competitive inhibitor. This facilitates a more regulated experimental approach to identifying the actual contribution of HAase activity. One such inhibitor is dextran sulphate (DxS) [26]. Dextran sulphate has been used extensively as a therapeutic due to its anticoagulant properties [27], its ability to enhance and suppress the immune system [28], and to prevent viral and bacterial attachment to mammalian cells [29,30]. Dextran sulphate has also been used as an anti-metastatic compound [31]. These biological activities have been attributed to the charge characteristics of DxS and its binding characteristics. Given the specificity of binding between DxS and HAase and its non-toxic nature, DxS appears to be an excellent candidate as a specific inhibitor of HAase activity. The use of DxS to inhibit HAase activity provides an opportunity to characterise the HA produced by anabolic processes, free from the influence of HAase activity.

Materials and methods

Establishing the inhibitory effect of dextran sulphate on exogenous *Streptomyces* hyalurolyticus and bovine testicular hyaluronidase activity in a cell-free system

The highest reported concentration of HA in a biological fluid is found in synovial fluid where concentrations approximate 2 mg/ml [32]. This concentration was therefore used as the substrate concentration for determining the optimal concentration of hyaluronidase and the corresponding inhibitory concentration of dextran sulphate. Titration of the inhibitory activity of dextran sulphate was determined by dissolving 2 mg/ml of 825 kDa HA (Contipro, Czech Republic) in PBS followed by the addition of 0, 1, 5, 10, 20, 40, 60, 80, 100, 150 and 200 TRU/ml of both bovine testicular hyaluronidase [E.C.3.2.1.53], 2120

units/mg protein; (Sigma, St Louis, MO, USA)] and *Streptomyces hyalurolyticus* hyaluronidase [E.C.4.2.99.1], 7460 units/mg; (Calbiochem, San Diego, CA, USA)]. The HA was incubated with the varying concentrations of hyaluronidases for 1 h at 37°C, after which the hydrolysis of HA was measured according to an adaptation of the Somogyi assay [33]. In brief, samples were oxidised by the alkaline copper reagent that was heated at 100°C for 15 min forming cuprous ions that reacted with the arsenomolybdate reagent to give a green colour. Tubes were allowed to stand for 2 min at ambient temperature before being diluted with distilled water to 1:10. Absorbance at 500 nm was recorded.

Testing the effect of various cell culture media on dextran inhibition of hyaluronidase

In order to assess the suitability of dextran sulphate as an inhibitor of hyaluronidase activity *in vitro* it was necessary to establish if the growth media commonly used in the culturing of human cells interfered with the hyaluronidase inhibitory activity of DxS. The following phenol red free media were purchased from Sigma, RPMI 1640, DMEM, SWIM and MEM. Hyaluronan (825 kDa) was dissolved at 2 mg/ml in the aforementioned growth media containing 10% v/v foetal calf serum, followed by the addition of 0, 1, 5, 10, 20, 40, 60, 100, 200, 400, 800, 1000 μg/ml dextran sulphate (500 kDa M_r and 17% sulphur-substituted; Pharmacia Fine Chemicals, Uppsala, Sweden). After addition of 100TRU testicular hyaluronidase or 60TRU *Streptomyces hyalurolyticus* hyaluronidase the samples were incubated for 1 h at 37°C. Hydrolysis of HA was measured as above using the Somogyi assay.

Determining the effect of dextran sulphate on human breast cancer cell proliferation and morphology

Culture of human breast cancer cells: Aneuploid human breast adenocarcinoma cell line MDA-MB 231 (American Tissue Culture Collection, Rockville, MD, USA) was selected based on its ability to produce 23.38 ± 2.16 μg HA/L/10^6 cells [34] and the differential expression of both HAS2 and HAS3 genes (data not shown). Cells were routinely grown and subcultured as a monolayer at 37°C in a humidity controlled incubator with 100% (v/v) air in Leibovitz L-15 Medium (Sigma), supplemented with 10% foetal calf serum (FCS) and antibiotic/antimycotic reagents.

Proliferation assay: When cultures reached 70% confluence, cells were trypsinised and plated at 2.5×10^5/25 cm^2. Cells were subjected to plating in 0 to 10 mg/ml DxS (M_r 500 kDa), alternatively, the DxS was added 24 h after attachment. After 5 days of growth, cells were detached using 0.25% w/v trypsin and cell number determined using a Coulter counter.

Determining the inhibitory effect of dextran sulphate on the degradation of hyaluronan by human breast cancer cells

Hyaluronan assay: Hyaluronan production was quantitated using an enzyme-linked HA binding protein assay (HABP) (Corgenix Inc, Westminster, CO, USA). The assay was performed according to the manufacturer's instructions. In brief, duplicate 100 μl of samples and the HA standards (0, 50, 100, 200, 500 and 800 ng/ml) were aliquoted into a 96 well plate coated with HABP, and incubated at room temperature (RT) for 60 min. Samples were washed four times with PBS. One hundred μl of HABP conjugated to horse-radish peroxidase was added and incubated at at RT for 30 min. After additional PBS washes, the reaction was visualised with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) after a 30 min, RT incubation. The reaction was stopped with 100 μl of 0.36 N sulphuric acid and read at 450 nm (650 nm reference) in a BioRad 350 microplate reader. Controls consisted of growth media ± DxS which had not been exposed to cells, consequently enabling the quantitation of endogenous HA as well as determining the effect of DxS on the assay.

Effect of dextran sulphate concentration on hyaluronan degradation: Breast cancer cells were seeded at 7.5×10^5 cells/75 cm^2 and were grown for 60 h in growth media ±50, 100, 200, 400, 600 and 800 μg/ml, and 1, 2, 4, 8 and 10 mg/ml of DxS. At 60 h the media were removed and analysed using the HA ELISA to demonstrate the concentration of DxS required to inhibit HA degradation *in vitro.* To confirm that the concentration of DxS required to inhibit the degradation of HA, the MDA-MB 231 cells were seeded at 7.5×10^5 cells/75 cm^2 and grown for 60 h in growth media containing 20 μg HMW [^{3}H]HA ±50, 100, 200, 400, 800 and 1000 μg/ml of DxS (M_r 500 kDa). The [^{3}H]HA was prepared as previously described [35] and had a specific activity of 420,000 dpm/μg. On completion of the 60 h incubation period the media were removed and subjected to size exclusion chromatography in a Superose 12 gel eluted in 0.15M NaCl/phosphate pH 7.25 which contained 19 mM NaH_2PO_4, 38 mM Na_2HPO_4 and 94 mM NaCl at 20 ml/h. Cells were harvested by trypsinization and counted using a Coulter counter. To ensure that trypsinization removed all of the ECM components from the flasks, flasks were stained with 1% w/v Alcian Blue in 3% v/v acetic acid. Media were used for quantitation of liberated HA. Cell-associated extracellular HA was released from ECM components and receptors using a pre-optimised method of centrifugation of the cell/trypsin fraction at $400g_{av}$ in a Beckman TJ-6 centrifuge. Hyaluronan metabolites were identified by comparison of elution volumes in a Superose 12 gel which had been calibrated with the following HA metabolites; [^{3}H] water (Kav 1), [^{3}H] acetate (Kav 0.83), HA-6 saccharide (Kav 0.69), HA-4 saccharide (Kav 0.72) and 10 kDa (Kav 0.41) and 20 kDa (Kav 0.30).

Determining the effect of dextran sulphate on the production of liberated and cell-associated HA: Triplicate cultures of human breast cancer cell line MDA-MB 231 were seeded at 7.5×10^5 cells/75 cm^2 and were grown with and without 400 μg/ml of DxS for 4, 12, 24 and 48 h during which cell cultures reached 85% confluence, and then for a further 24 h until cell contact inhibition was observed. At the conclusion of the incubation period, cells were harvested by trypsinization and

counted using a Coulter counter. Media were used for quantitation of liberated HA. Cell-associated extracellular HA was released from ECM components and receptors using a pre-optimised method of centrifugation of the cell/trypsin fraction at $400g_{av}$ in a Beckman TJ-6 centrifuge. The supernatant was quantitated for HA. Intracellular HA concentration was determined by treating the cell pellet as follows: the cell pellet was lysed under hypotonic conditions by resuspension in 10 mM HEPES pH 7.2 followed by disruption in a Dounce homogeniser using 20 strokes every 15 min. Cell lysis was confirmed by Giemsa stain and examination by light microscopy. To dissociate the HA from binding proteins, the cell lysate was heated to 37°C with 0.5%v/v Triton X-114 in 10 mM HEPES buffer pH 7.2 [14]. The HA/detergent micelles were centrifuged at $1500g_{av}$ for 5 min and the upper aqueous phase was analysed for HA. The individual analyses of the intra and extracellular HA fractions were not within the detection limits of the HA ELISA (>50 ng/ml), therefore the extracellular and intracellular fractions were pooled and characterised for HA concentrations.

Characterisation of the molecular weight of hyaluronan produced by human breast cancer cells after inhibition of hyaluronidase by dextran sulphate

Cells were seeded at 7.5×10^5 cells/75 cm^2 and were grown for 24 h in growth media containing 400 μg/ml DxS and 250 μCi D-[6-^{3}H]glucosamine hydrochloride (Perkin Elmer, Boston, MA, USA). At the conclusion of the 24 h incubation period, the media were removed and exhaustively dialysed (M_r exclusion of 6 kDa) against 10 mM Tris-HCl/0.15M sodium chloride/0.02% sodium azide pH 7.4 at 4°C. The dialysate and dialysis fluid were chromatographically analysed for the identification of [^{3}H]HA and its degradation products. As a control, to ensure that the 4 days of dialysis did not result in degradation of any high MW HA, [^{3}H]HA (5,000 kDa) was dialysed and analysed in an identical manner to test samples. [^{3}H]HA of >5 kDa was subjected to size exclusion chromatography in a Sephacryl S-1000 gel eluted in 0.15M NaCl/phosphate pH 7.25 which contained 19 mM NaH_2PO_4, 38 mM Na_2HPO_4 and 94 mM NaCl at 13.6 ml/h. The dialysis fluid (molecules <5 kDa) was subjected to size exclusion chromatography in a Superose 12 gel eluted in the above-mentioned buffer at an elution rate of 20 ml/h. Molecular weight estimations were calculated using calibration data for HA in Sephacryl S-1000 and Superose 12 data generated from commercially purchased HA fractions of high monodispersity (Contipro). To ensure that [^{3}H] non-dialysable (molecules >5 kDa) dpm was HA, the samples were subjected to digestion by 10 TRU of *Streptomyces* hyaluronidase at pH 6, 37°C for 24 h. Digested material was subjected to chromatography in both Sephacryl S-1000 and Superose 12, where profiles were compared to equivalent undigested sample. Any [^{3}H] material not digested by hyaluronidase was excluded from the chromatography profiles. For the calculation of column recoveries, counts in each fraction were taken as significant when >3 S.D. above the mean background dpm, with the background determined taking an equal number of sample points before and after V_o and V_t, where the average number taken was 20.

Effect of dextran sulphate on HYAL 1, HYAL 2, hyaluronan synthase and CD44

The expression of hyaluronidases, HA synthases and CD44 proteins were quantitated using Fluorescence-Activated Cell Sorter Analysis (FACS). In brief, the MDA-MB 231 cells were grown in ±400 μg/ml DxS for 24 h. Cells were removed by scraping and resuspended to 2×10^7 cells/ml in cold 0.25% paraformaldehyde, followed by fixation for 30 min at 4°C. After washing several times with PBS containing 0.1% sodium azide and 2% FCS, the fixed cells were permeabilised with PBS containing 0.05% Tween 20 and 2% FCS for 15 min at 37°C. Fifty μl of cells (1×10^6 cells) were then added to 50 μg of CD44s monoclonal antibody (Hybridoma Bank, USA), 50 μg HYAL 1 and 50 μg HYAL 2 polyclonal antibodies (kindly donated by Dr R. Stern, University of California, USA) or 25 μg HA synthase polyclonal antibody which recognised HAS1-3 isoforms (Hyaluronan Laboratory, Melbourne, Australia). The cells and antibody were incubated for 45 min at 4°C. Cells were washed twice by resuspending in 2 ml of wash buffer (4°C) followed by centrifugation in a Beckman TJ-6 centrifuge at $400g_{av}$ for 5 min to remove all unbound antibody. Following washing, fluorescent secondary antibodies were added and incubation continued at 4°C for 45 min. Cells were then washed as previously described to remove unbound secondary antibody and resuspended in 0.5 ml 10% formaldehyde/PBS solution and stored at 4°C until analysed. Data were collected using a FACS-Calibur™ analytical instrument (Becton Dickinson, San Jose, CA, USA) with subsequent analysis being performed with CellQuest™ software. Log fluorescence channel versus cell number per channel was plotted for samples of 10,000 cells at laser excitation of 488 nm. The quantitation of epitope expression was calculated by the shift in peak location on the x-axis as indicated by increased mean cell fluorescence. The assay controls consisted of any background fluorescence associated with the relevant secondary antibody. Any fluorescence associated with the controls was deducted from the test samples. The data are presented as the % cell population that displayed epitope associated fluorescence.

Results

Inhibition of hyaluronidase activity by dextran sulphate in a cell-free system

Incubation of varying concentrations of *Streptomyces* and testicular hyaluronidase with 2 mg/ml HA demonstrated that complete digestion of the HA occurred with 100TRU of testicular and 60TRU of *Streptomyces* hyaluronidase, therefore these enzyme concentrations were used in subsequent *in vitro*

validations. To demonstrate that the DxS inhibition of hyaluronidase and HA degradation could be used with several growth media and therefore adapted to experimentation with numerous cell types the catalytic activity of both testicular and *Streptomyces hyalurolyticus* hyaluronidase was determined in the presence of exogenous protein (10% v/v FCS), various cell growth media, pH 7.0 and distilled water, pH 5.8. The catalytic activity of both testicular and *Streptomyces hyalurolyticus* hyaluronidase were totally inhibited with $\geq$400 μg/ml ($>$7.2 μM) of DxS even in the presence of exogenous protein (10% v/v FCS), various cell growth media, pH 7.0 and distilled water, pH 5.8 (Figure 1 panel A and B respectively). The low concentration of DxS required to produce complete inhibition is consistent with the finding that it is a highly specific inhibitor of hyaluronidase activity. This resembles the competitive mode of action observed by Zimmerman of DxS on bovine testicular HAase [26].

Dextran sulphate inhibition of hyaluronidase does not affect human breast cancer cell proliferation or morphology

High concentrations of DxS (10 mg/ml) did not exert a significant effect on cell proliferation or morphology (data not shown). Cell cultures underwent cell doubling within 28 h in the presence and absence of DxS. Thus, DxS did not have an effect on cell cycle. Previous researchers have demonstrated that when fibrosarcoma cells [36], pulmonary tumour cells [37] or fibroblasts [38] are grown in the presence of DxS, increased electrostatic repulsion occurs through an increase in surface negative charge, manifested as cells growing in clumps with reduced number of cell to cell focal points. These morphological and growth patterns were not observed in this cancer cell line, suggesting that the DxS did not alter cell surface charge of these particular cells.

Effect of dextran sulphate concentration on hyaluronan degradation

The MDA-MB 231 cell line grown in the presence of varying concentrations of DxS demonstrated that HA degradation was completely inhibited at between 200–400 μg/ml (Figure 2A). This finding is supported by an apparent 2 to 3-fold increase in HA concentration when compared to cell cultures lacking DxS. After the exogenous addition of 20 μg of [^{3}H] HA, chromatographical analysis of media from cultures containing less than 200 μg/ml of DxS contained HA degradation products of [^{3}H] water, [^{3}H] acetate and intermediate metabolites of approximately 20 kDa, while [^{3}H]HA in cultures containing greater than 200 μg/ml of DxS did not demonstrate any depolymerisation or degradation (Figure 2B). Analysis of the cell-associated [^{3}H]HA indicated that $>$400 μg/ml of DxS was required to inhibit the degradation of the [^{3}H]HA to the 20 kDa, acetate and water (Figure 2C).

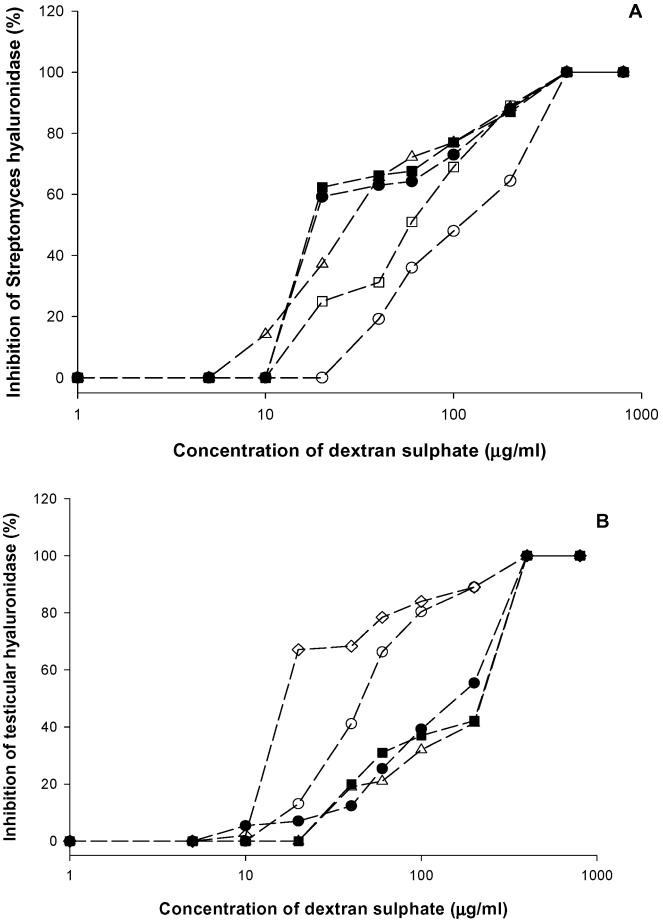

Figure 1. *Effect of various culture media on the dose-dependent inhibitory effect of dextran sulphate on exogenous hyaluronidase activity in a cell-free system.* Hyaluronan (2 mg/ml) was dissolved in RPMI 1640, DMEM, SWIM and MEM growth media containing 10% v/v foetal calf serum. Dextran sulphate (M_r 500 kDa) was added to a final concentration of 0, 1, 5, 10, 20, 40, 60, 100, 200, 400, 800, 1000 μg/ml, followed by the addition of 100TRU testicular or 60TRU *Streptomyces hyalurolyticus* hyaluronidases. Samples were incubated for 1h at 37°C after which the hydrolysis of HA was measured as above using the Somogyi assay. Dextran sulphate inhibited both *Streptomyces hyalurolyticus* hyaluronidases (A) and testicular hyaluronidase (B). Varying degrees of hyaluronidase inhibition were observed; distilled water (●---●); RPMI 1640 (△---△); SWIM medium (□---□); DMEM (■---■) and MEM (○---○).

Growth of breast cancer cells in dextran sulphate inhibits the degradation of both liberated and cell-associated hyaluronan

Analyses of cells grown with and without DxS demonstrated that 400 μg/ml DxS did not affect cell proliferation (Figure 3A). The accumulation of HA in the media and cell-associated fraction of the breast cancer cells was also significantly increased in the presence of 400 μg/ml DxS (Figures 3B and C). The HA quantitation assay utilises a bovine nasal cartilage HABP [39], with MW detection limit of $>$2000 Da where the presence of

up to 10 mg/ml DxS did not alter the specificity or accuracy of the assay. The MW exclusion of the HA assay ensures that the end-products of HYAL 1 and PH-20 after terminal degradation (~1200 Da) would not be detected. Accordingly, these data reflect an accurate estimation of HA synthesis and degradation within a 60 h period. When comparing the quantity of HA synthesised by cells grown ±DxS, it became evident that peak levels of HA liberated into the media of DxS cultures occurred in the 48 to 60 h interval, when there was a 7-fold difference ($p < 0.001$, student t-test) compared to DxS negative cultures. The difference between the accumulated HA ± DxS could potentially demonstrate the actual turnover of HA by the breast cancer cells, making this an excellent method of determining HA cellular turnover. In the cell-associated HA, maximum levels were noted at 4 h where the quantity of HA in the cell-associated fractions remained constant for the remainder of the experimental period.

Dextran sulphate inhibits the enzymic degradation of high molecular weight hyaluronan

Incubation of the cell cultures with DxS inhibited the enzymic degradation of newly synthesized [^{3}H] HA as indicated by the quantitative and qualitative analysis of both the dialysable (<5 kDa) and non-dialysable (>5 kDa) breast cancer [^{3}H] HA. Chromatographic analysis of the [^{3}H] molecules contained within the dialysate (<5 kDa) of the control cell cultures (minus DxS) demonstrated that approximately 20% of the synthesised liberated HA and 46% of the cell-associated HA had been degraded. The dialysis fluid from cell-associated fractions contained the metabolic end products of [^{3}H] tetrasaccharide, acetate and water; while the media only contained acetate and water. No other metabolic intermediates were identified. In contrast, cells exposed to DxS did not contain any [^{3}H]HA metabolites in the dialysate, indicating that enzymic HA degradative processes had been inhibited.

When the non-dialysable [^{3}H] fraction (>5 kDa) of the liberated and cell-associated macromolecules were subjected

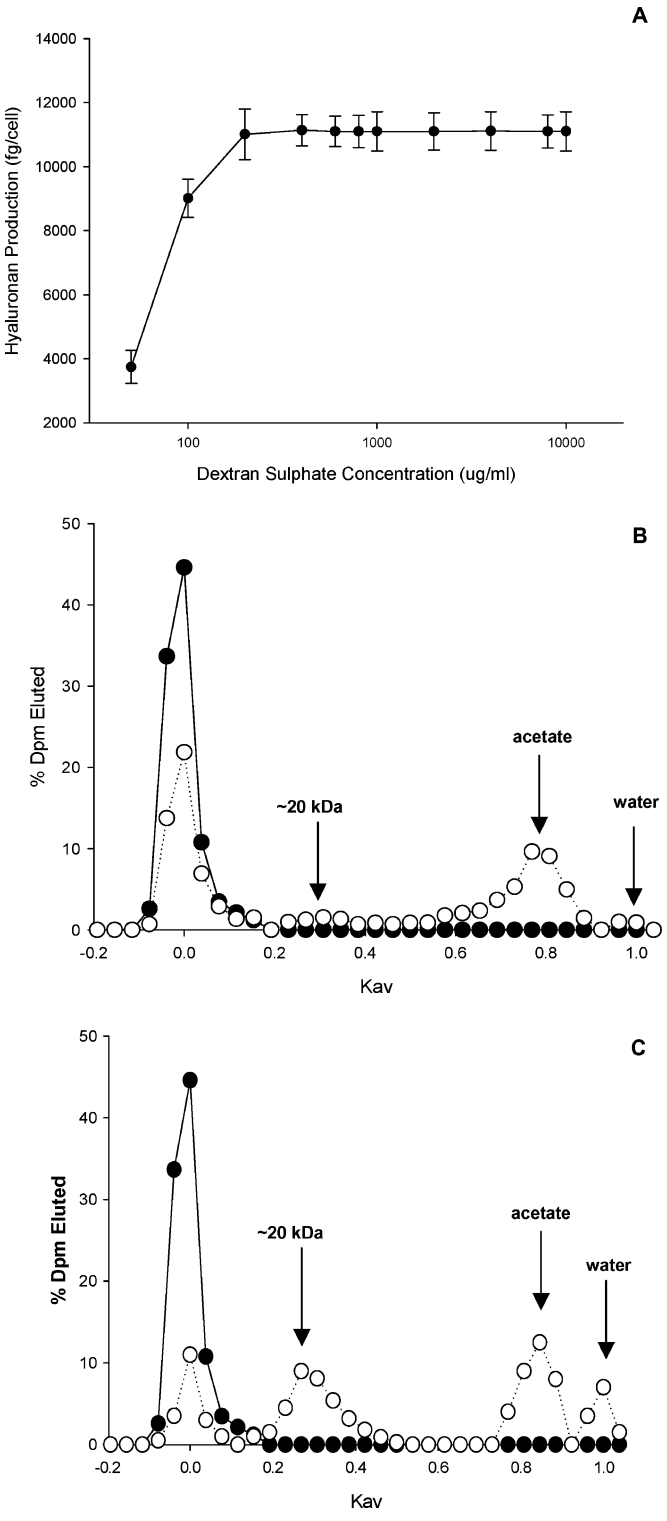

Figure 2. *Determining the concentration of dextran sulphate that inhibits hyaluronan degradation by breast cancer cells.* (A) Breast cancer cell line MDA-MB 231 was seeded at 7.5×10^5 cells/75 cm^2 and grown for 60 h in growth media ±50, 100, 200, 400, 600 and 800 μg/ml, 1, 2, 4, 8 and 10 mg/ml of DxS (M_r 500 kDa). At 60 h the media were removed and the HA was quantitated using a using an enzyme-linked HABP assay. Each point is the mean ± SD of three independent determinations. (B) Breast cancer cell line MDA-MB 231 was seeded at 7.5×10^5 cells/75 cm^2 and grown for 60 h in growth media containing 20 μg HMW [^{3}H]HA ± 50, 100, 200, 400, 800 and 1000 μg/ml of DxS (M_r 500 kDa). At 60 h the media was removed and the identification of [^{3}H]HA degradation products was detected after chromatography in Superose 12 size exclusion gel. [^{3}H]HA degradation products were observed in the media from cultures incubated with 50 and 100 μg/ml DxS (○---○), while no degradation products were observed in media from cells incubated with 200–1000 μg/ml DxS (●---●). (C) The cell-associated [^{3}H] Dpm was subjected to chromatography in Superose 12 size exclusion gel. [^{3}H]HA degradation products were observed in cells incubated with 50–200 μg/ml DxS (○---○), while no degradation products were observed in media from cells incubated with 400–1000 μg/ml DxS (●---●).

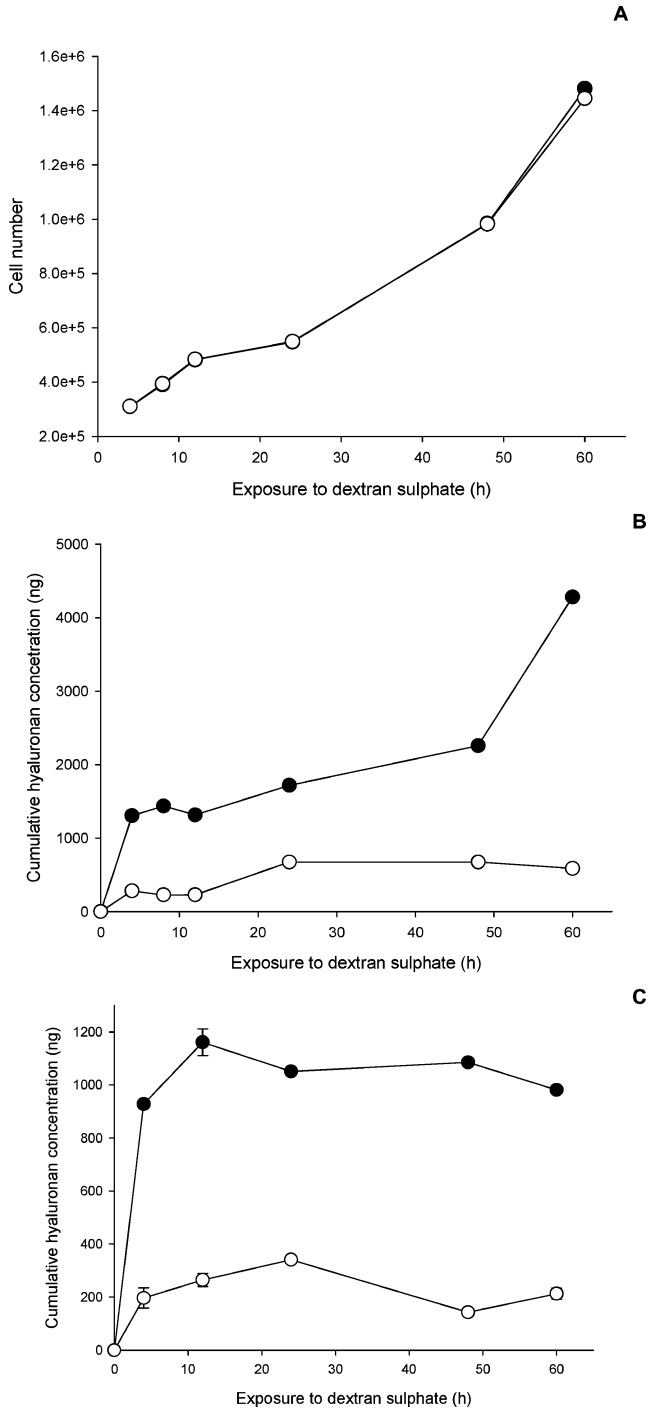

Figure 3. *Dextran sulphate inhibits the degradation of liberated and cell-associated hyaluronan.* MDA-MB 231 cells were plated at 7.5 $\times$ 10^5 cells/75 cm^2 and grown with (●---●) and without (○---○) 400 μg/ml of DxS for 4, 12, 24, 48 and 60 h. At the conclusion of the incubation period, cells were counted using a Coulter counter (A). The media were used for quantitation of liberated HA, while the cell-associated HA was quantitated in the trypsin/cell fraction. The individual analyses of the liberated (Figure 3B) and cell-associated (Figure 3C) HA was determined using an enzyme-linked HABP assay. Each point on all graphs is the mean $\pm$ SD of three independent determinations.

to *Streptomyces* hyaluronidase digestion size exclusion chromatography showed that 95–96% of the [^{3}H]-glucosamine was incorporated into HA (data not shown), where any non-digested [^{3}H] dpm were subtracted from [^{3}H] dpm associated with digested material. The non-dialysable fraction (>5 kDa) of the liberated HA recovered from DxS-treated cells contained 20% more [^{3}H]HA than the counterpart that had not been treated with DxS, while the cell-associated fraction contained approximately 45% more [^{3}H]HA. Chromatographic characterisation of the liberated and cell-associated [^{3}H]HA in both Sephacryl S1000 and Superose 12 size exclusion gels demonstrated that the high MW HA extruded into the media consisted of four distinct populations of HA of modal MW of >3000, 100 and 60 kDa (Figure 4A). The equivalent sample from cells grown without DxS exhibited depolymerisation of the liberated HA into fractions of 500, 40 and 20 kDa, suggesting a potential combination of ROI-mediated depolymerisation and/or HYAL 2 activity. A similar result was observed in the cell-associated HA, where cells grown in the presence of DxS extruded 660, 500 and 200 kDa HA, compared to DxS absent cultures where smaller modal MW populations of 70, 40 and 20 kDa were observed (Figure 4B).

Dextran sulphate down-regulated CD44 and HA synthase expression while up-regulating HYAL 1 and HYAL 2 enzymes

Fluorescence Activated Cell Sorter Analysis of the surface CD44 and synthase proteins demonstrated that growth of cells in 400 μg/ml DxS for 24 h resulted in the down-regulation of the protein expression, yet a significant increase in both the HYAL 1 and HYAL 2 enzymes (Figure 5). For exponentially growing cells that were grown without DxS equilibrium between HA degradation and synthesis was established until contact inhibition (60 h) had occurred; at which stage HA synthesis/cell appeared to decrease (data not shown). Cells treated with DxS exhibited a continual decrease in liberated HA. This finding, together with the knowledge that HA degradation was inhibited, suggested a gradual decrease in HA synthesis. This is substantiated by the quantitation of the HA synthase proteins (Figure 5). The contrasting 25–35% increase in hyaluronidase enzymes appears to be a compensatory mechanism for the down-regulation of CD44, yet increased levels of environmental HA.

Discussion

Previous studies attempting to quantify rates of HA synthesis in tissues and cells have not considered the dynamic balance between the continual production and degradation of HA. Failure to take this into account can lead to misleading results and an underestimate of HA anabolic processes. As a means of establishing the role of HA production and the relevance of MW to breast cancer invasiveness, it was necessary to identify and validate a means of inhibiting the normal HA degradative processes that occur concurrently with synthesis. Several sulphated polymers have been found to be effective inhibitors of testicular HAase,

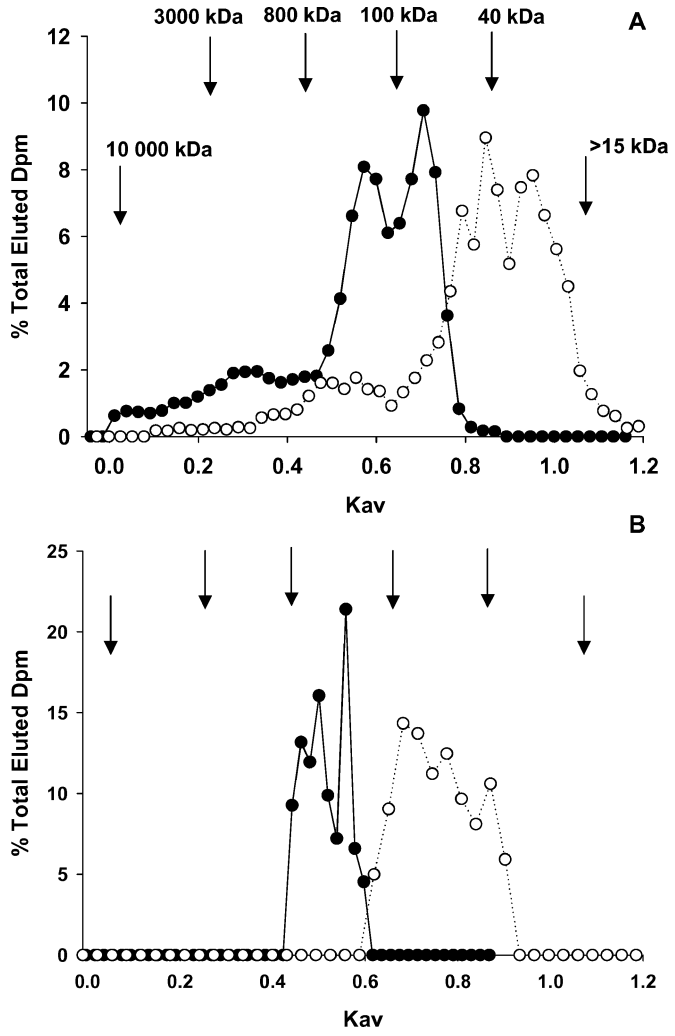

Figure 4. *Dextran sulphate inhibits the degradation of high molecular weight HA* MDA-MB 231 cells were seeded at 7.5 $\times$ 10^5 cells/75 cm^2 culture flask and were grown for 24 h in growth media containing $\pm$400 μg/ml DxS and 250 μCi D-[6-^{3}H]glucosamine. At the conclusion of the incubation, the media and cell-associated HA were removed and dialysed (M_r exclusion of 5 kDa). After substantiation that the non-dialysable Dpm was HA (as determined by *Streptomyces* hyaluronidase digestion) it was subjected to size exclusion chromatography in a Sephacryl S-1000 gel eluted in 0.15 M NaCl/phosphate buffer, pH 7.25 at 13.6 ml/h. Differences in the MW of liberated (Figure 4A) and cell-associated HA (Figure 4B) and produced by cells that were treated with DxS (●---●) and without DxS (○---○) are demonstrated. Mean column recovery was 98%.

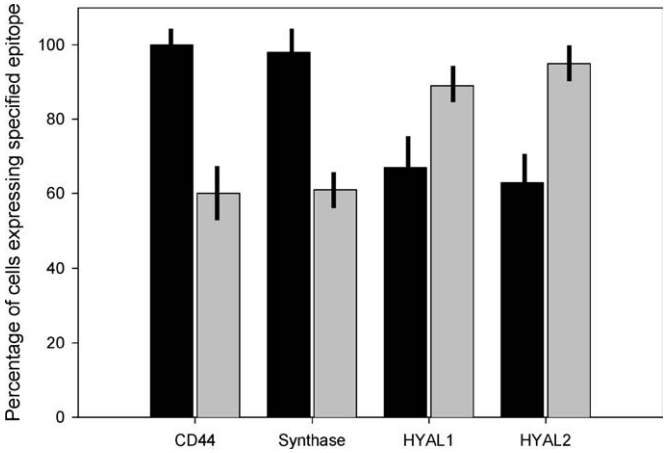

Figure 5. *Dextran sulphate down-regulates CD44 and HA synthase activity while up-regulating HYAL1 and HYAL2 protein.* MDA-MB 231 cells were grown in $\pm$400 μg/ml DxS for 24 h. Cells were scraped and incubated with 50 μg of CD44s MAb, 25 μg HA synthase polyclonal Ab, 50 μg HYAL 1 polyclonal Ab and 50 μg HYAL 2 polyclonal Ab. The quantitation of epitope expression was calculated by the shift in peak location on the x-axis as indicated by increased mean cell fluorescence. Controls consisted of background fluorescence associated with the relevant secondary antibody and was deducted from test samples. The data is presented as % of the cell population that displayed epitope-associated fluorescence, where ▭ and ■ represent cells treated with and without DxS respectively. Each point is the mean $\pm$ SD of three independent determinations.

including heparin. Heparin is a non-competitive inhibitor that does not bind specifically to the HAase catalytic site [13]. Dextran sulphate, also a HAase inhibitor, is a competitive inhibitor with high affinity for the HAase active site [26]. This study has utilised high MW DxS as a means of altering breast cancer cell-associated HAase activity and subsequent inhibition of HA degradation enabling a full quantitation and characterisation of the HA produced by a highly invasive and progressive breast cancer cell line, while presenting a preliminary insight into the mechanisms involved in its mode of inhibition.

Dextran sulphate inhibited both testicular and Streptomyces HAase in a dose-dependent manner. No other studies have demonstrated that a sulphated GAG can exert an inhibitory effect on both classes of HAases. In fact heparin has been demonstrated to be inactive in the inhibition of *Streptomyces* HAase [13]. The bacterial and vertebrate eliminase and hydrolase classes of HAases, respectively, demonstrate very different mechanisms of action. It is most likely that DxS inhibits the two classes of enzymes by entirely different and unrelated mechanisms. At present very little is known about the active site of lyases, Yudin *et al.* [40] have identified putative catalytic sites centred on either glutamic acid or aspartic acid regions of PH-20. These sites are thought to act as either an acid catalyst site (amino acid sequence 277–297) or as a nucleophilic site (amino acid site 142–172) that could potentially modulate the kinetics of hydrolase HAase activity over a broad pH range. Through highly negatively charged DxS could be exerting its inhibitory action on the transglycosidase activity by interrupting the nucleophilic substitution through scavenging of electrons during the intramolecular electron transfer cascade. Another potential inhibitory mechanism could relate to the cation requirement of testicular and several *Streptococcus* bacterial HAases [41] where the potent polyanionic DxS could inter-

act with cations, depleting the HAase of essential co-factors. Increased sulphation of the glycan chain increases molecular rigidity, hampering any stabilisation effect on the HAase [42]. A more simple explanation for the inhibitory activity of DxS against both the vertebrate and bacterial forms of HAase could rely on the fact that both enzymes retain a similar carbohydrate-binding groove, [43,44] whereby the deposition of the DxS in the binding groove could sterically hinder the binding of other substrates.

It is currently proposed that cellular degradation of HA occurs by the concerted effort of the predominant somatic HAases, HYAL 1 and HYAL 2, where the high MW HA binds to CD44, and in co-operation with the GPI-anchored HYAL 2 is internalised and degraded in unique acid endocytic vesicles to a 20 kDa HA fragment. The 20 kDa fragments are then transported intracellularly by an unknown process, and further digested by HYAL 1 together with two β-exolycosidases, β-glucuronidase and β-*N*-acetyl glucosaminidase, resulting in low-MW oligosaccharides [17]. The observations that the media and cell-associated fraction of DxS treated cultures did not contain the HA metabolites of acetate, water, tetrasaccharides and 20 kDa HA and the significant increase in the levels of detectable HA concomitant with a down regulation of the HA synthase protein provides strong evidence that DxS can inhibit HA degradation. The up-regulation of both HYAL 1 and HYAL 2 enzymes after a 24 h exposure to DxS did not influence the turnover rate of the HA, as the inability to detect HA metabolites, which were detected in the DxS treatment group confirms the total inhibition of the HAase activities. The continual decline in liberated HA in DxS-treated cultures suggests a reduction in synthesis, where the 40% down-regulation of HA synthase at 24 h (Figure 5) correlated exactly with the 38% decrease in liberated HA (data not shown). It is well established that HA is retained on the membrane-bound synthase complex during chain elongation and can be released from the cell surface as an intact molecule [45] or it can be cleaved via hyaluronidases [46], where the synthase is activated upon release of the HA chains [47]. The steady, decrease in liberated HA could also be representative of uncleaved HA chains which signalled back to the cell to reduce HA synthesis via down-regulating of the protein.

Dextran sulphate inhibition of HA degradation enabled an accurate characterisation of the synthesised and liberated HA. The observed MW was consistent with the reported synthetic activity of a *HAS2* transfected COS cell line which generated $>$2000 kDa HA [16]. The presence of the 60–100 kDa HA could be the result of interrupted chain elongation or the activity of radical oxygen intermediates. Human tumour cell lines generate up to 0.5 nmol/10^4 cells/h of hydrogen peroxide [48]. Hydrogen peroxide can react with metal ions in cell culture media to generate hydroxyl radicals. These radicals can randomly cleave HA to generate polydisperse MW HA [22]. It is postulated that ROI prepares the HMW HA for presentation to the HYAL 2 enzyme and CD44 receptor that exists within caveolin-rich membrane microdomains [49]. This mechanism has previously been suggested by Knudson *et al*. [50] who hypothesised that extracellular HAases may also cleave HA into smaller fragments allowing their uptake. The data from this study do not totally discount the possibility of this process; however, it is more indicative of the ROI contribution to this process due to the evidence of the inhibition of HAase activity.

The cell-associated HA contained MW species of 660, 500 and 200 kDa. The continual increase in the HA concentration of cell-associated HA suggests that the normal mechanism associated with HA release from the synthase or cell surface receptors was not occurring in the presence of DxS. Alternatively, the high levels of environmental HA and its ability to act as a free radical scavenger [51] may have rendered the ROI burst insufficient to cleave the HA chains.

The inhibitory effect of DxS on HA degradation and the resultant increase in both liberated and cell-associated HA exerted a negative feedback on the expression of CD44 and synthase expression. A previous study highlighting the modulation of CD44 by HAase [52] demonstrated that cells are able to modulate CD44 expression in response to environmental signals. This is substantiated in dermal fibroblasts where the addition of exogenous HA decreased expression of CD44 [53]. The finding that the decrease in liberated HA may have led to a dynamic feedback signal, subsequently reducing the HA synthesis via down-regulation of the HA synthase, then it would logically follow that hyaluronidase treatment of cells may cause an increase in CD44 and HA synthase expression. This phenomenon has been observed in fibrocartilage cells, where treatment with hyaluronidase increased CD44 and HA synthase expression as well as enhanced binding to HA [54].

Previous studies centred on the ability of DxS to reduce peritoneal [31] or pulmonary metastasis [37]. These studies used both low (7 kDa) and high (500 kDa) MW DxS to reduce adherence of melanoma or lung tumour cells to endothelium of lung and peritoneum. Dextran sulphate was not toxic to cancer cells, therefore the anti-metastatic capabilities were thought to be due to the polyanionic nature of the DxS. It was suggested that DxS binding to both tumour and endothelial cells rendered them more negatively charged. This increased electrostatic repulsion altered the adhesiveness between the tumour cells and the vascular endothelium. This alteration in adhesion was thought to reduce or inhibit the lodgement of the tumour cells in the capillaries of organs resulting in a subsequent reduction in metastasis. The ability of DxS to competitively inhibit hyaluronidase was not invoked. The findings of Zimmermann [26] and those reported in the present study document the ability of DxS to inhibit enzymic degradation of HA. Hyaluronidases have been previously implicated in tumour progression and metastasis. The concerted action of CD44, endocytosis of HA, HYAL 1 and HYAL 2 result in low MW oligosaccharides that are highly angiogenic [2,55]. Therefore it could be postulated that the inhibition of HAase *in vivo* would act as an anti-angiogenic agent, inhibiting tumour growth and neo-vascularisation. The findings of reduced metastasis after intravenous or intraperitoneal

administration of DxS could easily be attributed to the hyaluronidase inhibitory action of DxS as well as an anti-adhesive mechanism of action. The administration of DxS may have interfered with tumour cell lodgement in the vascular endothelium. Additionally the DxS may have inhibited inter- and intracellular HAase. The reduced levels of HA oligosaccharides decreased tumour vascularization and as tumours reached critical mass insufficient supplies of oxygen and nutrients limited tumour progression.

This study was designed to establish and validate the applicability of growing cells in DxS as a means of inhibiting cellular metabolism and depolymerisation of HA. Dextran sulphate was shown to be non-toxic to cells. The application of DxS to growth media will enable researchers to accurately determine the relationship between HA synthase isoform expression and MW. True turnover rates in specific cell types can be assessed allowing elucidation of anabolic and catabolic rates. Through these experiments insights into the complex interconnections between synthesis and degradation of HA in cells has been achieved.

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