Chemotaxis towards hyaluronan is dependent on CD44 expression and modulated by cell type variation in CD44-hyaluronan binding

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Accepted 4 August 2005
Journal of Cell Science 118, 5119-5128 Published by The Company of Biologists 2005
doi:10.1242/jcs.02629

Summary

The accumulation of the extracellular matrix glycosaminoglycan hyaluronan by tumours and tumour-associated stroma promotes cancer cell invasion and metastasis. Using the Dunn chamber chemotaxis assay, we demonstrate for the first time that high molecular mass hyaluronan acts as a soluble chemoattractant promoting the directional migration of MDA-MB-468 and MDA-MB-231 breast cancer cells. Moreover, chemotaxis towards hyaluronan, but not foetal bovine serum, can be abrogated following treatment of the cells with siRNA oligonucleotides to downregulate CD44 expression. These data indicate that CD44 is the principal receptor mediating this response and that CD44 expression is not a general requirement for cell migration and gradient sensing, rather it elicits a ligand-specific response. However, expression of CD44 alone is not sufficient to drive chemotaxis towards hyaluronan, as NIH-3T3 fibroblasts were unable to respond to a hyaluronan gradient even when transfected with high levels of human CD44. For NIH-3T3 cells to bind exogenous hyaluronan, it was necessary to both increase the level of receptor expression and remove a hyaluronan pericellular matrix. Together, these studies reveal a direct mechanism for promoting cell invasion into the hyaluronan-rich matrix and predict that in the complex multicellular environment in vivo, multiple mechanisms exist to regulate the ability of a cell to respond to a chemotactic hyaluronan gradient.

Key words: Hyaluronan, CD44, Chemotaxis, Migration, Breast cancer

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Introduction

Hyaluronan is a ubiquitous glycosaminoglycan composed of an unbranched linear chain of repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units, which commonly reaches a size of 4 MDa. Unlike other glycosaminoglycans, hyaluronan is never covalently attached to a protein core or modified by sulphation yet despite this simplicity of structure, hyaluronan has a surprisingly wide range of physiological roles (Laurent and Fraser, 1992) (for reviews, see *Hyaluronan Today* at http://www.glycoforum.gr.jp). In general, these can be divided into two broad but overlapping categories. First, hyaluronan has a structural role within the extracellular matrix because of its expanded coil conformation, which in solution can assume a hydrated sphere morphology. This allows hyaluronan to create and fill extracellular space thus regulating cell movement and the transport of extracellular components (Laurent and Fraser, 1992; Lee and Spicer, 2000; Toole, 2004). In addition, hyaluronan can associate non-covalently with proteoglycans such as aggrecan or versican to form large aggregates that are stabilised by the hyaluronan-binding link proteins (Day and Prestwich, 2002). Although these properties of hyaluronan regulate various aspects of cell behaviour, in particular cell migration, it is increasingly clear that hyaluronan has a second, more direct role in controlling cellular responses via its ability to bind to specific hyaluronan receptors on the cell surface (Ponta et al., 2003; Turley et al., 2002).

To date, CD44 is the best characterised transmembrane hyaluronan receptor and because of its wide distribution is considered to be the major hyaluronan receptor on most cell types. Work from a large number of laboratories has established that CD44 can function as an adhesion receptor and can mediate rolling, attachment and migration of cells on a hyaluronan substratum. In addition, as has been demonstrated for all of the other major adhesion receptors, binding of ligand can result in CD44-mediated transduction of intracellular signals leading to changes in cell proliferation, survival and differentiation (Ponta et al., 2003; Thorne et al., 2004; Toole, 2004). More recently, a number of other transmembrane hyaluronan receptors have been identified (Day and Prestwich, 2002), indicating that cells may use more than one mechanism to respond to ligand and that when interpreting cellular responses to hyaluronan it is important to identify the particular receptor(s) involved.

Much of the interest in hyaluronan has arisen from its association with pathological processes, particularly cancer (Toole, 2004). Accumulations of hyaluronan have been demonstrated in association with many malignant tumours and there is now extensive data correlating hyaluronan deposition with enhanced metastatic cell behaviour and poor patient

survival (Anttila et al., 2000; Auvinen et al., 2000; Lipponen et al., 2001). Hyaluronan is produced by hyaluronan synthases localised at the cell surface (HAS1, HAS2 and HAS3) and, in tumours, increased production by the tumour-associated stromal cells or by the tumour cells themselves is commonly observed. Experimental evidence that hyaluronan has a role in tumour progression has come from studies in which hyaluronan levels have been manipulated. For example, overexpression of a hyaluronan synthase in prostate and mammary carcinoma cell lines results in enhanced tumour growth (Liu et al., 2001), loss of contact inhibition and promotion of cell migration (Itano et al., 1999). Conversely, transfection of prostate cancer cells with HAS2 or HAS3 antisense constructs or intravenous administration of mice bearing human breast cancer xenografts with hyaluronidase, results in reduced tumour growth (Shuster et al., 2002; Simpson et al., 2002). In vitro studies on similarly manipulated cell lines have indicated that the increased tumour cell invasion associated with an increased level of hyaluronan in the matrix results from the hyaluronan clearing a migratory path by excluding crosslinked fibrous proteins from the matrix and acting as a scaffold for migrating cells (Toole et al., 2002). However, there is also increasing evidence that the signalling functions of hyaluronan are important in these events, both for promoting tumour cell growth and survival and as a chemoattractant source for the directional migration of tumour cells and infiltrating angiogenic endothelial cells. What is less clear is whether these signalling events are mediated by specific forms of hyaluronan, in particular by hyaluronan size. As stated above, hyaluronan is synthesised as large 100-1000 kDa unbranched chains (high molecular mass hyaluronan) but it can be degraded in the matrix by hyaluronidases or the action of reactive oxygen species to give rise to intermediate size hyaluronan (~10 to ~100 kDa) or small fragments (4-25 disaccharides) (Stern, 2003). Interest in these breakdown products has come from the demonstration in vitro that cells respond differently to variously sized hyaluronan preparations (Noble, 2002; Rooney et al., 1995; Tammi et al., 2002) and in particular that the angiogenic response to hyaluronan is size dependent (Lees et al., 1995; West et al., 1985). In addition, increased levels of small hyaluronan fragments have been demonstrated in prostate tumours (Lokeshwar et al., 2001), the saliva of patients with head and neck squamous cell carcinoma (Franzmann et al., 2003) and the serum of patients with renal cancer (Kumar et al., 1989). However, in the majority of studies there has been no systematic attempt to determine whether the small hyaluronan fragments identified in association with human tumours play any biological role or that their presence simply reflects an increased rate of hyaluronan production and turnover.

In order to gain a better understanding of the role of hyaluronan in promoting tumour progression we investigated whether hyaluronan could act as a chemotactic signal for tumour cells. Further, as the predominant hyaluronan present associated with human tumours in vivo is of high molecular mass (Franzmann et al., 2003; Lokeshwar et al., 2001) we focussed our studies on this hyaluronan species.

Materials and Methods

Cell lines and antibodies

MDA-MB-468 and MDA-MB-231 cells were obtained from ATCC and cultured in DMEM plus 10% foetal bovine serum (FBS). NIH-3T3 cells were obtained from C. Marshall (Institute of Cancer Research, London, UK) and were cultured in DMEM plus 10% donor calf serum (DCS). Cells were transfected with either pSRα vector alone or pSRα vector containing the standard form of human CD44 (pSRα-CD44) (Neame and Isacke, 1993) using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected in 0.6 mg/ml G418 (Invitrogen) and a population of cells expressing equivalent levels of human CD44 selected by FACS. Monoclonal antibodies used were E1/2.8 specific for human CD44 (Isacke et al., 1986), IM7.8.1 recognising human and mouse CD44 (Trowbridge et al., 1982), KM201 specific for mouse CD44 (Miyake et al., 1990), anti-DAF antibody IA10 (BD Biosciences) and anti-α-tubulin (Sigma). Chimaeric constructs in which either the human CD44 extracellular domain or the Endo180 extracellular domain (Endo180ΔCTLD5-8-Fc) (Wienke et al., 2003) were fused in-frame with the Fc tail of human IgG_1 were expressed in COS cells, purified on Protein G-Sepharose as previously described (Wienke et al., 2003) and used at 5 μg/ml for flow cytometry. Alexa-conjugated secondary antibodies were purchased from Molecular Probes. HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch, phycoerythrin (PE)-conjugated anti-human Fc was purchased from Sigma. Western blotting, immunofluorescence staining and confocal microscopy were performed as previously described (Sullivan et al., 2003).

Hyaluronan and hyaluronidase

Hyaluronan from human umbilical cords with a peak molecular mass of ~200 kDa (McKee et al., 1996) was purchased from ICN. Streptococcal hyaluronan with a molecular mass of ~760 kDa was purchased from Calbiochem. Streptococcal hyaluronan was directly conjugated to FITC as previously described (de Belder and Wik, 1975) and used for flow cytometry and immunofluorescence at 10 μg/ml. Hyaluronidase (type IV-S or from *Streptomyces hyalurolyticus*, Sigma) was added to the cells at 30 U/ml in DMEM with 10% FBS and incubated for 1 hour at 37°C. Cells were then washed twice with PBS.

siRNA

The following small interfering (siRNA) oligonucleotides were used: CD44 siRNA oligonucleotides, 5′-GUAUGACACAUAUUGCUUC dT dT-3′ and 5′-GAAGCAAUAUGUGUCAUAC dT dT-3′ (Roscic-Mrkic et al., 2003); control oligonucleotides, 5′-UCUACUCUUC-CUUCUGCAACCC dT dT-3′ and 5′-GGGUAGCAGAAGGAGU-AGA dT dT-3′. Annealed siRNA oligonucleotides (0.2 nM) were transfected into MDA-MB-468 cells seeded onto glass coverslips (30-50% confluent) with 100 μM oligofectamine (Invitrogen) in Opti-MEM reduced-serum medium (Invitrogen). Cells were incubated at 37°C for 4 hours before the addition of FCS to 10% and cultured for a further 72 hours before the commencement of further assays.

Chemotaxis assays

Chemotaxis was measured by direct observation and recording of cell behaviour in stable concentration gradients using Dunn chemotaxis chambers (Hawksley Technology, Lancing, UK) as described previously (Allen et al., 1998; Sturge et al., 2002; Zicha and Dunn, 1995). Glass coverslips were coated with either 1% Matrigel (BD Biosciences) in DMEM (MDA-MB-468 and MDA-MB-231) or 1 μg/ml laminin (BD Biosciences, cat. no. 354232) in PBS (NIH-3T3) for 1 hour at room temperature and then washed twice with DMEM before cell plating. In all cases, cells were starved in DMEM with 0.25% of the appropriate serum for 12 hours before setting up the chemotaxis chambers. Gradients of FBS (MDA-MB-468 and MDA-MB-231) or DCS (NIH-3T3) were formed by placing DMEM

Journal of Cell Science

containing 10% of the relevant serum in the outside well and 0.25% in the inside well. Umbilical cord or streptococcal hyaluronan gradients were formed by placing a 400 μg/ml dilution of hyaluronan in DMEM with 0.25% serum in the outside well and DMEM with 0.25% serum in the inside well. For no gradient control chambers, DMEM with 0.25% serum was placed in both inside and outside wells. Fields were selected and filmed with a time-lapse interval of 10 minutes for 8 hours and Kinetic Imaging (Nottingham, UK) Motion Analysis software used to register cell tracks. Data were then analysed using a Mathematica (Wolfram Research, Illinois, USA) notebook written by D. Zicha (Cancer Research UK, London, UK). In determining the directional responses, only cells migrating to a certain 'horizon' distance were included in the calculation. The horizon distance was defined as the distance migrated from the starting position by 90% of cells, a value determined arbitrarily to enable a highly stringent statistical test (see below). For cells passing the horizon, *x*/*y* coordinates from each movie frame were used to calculate the mean angle of movement. These angles were then displayed in a circular histogram where each bar represents the total number of cells with an angle of migration lying within an 18° interval. The Rayleigh test for unimodal clustering of directions was used to determine whether there was a significant chemotactic response. This statistical test verifies the significance of the mean direction in a circular histogram. Note, this analysis only provides a measurement of statistical significance and not a measurement of the strength of the chemotactic response. A uniform distribution (random cell motility) of the data was assumed if the P-value for the calculation was greater than 0.05. A significant chemotactic response is indicated on the circular histogram by a red arrow showing the mean angle of migration and green sector indicating the 95% confidence interval.

Isolation of detergent-insoluble raft domains

Biochemical isolation of detergent-insoluble raft domains was performed essentially as previously described (Thorne et al., 2000) but with minor modifications. Briefly, cells were grown to 80% confluence and then further incubated in low-serum medium (0.25% FBS) for 2-16 hours before addition of 50-100 μg/ml hyaluronan for 5-10 minutes at 37°C. Cells were then lysed in 1% Triton X-100 in MES-buffered saline (pH 6.8) and the sample adjusted to 40% sucrose (w/v) before fractionation by ultracentrifugation in a discontinuous 5/30/40% sucrose gradient. The detergent-insoluble material was collected from the 5/30% sucrose interface together with a sample of soluble lysate from the 40% sucrose layer. The protein concentration of each sample was determined using the BCA assay (Pierce Chemical Company) and equivalent protein amounts subjected to electrophoresis and western blotting.

Results

High molecular mass hyaluronan can mediate cell chemotaxis

Increased accumulation of hyaluronan around solid tumours is associated with increased cancer cell invasion and metastasis. It has been suggested that this, at least in part, reflects the ability of hyaluronan to promote chemotaxis. Although the majority of hyaluronan in the extracellular matrix is of high molecular mass, most studies examining the pro-chemotactic role of hyaluronan have focussed on small hyaluronan fragments. To the best of our knowledge, the studies performed here are the first systematic attempt to determine whether high molecular mass hyaluronan can promote directional cell migration. Initially, the breast cancer cell lines, MDA-MB-468 and MDA-MB-231, were tested for their ability to migrate directionally up a gradient of a known chemoattractant, FBS

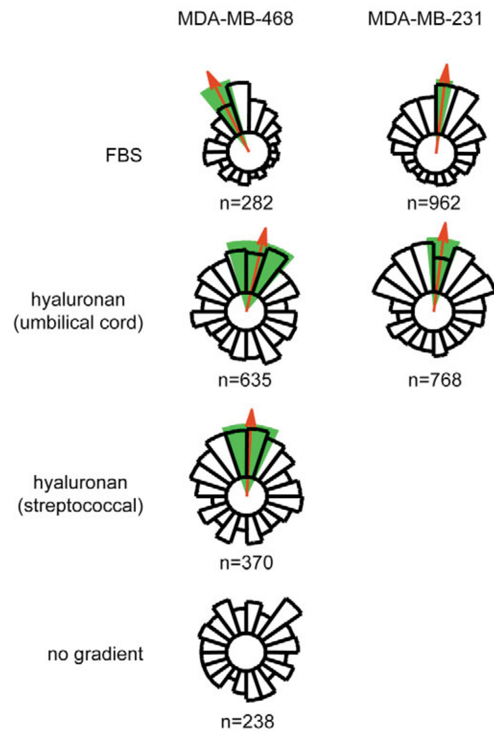

Fig. 1. Directional migration of cells in a hyaluronan gradient. The directional migration of MDA-MB-468 and MDA-MB-231 cells was assayed in a Dunn chemotaxis chamber. The gradients employed were 0.25% to 10% FBS, 0 to 400 μg/ml umbilical cord hyaluronan, 0 to 400 μg/ml streptococcal hyaluronan or no gradient (0.25% FBS in both chambers). The data are presented as a circular histogram in which each 18° segment represents the total number of cells with an average angle of migration falling within that particular interval. The Rayleigh test for unimodal clustering is used to verify directional migration. If cells show statistically significant directional migration, the mean migration angle is displayed as a red arrow and the 95% confidence interval as a green sector. The number of cells analysed for each treatment is given.

or hyaluronan using a Dunn chemotaxis chamber. In this system, cells are directly viewed and filmed over an 8-hour period, following which the direction of migration can be measured by tracking the movement of individual cells. The advantage of this approach is that cell directionality is measured independently of cell speed. As expected, both cell lines migrated in a directional manner towards FBS. In addition, both cell lines also migrated in a directional manner when plated in a gradient of high molecular mass (~200 kDa) umbilical cord hyaluronan (Fig. 1). To determine whether this was an artefact due to the particular hyaluronan employed, it was also demonstrated that MDA-MB-468 cells showed directional migration towards streptococcal hyaluronan (~760 kDa) but only displayed random migration when no gradient was present.

Hyaluronan-dependent chemotaxis requires expression of the hyaluronan receptor CD44

Directional migration requires that cells sense a gradient of chemoattractant and that this in turn leads to their polarization.

To date, the best characterised hyaluronan transmembrane receptor is CD44 and flow cytometric staining demonstrated that both MDA-MB-468 and MDA-MB-231 cells express uniformly high levels of CD44 (Fig. 2A). Immunofluorescence staining also revealed a typical CD44 distribution with the receptor strongly expressed at the plasma membrane and little or no intracellular staining (Fig. 2B). Using hyaluronan conjugated to FITC, it was demonstrated that MDA-MB-468 and MDA-MB-231 cells could bind hyaluronan as assayed both by flow cytometry (Fig. 2A) and fluorescence microscopy (Fig. 2B) although, as discussed later, despite similar levels of CD44 expression MDA-MB-231 cells bind less hyaluronan than MDA-MB-468 cells. A number of transmembrane receptors, other than CD44, which contain the hyaluronan-binding Link module have been reported (Day and Prestwich, 2002). These include Lyve-1, which is expressed predominantly on lymphatic endothelium (Jackson, 2003; Prevo et al., 2001), members of the stabilin family, which are reported to function as hyaluronan internalisation receptors on hepatic sinusoidal endothelial cells (Harris et al., 2004; Politz et al., 2002), potentially Toll-like receptor 4 (TLR4) (Taylor et al., 2004; Termeer et al., 2002) as well as a cell-associated hyaluronan binding protein, RHAMM (Turley et al., 2002). To address the possibility that a non-CD44 hyaluronan receptor might be responsible for sensing the hyaluronan gradient, we used small interfering double stranded RNA (siRNA) to downregulate CD44 expression. By flow cytometric analysis, siRNA directed towards CD44 resulted in a substantial decrease in CD44 expression whereas no effect was seen in mock-transfected cells or cells transfected with a control siRNA (Fig. 3A). Importantly, this downregulation of CD44 expression also resulted in a substantial reduction in FITC-hyaluronan binding as monitored both by flow cytometry (Fig. 3A) and fluorescence microscopy (Fig. 3B). Treatment of cells with CD44 siRNA, but not with control siRNA, also resulted in loss of the ability of the cells to migrate in a directional manner towards a hyaluronan source. This was not a global impairment of chemotaxis however as cells treated with CD44 siRNA retained their ability to migrate directionally up a gradient of FBS (Fig. 3C).

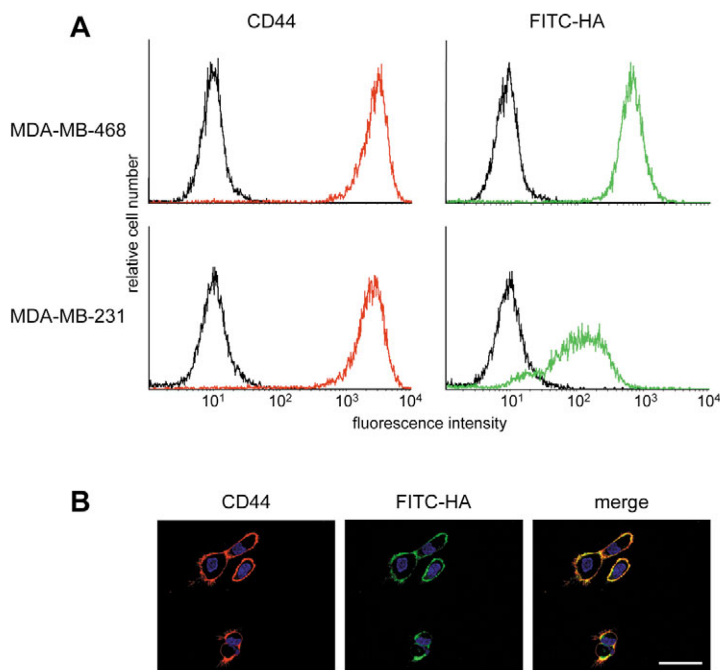

Fig. 2. MDA-MB-468 and MDA-MB-231 cells express CD44 and exhibit constitutive hyaluronan binding. (A) Cells cultured overnight were detached and stained for flow cytometry. For CD44 expression, cells were incubated either with anti-CD44 monoclonal antibody E1/2 followed by Alexa555 anti-mouse Ig (red graph) or with secondary antibody alone (black graph). For hyaluronan binding, cells were either incubated with FITC-conjugated hyaluronan (FITC-HA, green graph) or medium alone (black graph). (B) MDA-MB-468 cells cultured overnight on glass coverslips were incubated with FITC-hyaluronan for 1 hour at 37°C (green) and then fixed, permeabilised and stained with the anti-CD44 monoclonal antibody E1/2 followed by Alexa555 anti-mouse Ig (red). Nuclei were visualised with TO-PRO-3 (blue). Bar, 50 μm.

Hyaluronan binding does not alter the distribution of CD44 in detergent-insoluble lipid raft domains

Recently, it has been demonstrated that lipid rafts can play an important role in providing the spatial cell polarity required for chemotaxis (Gomez-Mouton et al., 2004; Manes et al., 2003). CD44 is known to partition into detergent-insoluble raft domains in a cell type-specific manner (Neame et al., 1995; Perschl et al., 1995a) and suggestively, in immortalised mammary epithelial cell lines, this association was demonstrated to stabilise the interaction of CD44 with the actin cytoskeleton (Foger et al., 2001; Oliferenko et al., 1999). We therefore sought to determine if CD44 was partitioned into the lipid rafts of the breast cancer cell lines studied here and if so, whether the proportion of CD44 in the rafts was altered following hyaluronan binding. MDA-MB-468 cells were lysed in Triton X-100 buffer and fractionated on sucrose density gradients to prepare detergent-insoluble raft and detergent-soluble fractions as described in the Materials and Methods. Western blotting of equal amounts of protein from each fraction revealed that in MDA-MB-468 (Fig. 4) and MDA-MB-231 cells (data not shown) CD44 was enriched in the raft fraction. To validate the isolation procedures, western blotting of replicate samples revealed that decay accelerating factor (DAF) was found exclusively in the detergent-insoluble raft fraction as would be expected for a GPI-anchored protein, whereas α-tubulin was located exclusively in the detergent-soluble fraction. Addition of umbilical cord hyaluronan (Fig. 4) or streptococcal hyaluronan (data not shown) under any of the conditions tested did not increase the association of CD44 with lipid rafts indicating that, at least in these breast cancer cell lines, further raft recruitment of CD44 is not required for cell chemotaxis.

The chemotactic response to hyaluronan is dependent on cell type

The data presented here strongly suggest that the migration of breast cancer epithelial cells towards hyaluronan is dependent on CD44 expression (Fig. 3). To further investigate whether the chemotactic response towards hyaluronan is a common feature of CD44-

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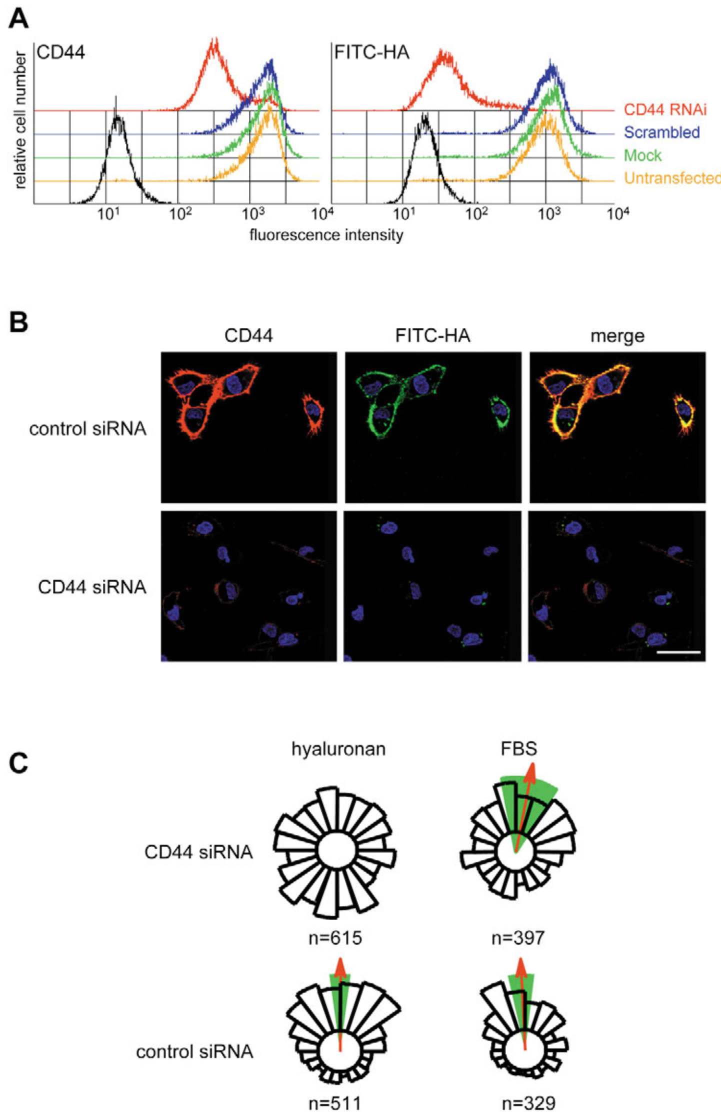

Fig. 3. CD44 expression is required for sensing a hyaluronan gradient. (A,B) MDA-MB-468 cells were left untreated, mock transfected, transfected with control siRNA oligonucleotides or transfected with CD44 siRNA oligonucleotides. 72 hours later cells were detached from dishes and CD44 expression and hyaluronan binding assessed by flow cytometry or fluorescence microscopy as described in Fig. 2. In A, black lines represent binding of second antibody alone (CD44) or medium alone (FITC-HA) to untreated cells. (C) MDA-MB-468 cells transfected with CD44 or control siRNA oligonucleotides were assayed for their ability to migrate directionally in a gradient of FBS or umbilical cord hyaluronan as described in Fig. 1. Bar, 50 μm.

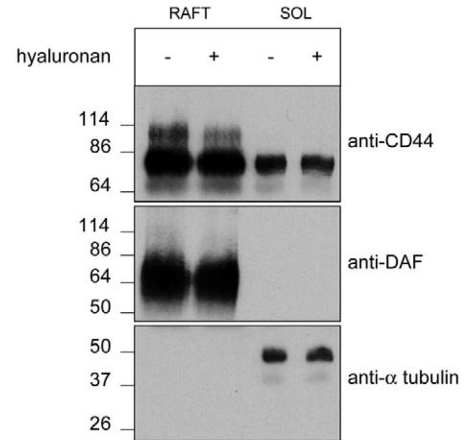

Fig. 4. Effect of hyaluronan binding on the partitioning of CD44 into detergent-insoluble lipid rafts. MDA-MB-468 cells were incubated without or with 100 μg/ml umbilical cord hyaluronan before preparation of detergent-insoluble lipid rafts (RAFT) and soluble fractions (SOL) as described in the Materials and Methods. Protein equivalents (5 μg) were separated by electrophoresis and western blotted for CD44 (monoclonal antibody E1/2), DAF and α-tubulin. Positions of molecular size markers are indicated in kDa.

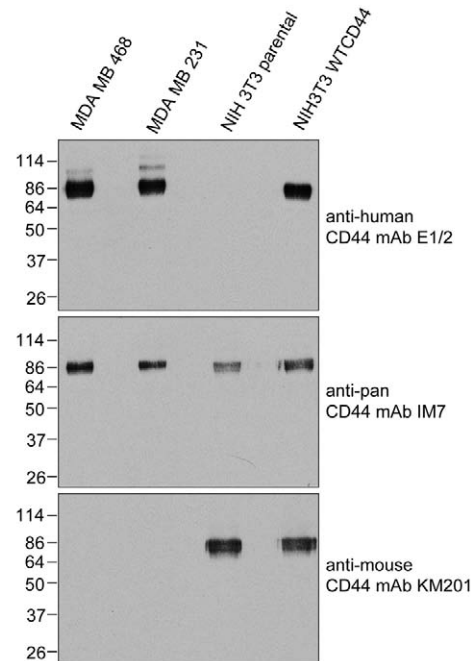

Fig. 5. Generation of a murine fibroblast line overexpressing human CD44s. MDA-MB-468 cells, MDA-MB-231 cells and NIH-3T3 cells transfected with pSRα vector alone and NIH-3T3 cells expressing human CD44s were lysed and subjected to western blotting with anti-human CD44 monoclonal antibody E1/2, monoclonal antibody IM7, which recognises both human and murine CD44, or anti-murine CD44 monoclonal antibody KM201. Positions of molecular size markers are indicated in kDa.

expressing cells, we evaluated NIH-3T3 fibroblasts in the Dunn chemotaxis chamber assay. Western blot analysis using monoclonal IM7 antibody, which recognises both human and mouse CD44, and monoclonal antibody KM201, which is specific for mouse CD44, demonstrated that like MDA-MB-468 and MDA-MB-231 cells, NIH-3T3 cells predominantly express CD44 of a molecular mass consistent with the standard CD44 isoform, CD44s (Fig. 5).

Analysis of the NIH-3T3 cells in the hyaluronan chemotaxis assay unexpectedly revealed that although these cells were able to migrate towards a gradient of DCS, only random migration was observed when these cells were plated in a gradient of

hyaluronan (Fig. 6A). Further analysis of these cells by flow cytometry demonstrated that despite CD44 being readily detected at the cell surface, there was essentially no binding of FITC-hyaluronan (Fig. 6B). This lack of hyaluronan binding provides an explanation as to why these cells do not migrate in a hyaluronan gradient, but raises the issue as to why a CD44-expressing cell line does not bind ligand. It is well recognised that a threshold of CD44 expression is required before hyaluronan binding is observed (Perschl et al., 1995b; Tzircotis et al., 2004; Uff et al., 1995) and it was notable by western blotting that the level of CD44 expression in NIH-3T3 cells was lower than that observed in the MDA-MB-468 and MDA-MB-231 breast cancer lines (Fig. 5). To address this, NIH-3T3 cells were transfected with a human CD44s construct and by FACS sorting with the human CD44 specific monoclonal antibody E1/2, a population of cells which stably expressed human CD44 at a level equivalent to that found in the breast epithelial lines was selected (Fig. 5 and Fig. 6B). Western blot analysis of these human CD44s-transfected NIH-3T3 cells

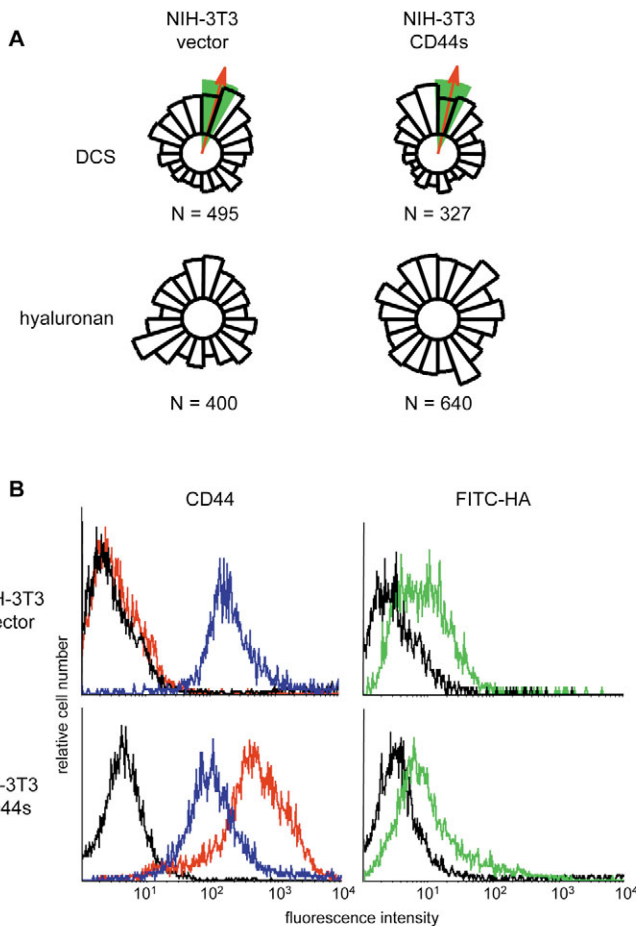

Fig. 6. NIH-3T3 fibroblasts do not migrate towards hyaluronan. (A) NIH-3T3 fibroblasts transfected with vector alone or human CD44s were assayed for their ability to migrate towards DCS (0.25-10.00% gradient) or umbilical cord hyaluronan (0-400 μg/ml gradient) as described in Fig. 1. (B) Cells were subjected to flow cytometry to monitor expression of murine CD44 using monoclonal antibody KM201 (blue), expression of human CD44 using monoclonal antibody E1/2 (red) and FITC-HA binding (green) as described in Fig. 2.

provides further evidence that both the NIH-3T3 cells and the breast cancer cell lines predominantly express CD44s (Fig. 5). Despite this increased expression, no directional migration of the CD44s-transfected NIH-3T3 cells in a hyaluronan gradient was observed (Fig. 6A) and surprisingly, binding of FITC-hyaluronan remained at background levels (Fig. 6B). This inability of the exogenously expressed human CD44s to bind ligand is not due to a defect in the human CD44s cDNA, as transfection of CD44-negative human melanoma cells and mouse T lymphoma cells with the same construct has previously been demonstrated to result in CD44s expression and efficient hyaluronan binding (Peck and Isacke, 1996; Uff et al., 1995). Consequently, it is valid to conclude that CD44 is modified in a cell type-specific manner to modulate ligand binding and hence cellular responses to hyaluronan.

To date, the best characterised mechanism for regulating hyaluronan binding to CD44s is variable extracellular domain glycosylation (Lesley et al., 1997) and it is evident that the relative role of N- and O-linked glycosylation and modification by chondroitin sulphate in this process is dependent on cell type (Bartolazzi et al., 1996; Bennett et al., 1995; Lesley et al., 1995; Skelton et al., 1998). Although there is a strong likelihood that CD44 glycosylation plays a role in modulating hyaluronan binding to NIH-3T3 cells, we wished to investigate whether additional mechanisms preventing binding were involved. Fibroblasts, which are known to synthesise hyaluronan, may have assembled a hyaluronan-containing matrix on their surface, which prevents binding of exogenously added ligand. Vector alone and human CD44s expressing NIH-3T3 cells pretreated with or without hyaluronidase were assessed for their ability to bind a CD44-Fc chimaeric protein which serves as a hyaluronan binding probe (Peach et al., 1993) (Fig. 7A). As a control, cells were incubated with an Fc chimaera containing the extracellular domain of an unrelated receptor, Endo180 (Wienke et al., 2003). In both vector alone and CD44s transfected cells, an equivalent level of CD44-Fc binding was observed which was significantly reduced in hyaluronidase-treated cells, indicating that these cells are surrounded by a hyaluronan-containing pericellular matrix. Additional control experiments demonstrated increased CD44-Fc binding to MDA-MB-468 cells following preincubation with exogenously added hyaluronan and that hyaluronidase treatment of these cells did not alter CD44 expression levels (data not shown). Although both vector alone and CD44s-expressing NIH-3T3 cells have a hyaluronan coat, only the CD44s-expressing cells show an increase in FITC-hyaluronan binding following hyaluronidase treatment. In addition, the flow cytometric analysis indicates that this increase is variable within the population. These data were confirmed by confocal microscopy (Fig. 7B) where it is shown that following hyaluronidase treatment, only cells expressing human CD44s were able to bind exogenously added FITC-hyaluronan. Note that NIH-3T3 cells sit flatter on the substratum than MDA-MB-468 cells (see Fig. 2) and therefore the plasma membrane expression of CD44 and the binding of FITC-hyaluronan to the NIH-3T3 cell surface is observed in a single low power confocal section. However, clearly not all transfected cells exhibited efficient binding, indicating that multiple mechanisms exist to regulate hyaluronan binding to these cells. Finally, it was important to determine whether this blocking of exogenous hyaluronan binding by a hyaluronan pericellular

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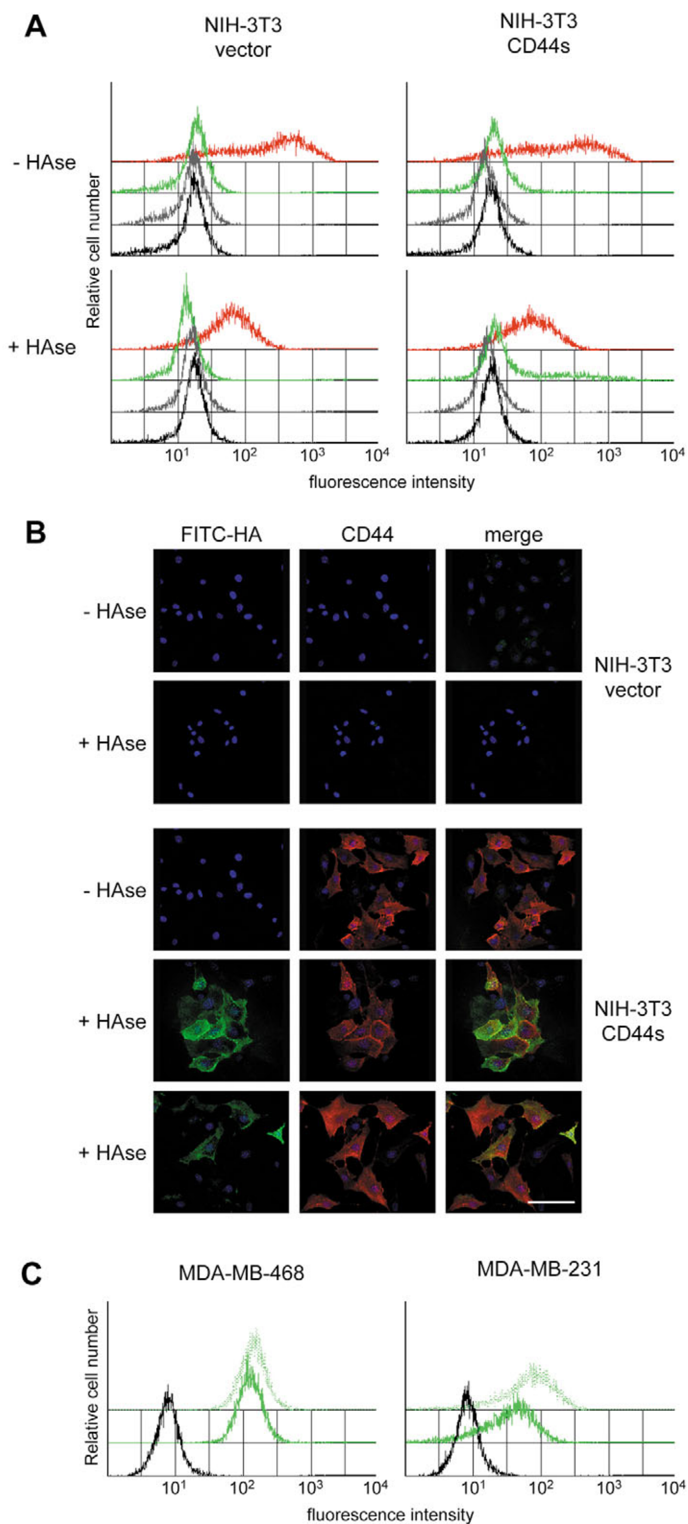

Fig. 7. Hyaluronan binding of human CD44s expressing NIH-3T3 cells after hyaluronidase treatment. (A) NIH-3T3 cells transfected with vector alone or human CD44s were detached from culture dishes, resuspended in DMEM plus 10% DCS and treated with *S. hyalurolyticus* hyaluronidase. After washing twice in PBS, cells were incubated either with FITC-hyaluronan (FITC-HA, green) or CD44-Fc (red). As controls, cells were also incubated with Endo180-Fc (grey) or medium alone (black). Fc chimaeras were detected with PE-anti-human Fc. (B) NIH-3T3 cells transfected with vector alone or human CD44s were cultured on coverslips overnight and then treated with type IV-S hyaluronidase. After washing, cells were stained with FITC-hyaluronan (green) and anti-human CD44 monoclonal antibody E1/2 (red) as described in Fig. 2. Nuclei were visualised with TO-PRO-3 (blue). To illustrate the variation in FITC-hyaluronan binding following hyaluronidase treatment, two separate fields of view are shown (lower two rows). (C) MDA-MB-468 and MDA-MB-231 cells were analysed by flow cytometry for FITC-HA binding following treatment with either medium alone (green) or treatment with *S. hyalurolyticus* hyaluronidase (dotted green). Black lines represent binding of secondary antibody alone. Bar, 100 μm.

matrix is a feature of other cell types. MDA-MB-468 cells, which constitutively bind high levels of hyaluronan (Fig. 2), showed essentially no increase in hyaluronan binding following hyaluronidase treatment (Fig. 7C). MDA-MB-231 cells which constitutively bind comparatively lower levels of hyaluronan exhibited a twofold increase in binding following hyaluronidase treatment although this binding did not reach the levels shown by the MDA-MB-468 cells (Fig. 7C).

Discussion

Directional migration of breast cancer cells towards hyaluronan is dependent on CD44

In this report, we establish that high molecular mass hyaluronan can act as a chemoattractant in a rigorous directional migration assay. In particular, we have demonstrated that human breast cancer cells will migrate directionally when placed in a gradient of either umbilical cord or streptococcal hyaluronan. Moreover, using siRNA oligonucleotides directed against CD44 we have demonstrated that this migration is dependent on CD44. This latter experiment also established two other important issues. First, the impairment of chemotaxis in cells treated with CD44 siRNA oligonucleotides demonstrates that the directional migration observed does not result from a pro-chemotactic contaminant in the hyaluronan preparations used. This is important given previous reports that responses ascribed to hyaluronan have been due to impurities in the preparations (Filion and Phillips, 2001). It should also be noted that although NIH-3T3 cells display a chemotactic response to serum they do not migrate directionally in a hyaluronan gradient, further supporting the assertion that the responses observed in the breast cancer cell lines are specific for hyaluronan. Second, as CD44 siRNA treatment did not impair chemotaxis towards a serum gradient (which contains a cocktail of chemotactic agents) it can be concluded that CD44 expression is not a general requirement for cell migration and gradient sensing, rather it elicits a ligand-specific response.

Like all the major classes of adhesion receptor, CD44 lacks intrinsic kinase activity, raising the question as to the mechanism by which it modulates cellular responses to a soluble ligand. Previous studies from our and other laboratories have shown that the cytoplasmic domain of CD44 can associate with members of the ERM (ezrin, radixin, moesin) family of actin binding linker proteins (Legg and Isacke, 1998; Morrison et al., 2001; Yonemura et al., 1998) and that this interaction is an important component of the chemotactic response (Legg et al., 2002). One mechanism that has been suggested to regulate the association of CD44 with the actin cytoskeleton is its

recruitment into lipid rafts (Foger et al., 2001; Oliferenko et al., 1999) and it has been reported that treatment of MDA-MB-231 cells with hyaluronan increased the proportion of detergent-insoluble CD44 in the cell (Bourguignon et al., 2004). To investigate whether such recruitment was occurring in our cells, we solubilised cells in cold detergent and fractionated the lysate by sucrose density centrifugation. Under these conditions, a substantial proportion of CD44 was detected in detergent-insoluble lipid rafts but this proportion did not change in cells previously incubated with hyaluronan. Although these data indicate that further recruitment of CD44 into rafts is not required for the chemotactic response, it does not preclude the hypothesis that a raft-associated population of CD44 is required for directional migration. In leukocytes, there is increasing evidence that the redistribution of specific membrane microdomains during cell migration is necessary for the establishment of cell polarity. This results from membrane rafts acting as a platform to assemble signalling complexes in a polarised fashion (Gomez-Mouton et al., 2004; Manes et al., 2003). Certainly, it will be important to assess whether hyaluronan does indeed cause a redistribution of raft-associated CD44 in polarised cells.

Cell type specificity of hyaluronan-dependent chemotaxis

Although we demonstrate here that CD44 expression is required for a cell to migrate towards a hyaluronan gradient, it is well accepted that not all CD44-positive cells are competent to bind hyaluronan (Lesley et al., 1993). To date, three major mechanisms have been reported which regulate the ability of CD44 to bind its ligand: the level of receptor expression (Perschl et al., 1995b; Uff et al., 1995), expression of particular alternatively spliced CD44 isoforms (Stamenkovic et al., 1991) and receptor glycosylation (Lesley et al., 1997). We show here that murine NIH-3T3 fibroblasts do not bind exogenously added hyaluronan and do not migrate directionally in a hyaluronan gradient. Moreover, as hyaluronan binding was also not observed in human CD44s-transfected NIH-3T3 cells, it can be concluded that the inability of these fibroblasts to bind ligand is not due to these cells expressing a non-binding or alternatively spliced CD44 isoform. What our data indicate is that binding of exogenously added hyaluronan to human CD44s-transfected NIH-3T3 cells is impaired by the presence of a hyaluronan containing pericellular matrix but that removal of the matrix does not restore efficient hyaluronan binding to all CD44s-expressing cells. This strongly suggests that binding of hyaluronan to these cells is regulated by additional mechanisms such as variable receptor glycosylation.

It was also observed in these experiments that NIH-3T3 cells transfected with vector alone had a hyaluronan coat, as evidenced by their ability to bind CD44-Fc, but were unable to bind FITC-hyaluronan following hyaluronidase treatment. This raises the issue as to how such a coat might persist if the CD44, or any other hyaluronan receptor, expressed on these cells cannot bind ligand. The most likely explanation for this apparent contradiction is that in these cells the hyaluronan matrix is not anchored to the cell surface by CD44. Hyaluronan is produced by hyaluronan synthases localized in the plasma membrane and it has been demonstrated that hyaluronan can be tethered to the cell surface by retention in the synthase, resulting in assembly of a CD44-independent pericellular matrix (Heldin and Pertoft, 1993; Spicer and McDonald, 1998). Finally, it is important to note that the presence of cell surface-associated hyaluronan does not necessarily result in a complete block of exogenous hyaluronan binding. In MDA-MB-231 cells, the presence of a pericellular matrix only results in a partial impairment of hyaluronan binding ability and untreated cells are able to migrate directionally up a hyaluronan gradient. It is therefore likely that mechanisms regulating hyaluronan binding, such as receptor modification and receptor blocking, are not mutually exclusive but may act in concert.

Hyaluronan-CD44 interactions in pathological states

The studies undertaken here have two important implications when considering the role of hyaluronan in tumour cell invasion. First, we demonstrate that high molecular mass hyaluronan can mediate tumour cell chemotaxis. This suggests that in addition to the well-established role for hyaluronan in promoting cell migration, there is a direct mechanism for promoting the infiltration of cells into the surrounding matrix particularly where large accumulations of hyaluronan occur in the tumour-associated stroma (Anttila et al., 2000; Auvinen et al., 2000). Second, in the breast cancer cell lines that showed a positive response to the gradient of high molecular mass hyaluronan, CD44 was demonstrated to be the sole hyaluronan receptor responsible for mediating these effects. However, as illustrated by the studies on NIH-3T3 fibroblasts, expression of CD44 per se is not sufficient to promote chemotaxis, rather the receptor must be competent to bind ligand. Here we demonstrate two mechanisms for modulating ligand binding in cells: levels of receptor expression and the presence of a hyaluronan pericellular matrix. Thus, we predict that multiple mechanisms will exist in an in vivo multicellular environment that regulate the ability of the cell to respond to a chemotactic gradient of hyaluronan.

The data presented initially may seem at variance with current trends to assign different cellular responses to hyaluronan of distinct sizes. In general, high molecular mass hyaluronan has been associated with structural roles (as a space-filling molecule, a component of the pericellular matrix and a scaffold for migrating cells) whereas it has been suggested that hyaluronan fragments are more biologically active with respect to stimulating signal transduction in cells. This stems, at least in part, from early demonstrations that hyaluronan oligosaccharides promote angiogenesis whereas high molecular mass hyaluronan does not (Rooney et al., 1995) and more recent reports that smaller hyaluronan fragments are more potent than higher molecular mass material in promoting gene transcription and random cell migration when added to cells in vitro (Sugahara et al., 2003; Turley et al., 2002). However, none of these studies rigorously assessed the role of hyaluronan in promoting cell chemotaxis and certainly high molecular mass hyaluronan can activate signalling events in a wide variety of cells. We propose that both specific effects of hyaluronan fragments and high molecular mass hyaluronan can act together in the complex multicellular environment in vivo as in pathological conditions, both hyaluronan production and the presence of hyaluronan fragments/oligosaccharides is increased. Given that hyaluronan oligosaccharides can displace

Journal of Cell Science

hyaluronan bound to the cell surface (Tammi et al., 1998), it is tempting to speculate that this may provide a mechanism by which cells non-responsive to hyaluronan switch to a responsive mode, activating signalling pathways leading to the chemotactic migration towards high molecular mass ligand.

We thank Daniel Zicha (Cancer Research UK, London Research Institute) for generously providing us with the Mathematica analysis notebook. We would also like to thank Justin Sturge for help with chemotaxis assays, Ian Titley for help with FACS sorting and Helen Yarwood and John MacFadyen for providing the Fc chimaeric proteins. This research was supported by the Association for International Cancer Research, Breakthrough Breast Cancer Research and the Biotechnology and Biological Sciences Research Council.

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