Histol Histopathol (2000) 15: 629-636
DOI: 10.14670/HH-15.629
http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Invited Review

Are hyaluronan receptors involved in three-dimensional cell migration?

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Summary. Hyaluronan (HA), an unbranched polysaccharide consisting of repeated glucuronic acid/N-acetylglucosamine disaccharide units, is ubiquitously present in the extracellular matrix of many tissues (for a more comprehensive review see: Fraser et al., 1997). Increased amounts of hyaluronan are produced by solid tumors and tumor-associated fibroblasts, and tumor-induced HA is correlated with poor prognosis. HA is well known to stimulate the migration of a large variety of cell types. Stimulation of cell migration by HA has been explained by different mechanisms. HA was shown to specifically bind to cell surface receptors, and inhibition of HA-receptor function was demonstrated to decrease cell migration and tumor growth. On the other hand, HA as a large hydrophilic molecule is also known to modulate the extracellular packing of collagen and fibrin, leading to increased fiber size and porosity of extracellular substrates. Hence a modified matrix architecture might similarly account for increased locomotion of cells. In this review, we attempted to summarize the available data on HA-induced cell migration, with particular emphasis on the role of HA receptors in three-dimensional cell migration. Although the HA receptor CD44 has been shown to mediate migration of cells over two-dimensional hyaluronan-coated surfaces in vitro, there is only little evidence that HA-binding to CD44 or other HA receptors has major impact on the locomotion of cells through three-dimensional matrices in vivo. We showed recently that the promigratory effect of HA in fibrin gels is largely due to HA-mediated modulation of fibrin polymerization. By increasing the porosity of fibrin gels, HA strongly accelerates cell migration. The porosity of matrices therefore appears as an important and probably underestimated determinant of cell migration and tumor spread.

Key words: Hyaluronan, Hyaluronan receptor, CD44, Migration, Matrix, Porosity, Fibrin, Tumor

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Hyaluronan receptors

CD44

The first cell surface receptor identified for hyaluronan (HA) was **CD44**, a broadly distributed glycoprotein which plays multiple roles in lymphocyte homing, cell activation, and cell migration (Underhill, 1992; Stamenkovic et al., 1991; Lesley et al., 1993; Sherman et al., 1994). The gene for CD44 encodes both a transmembrane domain and three HA binding regions, two in the extracellular domain and one in the cytoplasmic tail (Peach et al., 1993; Yang et al., 1994). Probably more than 20 **isoforms** can be generated by alternative splicing and by different patterns of N- and **O-glycosylation**, giving rise to CD44 proteins with molecular weights ranging from 85-230 **kD**. The standard isoform **CD44s** does not include any of the variant exons and is found ubiquitously expressed in many cell types. The CD44 isoforms containing variant exons **(CD44v)** show a more restricted expression, and, most interestingly, have been linked to metastasis and tumor progression (Gunthert et al., 1991; Herrlich et al., 1993; reviewed by **Günthert** et al., 1995). **CD44s** does not exclusively bind to HA but also to several other ligands including fibronectin (Jalkanen and Jalkanen, 1992), collagen type I and IV (Carter and Wayner, 1988), mucosal addressin, chondroitin sulfate (Aruffo et al., 1990; Stamenkovic, 1991) and fibrin (Svee et al., 1996).

There is ample evidence that CD44 mediates adhesion to and migration of cells over surfaces coated with hyaluronan in vitro (Thomas et al., 1992; Goebeler et al., 1996). It is also clear that inhibition of CD44 signaling by antibodies or soluble CD44-Ig fusion proteins inhibits cell migration and tumor growth in different experimental settings in vivo and in vitro (Gunthert et al., 1991; Sy et al., 1991; **Bartolazzi** et al., 1995; Koochekpour et al., 1995). However, it has not definitely been shown that HA binding is required for CD44-mediated cell migration under three-dimensional conditions and in complex matrices. Considering that CD44 interacts with a large number of ligands, it is not surprising that antibodies against **CD44**, for example,

block invasion of fibrin gels by fibroblasts (Svee et al., 1996). However, the role of HA binding to CD44 has been investigated in only a few studies.

What is the evidence that HA stimulates cell migration via CD44? The most convincing evidence with regard to this, as mentioned above, comes from two-dimensional in vitro studies investigating the migration of cells on HA-coated surfaces which can be inhibited by antibodies against CD44. However, matrices solely consisting of HA cannot be expected to occur in vivo. In living organisms, HA is found in complex matrices additionally containing a variety of matrix proteins as well as other glycosaminoglycans and proteoglycans (Toole et al., 1984; Weigel et al., 1986; Rooney and Kumar, 1993; Fraser et al., 1997). The frequently postulated concept that receptor-dependent cell adhesion to HA is responsible for accelerated migration also contrasts to observations showing that HA leads to anti-adhesion and detachment of a variety of cell types (Abatangelo et al., 1982; Tucker and Erickson, 1984; Brecht et al., 1986; Koochekpour et al., 1995; Delpech et al., 1997).

The hypothesis that direct interaction with HA is required for CD44-dependent tumor growth is supported by studies showing that growth of tumor cells which were stably transfected with deletion mutants in the hyaluronan binding domain of CD44 is impaired after injection in nude mice (Bartolazzi et al., 1994). However, there is no proof yet that better growth performance of HA-binding wild type cells is due to HA-induced cell migration. HA might have stimulated tumor growth by alternative mechanisms: by inducing CD44-dependent cytokine production or by affecting the proliferative rate of the cells. Since CD44 is known to be involved in cell-cell adhesion, it also appears debatable that CD44 mutants affect tumor growth by modulating intercellular adhesion (for review: Isacke, 1994).

There are rather conflicting results concerning the role of CD44 in tumor growth. Although some publications found that tumor growth is accelerated by a CD44-dependent mechanism (Sy et al., 1991; Bartolazzi et al., 1995), other authors found the reverse to be true. It was recently shown, for example, that the in situ growth of a highly aggressive prostate cancer cell line is impaired by stably transfecting the cells with CD44s (Gao et al., 1997, 1998) which led to an increase in the HA-binding capacity of the cells. The same effect, namely inhibition of tumor growth, was also observed for cells transfected with mutant CD44 unable to bind HA. These results suggest that metastasis suppression by CD44s involves ligands other than HA. It appears possible, therefore, that on a two-dimensional HA surface the net effect of CD44 may be adhesion and migration, whereas under complex three-dimensional conditions CD44 might bind with lower affinity to HA and with higher affinity to ligands other than HA, e.g. fibrin or collagen. Several authors found that HA surfaces lead to cell detachment (see references above) and it might be concluded that anti-adhesion accounts for acceleration of cell migration in three-dimensional matrices.

Meanwhile, further data have accumulated indicating that CD44 might even lead to inhibition of tumor growth. Downregulation rather than upregulation of CD44 was described for prostate carcinoma cells and squamous carcinoma cells during malignant transformation (Salmi et al., 1993; Kallakury et al., 1996; Gao et al., 1997). By overexpressing CD44s in colon carcinoma cells, reduced tumor formation was observed (Tanabe et al., 1995). For melanoma cell lines, it was shown that less aggressive and highly aggressive cells contain similar amounts of CD44s (Goebeler et al., 1996) although synthesis and turnover of CD44 was distinctly increased in the highly aggressive cell lines. Shedding of CD44 protein into the medium (Bazil and Horejsi, 1992; Bartolazzi et al., 1995; Günthert et al., 1996) was also strongly increased by more malignant cell lines (Goebeler et al., 1996).

The role of soluble CD44, which after shedding has a reduced molecular weight of about 65 kD, is unclear so far. Colon carcinoma cells were shown to detach from HA-coated surfaces by shedding of CD44 which was induced by CD95-mediated apoptosis (Günthert et al., 1996). It could be speculated therefore that shed CD44 might increase cell migration by fulfilling an anti-adhesive function: soluble CD44 might compete with membrane-bound CD44 for binding to HA or other ligands. This hypothesis would implicate that membrane-bound CD44 is inhibitory with respect to locomotion of cells, an idea which is supported by the studies cited above (Gao et al., 1998).

More recently, it was proposed that HA binding may be regulated by phosphorylation of the cytoplasmic domain of CD44. Serines 325 and 327 within the cytoplasmic domain were reported to be required for the constitutive phosphorylation of CD44 in T cells, and mutants containing a serine to glycine substitution at position 325 or serine to alanine substitution at position 327 were shown to be defective in HA binding (Puré et al., 1995). Other authors, in contrast, found that phosphorylation-defective mutants, inserted into murine fibroblasts or into a human melanoma cell line, were still capable of binding to immobilized HA (Peck and Isacke, 1996, 1998). However, although adhesion to HA-coated surfaces was not impaired, cells expressing CD44 mutants were severely inhibited to migrate over HA-coated surfaces. These findings suggest that phosphorylation of CD44 plays a pivotal role in CD44-mediated cell migration and, further, that CD44 phosphorylation is regulated by hitherto unknown intracellular components interacting with the CD44 cytoplasmic domain (Peck and Isacke, 1998). Some years back it was shown that the CD44 cytoplasmic domain binds to actin filaments, probably via ankyrin, and it was additionally demonstrated that ankyrin-binding to CD44 is crucial for HA binding (Lacy and Underhill, 1987; Lokeshwar et al., 1994). However, it is unknown so far whether ankyrin or other intracellular

molecules regulate CD44 phosphorylation. It appears possible that the phosphorylation-dephosphorylation cycle of the CD44 cytoplasmic domain triggers rapid changes of CD44 affinity to ligands other than HA and that such modulations may finally mediate cell migration. Since phosphorylation-defective mutants of CD44 were shown to be still capable of binding hyaluronan (Peck and Isacke, 1996, 1998), this interpretation would implicate that not HA but other ligands may be important for CD44-mediated cell migration.

Goebeler et al. (1996) demonstrated that migration of highly aggressive melanoma cell lines over HA-coated surfaces is faster as compared to less aggressive melanoma cells, with more malignant cells displaying higher levels of phosphorylated CD44. By considering the data discussed above, it appears possible that increased migration velocity is partially a consequence of increased CD44 phosphorylation (and CD44 shedding), but probably not of HA binding. Matrices constituted of other CD44 ligands, e.g. fibronectin or fibrin, quite likely would have disclosed similar differences in migration and CD44 phosphorylation patterns between aggressive and less aggressive melanoma cell lines.

IHABP (RHAMM) and ICAM-1

The HA binding protein named "receptor for hyaluronic acid mediated motility" (RHAMM) was originally identified by investigating the molecular basis of H-*ras*-promoted cell migration (Turley et al., 1991; Hardwick et al., 1992). By immunofluorescence labeling, RHAMM was found localized at the cell surface (Hardwick et al., 1992; Kornovski et al., 1994). Over a decade, many reports stated that HA-stimulated migration of a wide range of cell types (including fibroblasts, smooth muscle cells, astrocytes and sperm) is dependent on RHAMM (Turley et al., 1991, 1993, 1994; Hardwick et al., 1992; Kornovski et al., 1994; Savani et al., 1995). These findings were seriously challenged by recent investigations showing that murine and human RHAMM exclusively occurs intracellularly (Hofmann et al., 1998a,b; Assmann et al., 1998; Turley et al., 1998). It was therefore proposed that the name "RHAMM" should be substituted by "IHABP" for "intracellular hyaluronic acid binding protein". Provided that these corrections in "RHAMM-science" prove to be true, it appears unlikely that extracellular HA stimulates cell migration via direct binding to RHAMM/IHABP. However, it is still possible that HA reaches the cell interior, inducing migration by signaling cascades which include binding of intracellular HA to RHAMM/IHABP.

The third surface protein proposed as a receptor for HA was the intercellular adhesion molecule-1 (ICAM-1; McCourt et al., 1994). However, the same group recently reported nonspecific binding of ICAM-1 and HA to hexamethylene spacer arms (McCourt and Gustafson, 1997) which the authors used for affinity chromatography in their initial work. ICAM-1 was shown to bind via the hexamethylene linker (and not via HA) to the sepharose matrix and was elutable with HA oligosaccharides, leading to the erroneous conclusion that ICAM-1 is a receptor for hyaluronan.

Thus, in conclusion, of the three initially proposed molecules, CD44 apparently remains as the only known plasma membrane receptor for extracellular HA. However, it is also known that expression of CD44 is not sufficient for binding HA. There are several cell types known to express CD44 without having the ability to bind HA (Lesley et al., 1993), and even in those cells with CD44-mediated HA binding CD44 accounts only for a part of the total HA binding capacity. It therefore appears to be possible that more receptors for HA will be discovered in the future.

Receptor-independent membrane binding of HA

HA is a negatively-charged macromolecule which contains large hydrophobic patches (Scott, 1992). An important alternative mechanism to explain membrane binding of HA therefore is ionic interaction of HA with membrane lipids and/or proteins. Receptor-independent binding of HA to lipid bilayers could affect cell migration in several ways. First, HA might change membrane fluidity which probably is an important determinant of cell migration. In this regard, it appears interesting that HA has been shown to increase the flexibility of erythrocyte membranes (Clarke and Sirs, 1988). Second, by steric exclusion and/or ionic interactions, membrane-bound HA might modulate ligand binding of various cell surface receptors, e.g. integrins, other cell adhesion molecules or receptors for growth factors and cytokines. Third, extracellular HA might act as a scaffold for three-dimensional migration of cells. HA in physiological concentrations was shown to aggregate, forming supramolecular net-like structures (Scott, 1992), and at low pH, HA even forms elastic gels (Gibbs et al., 1968). However, since HA under physiological conditions presents as a fluid, it can be excluded that HA:HA interactions in the meshwork are sufficiently tight to resist cellular traction forces. It appears necessary therefore that HA first becomes immobilized by binding to other components of the extracellular matrix, and it appears at least doubtful that pure HA networks, without the participation of matrix proteins, can provide a matrix sufficiently stable to allow three-dimensional migration of cells. Other molecules, e.g. fibrin or collagen, most likely have to provide the scaffold, but HA may present as a "coat" on matrix fibers, and it appears possible that some cell types might predominantly interact with the coat (HA) and to a lesser extent with the scaffold proteins while migrating through tissues.

Hyaluronan-matrix interactions

HA is synthesized by hyaluronan synthases,

enzymes which are located at the inner side of plasma membranes (Weigel et al., 1997). The enzyme adds monosaccharide and disaccharide residues to the reducing, cytoplasmic ending of the polysaccharide while it protrudes through the plasma membrane (Prehm, 1989). The cDNAs for putative murine and human hyaluronan synthases were cloned recently (Itano and Kimata, 1996; Spicer et al., 1996; Shyjan et al., 1996; Watanabe and Yamaguchi, 1996). Increased production of HA appears to be a regular accompaniment of cell activation, and HA synthases were shown to be stimulated by a number of growth factors and cytokines, including PDGF, TGF-beta, interleukin-1 and many others (Laurent and Fraser, 1992).

HA as a hygroscopic and osmotically very active tissue expander was early assumed to promote cell migration by the creation of hydrous channels in the extracellular matrix (Tucker and Erickson, 1984; Laurent and Fraser, 1992; Rooney and Kumar, 1993). HA concentrations are highly elevated in inflamed tissues, in solid tumours and under almost all conditions which allow rapid migration of cells (Toole et al., 1979; Knudson et al., 1989; Waldenström et al., 1991; Delpech et al., 1997). The accumulation of HA in tumor tissue is correlated with poor prognosis (Zhang et al., 1995; Ropponen et al., 1998). HA in tumor tissue ranges from 50 to 800 μg/gr and in many cases is >250 μg/gr (Delpech et al., 1997). Especially high amounts of HA were found in invasive areas of tumours (Toole et al., 1979; Bertrand et al., 1992) which are simultaneously enriched in fibrin as a result of vascular leakage (Dvorak et al., 1992). The colocalization of these two major matrix components in areas of tumor expansion has focused many efforts on possible interactions. HA was shown to bind fibrinogen and to modulate fibrin polymerization, leading to thicker fibers and enhanced porosity of fibrin networks (LeBoeuf et al., 1986, 1987; Weigel et al., 1989). The mechanism which accounts for stimulation of fibrin polymerization by HA is most likely steric exclusion of macromolecules which thereby increases local concentrations of fibrin monomers. It is unkown so far whether HA-fibrin binding is important for stimulation of polymerization. The finding that other polysaccharides which physiologically do not occur in the organism, such as dextran, similarly increase fibrin polymerization (Dhall et al., 1976; Carr and Gabriel, 1980), apparently argues against this possibility.

We recently investigated the effect of an altered fibrin fiber architecture on cell migration. Migration of endothelial cells and glioma cells was drastically stimulated in gels consisting of thick fibrin fibers and large pores (Nehls and Herrmann, 1996; Hayen et al., 1999), as compared to compact fine fiber gels. The stimulation of three-dimensional cell migration in fibrin by HA was strongly correlated with the turbidity of the resulting gels, which is a measure of fiber size and pore diameter (Carr and Hermans, 1977; Nair et al., 1986; Blombäck et al., 1989). HA concentrations (1-30 μg/ml), which did not measurably affect the turbidity of the fibrin matrices, did not stimulate cell migration. We found that antibodies to the Hermes-1 epitope of CD44, which inhibited two-dimensional cell migration on HA matrices, did not decrease cell migration in complex three-dimensional substrates containing both fibrin and HA. Instead, migration in these gels was inhibited by antibodies to integrins, suggesting that adhesion to HA might be less important as compared to integrin contacts to fibrin fibers. We further realized that only the migration of cells *within* fibrin gels is stimulated by HA whereas surface migration is not different from fibrin gels polymerized in the absence of HA. These observations provided further credit to our hypothesis that the main effect of HA is to increase the pore width of fibrin gels, thereby reducing the need for cell-derived proteolytic activity. That the stimulatory effect of HA on cell migration requires modulation of fibrin polymerization was most convincingly demonstrated by our finding that HA had to be added to the fibrinogen solution prior to polymerization. HA remained ineffective when it was added to supernatants of already polymerized gels and, by further increasing the concentration of HA in supernatants, we even observed inhibition of cell migration (Hayen et al., 1999). This again strongly indicates a receptor-independent effect and underlines the importance of HA-mediated matrix remodeling.

It might be assumed that HA as a space-occupying macromolecule will not diffuse readily into collagen or fibrin gels. To exclude this possibility, we used fragments of HA or HA-oligosaccharides and observed that fragmented HA in higher concentrations (added to supernatants of ready polymerized gels) was even inhibitory for cell migration, probably due to anti-adhesion.

Other studies provided circumstantial evidence that HA also modulates the network structure of collagen gels, with consecutive increases in cell migration. By analysing the invasion of collagen gels by fibroblasts, Docherty et al. (1989) found that high concentrations of HA (1 mg/ml) were optimal to stimulate fibroblast invasion of collagen gels whereas lower concentrations were ineffective. Strong correlations were observed between cell migration and the collagen fiber structure. Collagen gels containing HA showed a more regular fiber structure and, at higher HA concentrations, more interfibrillar material. It was speculated by Docherty et al. (1989) that HA modulation of fibril packing might be important for cell migration. These data were supported later by results from Tsunenaga et al. (1992), showing that HA under certain conditions accelerates collagen fibrillogenesis. By injecting collagen/HA solutions into the dermis of rats, these authors showed that ingrowth of fibroblasts in collagen gels in vivo correlated with the rate of HA-induced fibril formation in vitro. Higher concentrations of HA, e.g. in the vitreous of the eye, were shown to prevent collagen fibrils from coalescing, thereby inhibiting the formation of thicker fibers and allowing the vitreous to remain transparent (Scott, 1990;

Rooney and Kumar, 1993). Turley et al. (1985) analysed the three-dimensional structure of arteficial extracellular matrices by electron microscopy and found that HA expands the volume of collagen gels and increases the space between collagen fibrils. In analogy to our results on fibrin fiber structure, we would expect that the process of expanding the interfiber space is decisive for cell migration.

HA binds with variable affinities to several other macromolecules of the extracellular matrix, including the proteoglycans aggrecan and versican, collagen, fibronectin, chondroitin sulfate proteoglycans and hyaluronectin (for review: Rooney and Kumar, 1993; Delpech et al., 1997; Fraser et al., 1997). The functional meaning of these associations in the context of cell migration, however, remains to become elucidated.

Conclusions

Different mechanisms have been proposed to explain stimulation of cell migration by HA. For decades, it was believed that HA expanded the interfiber space by its hygroscopic properties, providing hydrated channels for the locomotion of cells during embryogenesis, wound healing and tumor growth. In contrast to this rather old hypothesis, it appeared as a novel concept that HA accelerated migration by specifically binding to HA membrane receptors.

HA has repeatedly been shown to have profound anti-adhesive properties for a wide range of cell types, and it appears difficult to reconcile anti-adhesion with the hypothesis that cell migration follows receptor-dependent cell adhesion. During three-dimensional cell migration, the anti-adhesive effect of HA would lead to weakening of cell-matrix contacts and, since maximal migration requires cell-matrix contacts which are neither too strong nor too weak (Laufenburger and Horwitz, 1996), acceleration of migration.

Thus, we currently face two plausible and experimentally proven mechanisms to explain HA-induced migration. First, HA expands the interfiber space and thereby reduces the need for cell-derived proteolytic activity and, second, HA reduces the strength of cell attachment to substrates and thereby facilitates locomotion. None of these mechanisms requires binding of HA to membrane receptors. A third hypothetical mechanism, which also may deserve attention, is regulation of fiber strength by HA. The physical properties of extracellular matrices in general and the rigidity and strength of fibers in particular are important determinants of cell migration (Ingber and Folkman, 1989; McDonald, 1989). HA affects the fiber size of both fibrin and collagen and it appears likely therefore that cell migration also is regulated by HA-mediated modulation of fiber shape and stability. Fiber shape may result in different patterns of integrin clustering, and fiber stability is important to resist cellular traction forces during locomotion.

Clearly, there are also data indicating that HA receptors are involved in cell migration. The most convincing data were obtained so far for CD44 receptors in two-dimensional assays, but preliminary findings also indicate a role for CD44 in three-dimensional, HA-dependent migration. A major limitation of these studies lies in the fact that CD44 recognizes a variety of different macromolecules. Thus, theoretically, even by observing that cells are stimulated by HA to migrate through fibrin and, further, by observing that this effect is inhibited by antibodies to CD44, it would still be premature to conclude that the promigratory effect of HA was mediated by CD44. Bearing in mind that CD44 also recognizes fibrin as a ligand (Svee et al., 1996), among several others, an alternative explanation would be that HA increases migration by receptor-independent mechanisms, and that inhibition by anti-CD44 was a result of disrupting CD44-contacts to fibrin rather than to HA.

To definitely prove the participation of HA receptors in three-dimensional cell migration, future investigations should aim at avoiding matrix remodeling as a possible confounder, and the HA therefore should be added to supernatants of ready polymerized and crosslinked fibrin or collagen matrices. We would expect that under these conditions, depending on the matrix composition and the cell type under investigation, HA might even be inhibitory for cell migration. In case that HA stimulates locomotion of cells within these matrices, a possible anti-adhesion effect still has to be ruled out before involvement of HA receptors can be seriously considered.

Acknowledgements. We thank Dr. Matthias Goebeler for critically reading the manuscript and for helpful discussions.

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Accepted November 23, 1999