Hyaluronan, CD44 and its variant exons in human trophoblast invasion and placental angiogenesis

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Both hyaluronan and one of its receptors, CD44, can be demonstrated in the early human conceptus and in placental stroma. The variants of CD44 resulting from variable exon splicing are found in metastasizing human malignancies and are also involved in hyaluronan uptake and degradation. The resulting hyaluronan fragments are known to be highly angiogenic. We postulated that the self-limited process of trophoblast invasion of the uterine decidua results in part from the strategy of alternative splicing of CD44, similar to that used by invasive cancer cells in the course of metastatic spread and possibly angiogenesis. Monoclonal antibodies specific for CD44s and for an exon expressed during metastatic tumour progression, CD44v7-8, were used to examine this hypothesis. In this study we found human trophoblasts, for the first time, to express CD44. Intermediate trophoblasts of first and second trimester exhibited the standard form of CD44 while extravillous trophoblasts, which are responsible for the invading characteristics of the placenta, were positive for the alternatively spliced form, the CD44v7-8. Moreover, in the case of placenta accreta there was a prominent membrane staining of the trophoblasts that were embedded in the fibrin layer over the myometrium. The highly metastatic choriocarcinoma cells also expressed CD44v7-8. We propose, therefore, that the invading trophoblasts utilize the alternatively splicing machinery. These cells retain their invasive capabilities through the permissive ECM by carrying the CD44v7-8 isoform, which binds weakly to hyaluronan and thus prevents it from being degraded by intracellular hyaluronidase.

Key words: CD44/hyaluronan/invasion/placenta/trophoblast

Introduction

Implantation involves intimate interactions between the developing placenta and primed endometrium. The early human embryonic conceptus migrates through the predecidualized endometrium and penetrates the basement membrane underlying the luminal uterine epithelium to tap the maternal vessels. During this short and highly specific phase, the embryonic tissues are highly invasive as well as proliferative. This invasive potential is attributed to the ability of the trophoblast to penetrate the underlying stroma, once it has attached to the uterine epithelial surface. A number of mechanisms contribute to the subsequent loss of invasiveness, one of which is the remodelling of the extracellular matrix (ECM), with a shift from a permissive to a non-permissive state.

The process of ECM remodelling contributes simultaneously to the establishment of a functional placental unit. Both rodent and human embryos, dependent on the maternal environment for their continued development, utilize a haemochorial placenta, which allows direct contact between the invasive trophoblasts and maternal blood. This haemochorial structure is achieved by angiogenic activity in both the decidua and the chorionic villi. This process of angiogenesis comprises both endothelial sprouting and the creation of a new vascular meshwork. The presence of hyaluronan (HA) has been documented in association with this angiogenesis in uterine stroma, and up-regulation of HA deposition has been suggested to accompany the onset of angiogenesis (Brown and Papaioanou, 1995). It has also been established that HA fragments are intrinsically angiogenic, and highly mitogenic for endothelial cells.

HA, also known as hyaluronic acid, is a prominent component of the ECM, particularly in rapidly growing and remodelling tissues (Toole, 1991). HA has a strong negative charge, and the large volume of water of hydration associated with HA causes hydration and expansion of tissues, thus creating an environment which permits cell proliferation and migration. The transmembrane glycoprotein, CD44, is the predominant receptor for HA on the surface of cells (Underhill, 1992; Guenther, 1993; Lesley et al., 1993). CD44 exists in a short standard form, CD44s, and in a number of larger isoforms.

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containing additional exon inserts, all encoded by a single 19–20 exon gene (Jackson et al., 1992; Screaton et al., 1992) mapped, in the human, to the short arm of chromosome 11 (Goodfellow et al., 1982). These CD44 isoforms are implicated in a variety of cellular functions including lymphocyte activation and homing, cell motility, the migration of cells during wound healing, embryonic development, and in the metastatic spread of cancer cells (Sherman et al., 1994).

Our contention in these studies is that trophoblasts bear CD44, and that the invasive trophoblasts utilize the strategy found in invasive cancer cells, in that the conversion from the invasive to the non-invasive state might be reflected in their modulation of CD44 isotypes. Furthermore, we speculated that the pathology of normal conceptus implantation, as seen in partial and complete hydatidiform moles and in choriocarcinomas, might correlate with the persistent expression of invasive CD44 isotypes.

In this study, we examine the histological patterns of HA deposition during human embryo implantation and placental development, using an HA-specific biotinylated binding peptide, and of CD44 isotypes, using exon-specific monoclonal antibodies. The study includes normal specimens as well as a variety of placental pathologies, utilizing a spectrum of trophoblastic tissues. We examined first trimester and term placentae, as well as the implantation site following third trimester Caesarean hysterectomy in a case of placenta accreta. Other human trophoblastic tissues with a spectrum of invasive properties, ranging from partial to complete hydatidiform mole and choriocarcinoma, were also evaluated.

Materials and methods

Human tissues
Normal and abnormal trophoblastic tissues, as well as decidua, were obtained as surgical pathology specimens from the Department of Pathology, Hadassah University Hospital, Mt. Scopus, Israel. These samples underwent routine fixation in formalin, followed by processing and paraffin-embedding, and included first trimester, second trimester and term placental tissue, uterine wall with placenta accreta at 30 weeks gestation, partial hydatidiform mole, complete hydatidiform mole and choriocarcinoma. The latter sample included also fragments of proliferative endodermium.

Histological localization of HA
A biotinylated HA-binding peptide was utilized, derived from a trypsin digestion of bovine nasal cartilage and isolated by affinity chromatography using a column of HA-Sepharose (Tengblad, 1979). Slides were incubated in 3% normal goat serum for 30 min at 37°C, and then 0.3 ml of the diluted HA-binding peptide solution (1:200 of a 0.5 mg/ml preparation) was added and the samples were incubated overnight at 4°C. Slides were then washed for 10 min with phosphate-buffered saline-calcium-magnesium free (PBS-CMF). Endogenous peroxidase activity was blocked by incubating for 30 min at room temperature with 0.6% hydrogen peroxide in methanol. Slides were rinsed for 20 min in PBS-CMF, and then incubated for 45 min with the avidin-labelled horseradish peroxidase solution, prepared as specified by the manufacturer ( Vectastain ABC Peroxidase Kit PK-4000; Vector). After washing for 15 min in PBS-CMF, the slides were incubated for 5 min in the peroxidase substrate solution (Peroxidase Substrate Kit, DAB SK-4100, Vector), washed for 5 min in tap water, counterstained with haematoxylin, cleared and mounted with a coverslip. For controls, slides were preincubated following fixation, with 0.3 ml of a solution containing 0.1 M sodium acetate, pH 5.0, 0.05 M NaCl, 0.1 mg/ml bovine serum albumin (BSA), and 100 IU/ml Streptomyces hyaluronidase (CalBiochem, La Jolla, CA, USA), or the HA-binding peptide with preincubated with excess HA prior to application to the slide. Both controls gave comparable results.

Immunolocalization of CD44
Sections were cut from paraffin blocks and placed onto polysine-coated slides. Slides were deparaffinized first in xylene, followed by graded alcohol solutions. A microwave step was then used to enhance staining intensity. Slides were dipped in 10 ml prewarmed citrate buffer (pH 6.0) for 15 min, and then microwaved in a conventional domestic microwave oven at 50–60% of full power for 5 min. The slides were cooled, rinsed in distilled water, and then in PBS-CMF. To block non-specific binding sites, sections were incubated with 3% normal goat serum (Vector) in PBS-CMF for 30 min at 37°C. They were then incubated with the primary monoclonal antibodies [BM-CD44-UN (BMS 113) for the standard form, antiCD44s, and BM-CD44-V7 (BMS 117) for antiCD44v7 containing the additional exon inserts; BioSource International] at a dilution of 1:30 in 3% goat serum in PBS-CMF for 1 h at 37°C, and then overnight at 4°C. Slides, washed for 10 min in PBS-CMF, were then incubated with biotinylated goat, anti-mouse immunoglobulin (Ig)G (Vector) diluted 1:100 with 3% normal goat serum in PBS-CMF for 45 min at room temperature. Slides were again washed as above. Endogenous peroxidase activity was blocked as above, by incubating for 30 min at room temperature with 0.6% hydrogen peroxide in methanol. Slides were incubated with avidin-labelled horseradish peroxidase solution and peroxidase substrate solution and processed as described above. For negative controls for CD44v7, mouse IgGl from BioSource International was utilized at a dilution of 1:30 in 3% normal goat serum in PBS-CMF. Dilutions were used that provided comparable final protein concentrations.

Photography
Photographs were taken on an Olympus Vanox AHBT3 Microscope fitted with an integrated Olympus C-35AD-4 camera using Kodak Gold Plus 100 film.

Results

Placenta
Deposition of HA was confined to the villous stroma and was entirely absent in the varieties of trophoblast cell types. The stromal staining was uniform, and was most prominent in the mesenchymal villi of the first trimester placenta (Figure 1A). Staining was less intense in the large immature intermediate villi, where it was concentrated primarily around central blood vessels and under the trophoblast layer (Figure 1B). The intensity of HA staining became progressively weaker in second and third trimester placentae. In the second trimester, it was more pronounced in the smaller mesenchymal villi than in the immature intermediate villi (Figure 1C). In stem villi, staining was evident in the stroma, but was more concentrated around and in the walls of blood vessels (Figure 1D).

Staining for CD44s in the placenta was generally faint throughout, but that little which was present occurred in the villous stroma, most prominently in the immature intermediate...
HA and CD44 in human implantation

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Figure 1. Hyaluronan (HA) in normal placenta. (A) Staining for HA in the first trimester placenta is most prominent in the mesenchymal villi (brown colour) (original magnification ×1000). (B) Staining for HA in the large immature intermediate villi is concentrated primarily around central blood vessels, and in the stroma adjacent to the trophoblast layer (original magnification ×1000). (C) Staining for HA in the second trimester of pregnancy is more pronounced in the smaller mesenchymal villi than in the immature intermediate villi (original magnification ×500). (D) Staining for HA in stem villi is localized in the stroma, and more concentrated around and in the wall of blood vessels (original magnification ×250).

villi of the second trimester (Figure 2A). There were occasional prominent foci of staining in small groups of intermediate trophoblasts associated with villi (Figure 2B), but not in extravillous trophoblasts at the basal plate (Figure 2C). Staining for CD44v7–8 also was faint, in general, and was observed predominantly in syncytiotrophoblasts of first and second trimester placentae (Figure 2D). Faint staining was also observed in the extravillous trophoblasts of cell islands (Figure 2D and 2E). The trophoblasts of the cell columns were essentially negative, with only small foci of positive staining (not shown). In the term placenta, weak staining was also evident in blood vessels of stem villi (not shown). Only focal membrane staining was observed in extravillous trophoblasts of cell islands.

Placenta accreta

A placenta accreta from a 30 week gestational conceptus was examined. The HA staining within the placenta was more pronounced in the stroma of the larger intermediate villi and in the stem villi, comparable to the pattern observed in normal placenta. Remarkably intense staining was also evident in the stroma of villi embedded in the fibrin layer over the myometrium (Figure 3A). Staining for CD44s revealed a difference in the pattern of the villi in the major part of the placenta, where staining was localized predominantly in the villous stroma and in villi embedded in Nitbauch’s fibrin layer. In the latter, staining occurred in trophoblasts rather than in the villous stroma (Figure 3B). There was prominent staining for CD44v7–8 in the trophoblastic layer of the villi (Figure 3C) as well as membrane staining of the trophoblasts surrounding the villi that were embedded in the fibrin layer over the myometrium (Figure 3D).

Trophoblastic disorders

There was prominent staining for HA observed in the small mesenchymal and hydropic villi of a partial hydatidiform mole from the second trimester of pregnancy (Figure 4A). In the case of a complete hydatidiform mole from the first trimester, the staining pattern for HA was similar to that in normal first trimester placenta, and confined to the villous stroma. However, the staining was of much greater intensity in the complete hydatidiform mole (data not shown). The choriocarcinoma was essentially devoid of HA (Figure 4B).

There was weak stromal staining for CD44s in the second
trimester partial hydatidiform mole, with more pronounced staining occurring in the trophoblast layer surrounding villi, as well as in extravillous trophoblasts (Figure 4C). In the complete hydatidiform mole, staining was least pronounced in the clusters of proliferating trophoblasts of the kind typically observed in a complete hydatidiform mole (Figure 4D). Only weak staining was observed in the choriocarcinoma (Figure 4E).

Prominent staining for CD44v7–8 was observed in the cytotrophoblasts of a partial hydatidiform mole (Figure 4F). In the complete hydatidiform mole, staining patterns for CD44s (Figure 4D) and CD44v7–8 (Figure 4G) resembled each other. Prominent staining was also found in choriocarcinoma cells (Figure 4H).

Endometrium and myometrium

There was prominent staining for HA in the endometrial stroma during pregnancy, more intense in the spongy decidua than in the compact decidua (Figure 5A). There was staining in the endometrial stroma in the proliferative phase, and also in a few nuclei of glandular cells of the basal layer (Figure 5B). The myometrium was negative, but staining was observed in the fibrous tissue between muscle bundles and surrounding blood vessels (Figure 5C).

During pregnancy, prominent staining for CD44s occurred in the stroma of the decidua, and was particularly evident in the spongy decidua (Figure 5D). There was only weak staining of the stroma of the basal layer, in contrast with the pronounced nuclear staining of the basal endometrial glands (Figure 5E). Myometrial muscle bundles also stained positively, while the associated fibrous tissues were negative (Figure 5E). The staining for CD44v7–8 was prominent in the glandular epithelium of the proliferative endometrium (Figure 5F) and to a lesser extent in the decidua (Figure 5G). Weaker staining of the endometrium was evident.

Discussion

At implantation, the previously non-adhesive apical surface of the trophoectoderm becomes adhesive. By analogy with
lymphocyte extravasation, carbohydrate–lectin interactions might mediate the initial adhesion of the blastocyst, which then becomes stabilized by the binding of integrins to their ECM ligands (Burrows et al., 1995). Uterine glycosaminoglycans (GAGs) such as HA may also participate in the process of adhesion, since it has been observed that blastocysts in culture attach and spread on HA-coated dishes (Carson et al., 1987). In mice, attachment of the blastocyst to the receptive luminal epithelium triggers antimesometrial clearing of HA from the primary decidual zone of the underlying stroma (Brown and Papaioanou, 1992; 1995). This clearing process of the HA is part of an intrinsic maternal program of the
differentiating decidua, and is not dependent on embryo-specific signals. It is postulated therefore, that clearing of HA from the decidualizing antimesometrial stroma serves to restrict trophoblast invasion, by reducing the hydration of the matrix. This compaction permits closer cell–cell apposition, increased signalling between cells, and enhanced adhesive interactions between adjacent decidual cells.

CD44 is involved in receptor-mediated endocytosis of HA prior to its lysosomal degradation (Culty et al., 1992; Hua et al., 1993; Pavesant et al., 1994). The invading trophoblast was postulated to bear CD44 receptor molecules, based on the patterns of HA removal observed in early studies. However, more recent work from other laboratories failed to find CD44 expression in either mouse or human trophoblasts (St. Jacques et al., 1993, 1994; Behzad et al., 1994; Brown and Papaioanou, 1995; Campbell et al., 1995). In mice, it was concluded that CD44 cannot be involved in receptor-mediated endocytosis of HA during the process of decidualization, or in promoting the in-vitro outgrowth of trophoblasts that occur on HA-coated substrata (Brown and Papaioanou, 1995). Employing confocal microscopy and immunofluorescence histolocalization using CD44-specific monoclonal antibodies, examination of preimplanted human embryos demonstrated CD44 decorating the surface of trophoectoderm (Campbell et al., 1995). On the other hand, staining of first trimester human placentae demonstrated that the antibodies did not react with either cyto- or syncytiotrophoblasts (Campbell et al., 1995). It was proposed, therefore, that the presence of CD44 on the blastocyst and its subsequent loss from the trophoblast in the first trimester implied that the molecule was involved in the invasive phase of implantation, and thereafter became down-regulated.

In contrast with the clearing of HA in the antimesometrial stroma, substantial amounts of HA are retained in the angiogenic regions of the mesometrial decidua basalis (Brown and Papaioanou, 1992; Fenderson et al., 1993). Uterine CD44 expression is observed in decidual cells (Behzad et al., 1994) and in lymphocytes (Slukvin et al., 1994) of the decidua basalis, as well as in glandular cells throughout the endometrial stroma (Behzad et al., 1994; Yaegashi et al., 1995). These two components, the glandular epithelium and the decidual cells, are known to participate in active placental angiogenesis (Fenderson et al., 1993; Brown and Papaioanou, 1995). CD44 expression in glandular epithelium may be involved in the regulation of angiogenesis through growth factors (Ruoslahti and Yamaguchi, 1991) or cytokine release, or by the action of hyaluronidase. The adjacent stroma is rich in HA and the activity of such hyaluronidase may be involved in HA cleavage reactions that generate HA fragments of an appropriate size. High molecular weight HA is antiangiogenic (Feinberg and Beebe, 1983), while hyaluronidase cleavage products are highly angiogenic (West et al., 1985; West and Kumar, 1989). It is suggested, therefore, that HA and its catabolism by hyaluronidase plays a key role in the process of angiogenesis during implantation. In this model, stromal cells maintain a highly hydrated HA-rich matrix that prevents cell–cell adhesive interactions, by binding HA to cell surfaces using the CD44 receptors. Invading endothelial cells are then able to penetrate the large intercellular spaces, by degrading HA through the hyaluronidase reactions. The CD44 receptor may be involved in receptor-mediated endocytosis of HA, for delivery to the lysosomal compartment for degradation, as has been suggested (Culty et al., 1992; Hua et al., 1993; Pavesant et al., 1994).

The resulting endothelial–stromal cell interactions can mediate the cytoskeletal processing that endows the cell with motility, as well as stimulate signal transduction.

In this study we showed positive CD44 staining in human trophoblasts for the first time. Intermediate trophoblasts of first and second trimester exhibited the standard form of CD44 while extravillous trophoblasts, which are responsible for the invading characteristics of the placenta, were positive for the alternatively spliced form, the CD44v7–8. Moreover, in the case of placenta accreta, there was a prominent membrane staining of the trophoblasts that were embedded in the fibrin layer over the myometrium. The highly metastatic choriocarcinoma cells also expressed CD44v7–8.

A number of factors may be invoked for the lack of CD44 staining reported by other investigators. We routinely performed a microwave step. This provided an enhanced signal in each of our staining reactions. The particular antibodies used may provide an additional explanation. We observed that not all commercial preparations of monoclonal antibodies directed against CD44 and its exon variants gave positive staining reactions in formalin-fixed tissue. In our hands the antibodies received from BioSource were very reliable, while those purchased from Becton-Dickinson, Franklin Lakes, NJ, USA gave more variable results. Moreover, these latter antibodies are supplied with non-specific mouse IgG antibodies only as positive control, while those from BioSource have specific subtypes as controls. In screening a large number of preparations from a variety of companies, we observed that some gave no reaction at all in formalin-fixed tissues. The reagents described herein gave optimal results under our conditions, with routine specimens from surgical pathology archives.

In their review, Brown and Papaioanou (1995) failed to detect any trophoblastic signal for a CD44 isoform-conserved amino-terminal epitope in mice. They therefore concluded, that CD44 is involved in neither receptor-mediated endocytosis of HA during decidualization, nor in promoting the in-vitro outgrowth of trophoblasts on HA-coated substrata. Therefore, the puzzle documenting on the one hand clearing of HA from the antimesometrial stroma and on the other hand its retention in the angiogenic region of the decidua basalis, remains unsolved. It was recently shown that CD44 differential splicing provides a regulatory mechanism for HA binding (Bennett et al., 1995). Using melanoma cell transfectants, the authors showed a significantly better binding of the standard form to HA, when compared with the isoform containing the variably spliced exons v8–10.

Our first demonstration of CD44 expressed by trophoblasts from different gestational ages seems to supply the missing link in the model proposed by Brown and Papaioanou (1995). We propose that the invading trophoblasts found in the above described specimens of normal placentas, placenta accreta and choriocarcinoma utilize the alternatively splicing machinery. These cells retain their invasive capabilities through the permissive ECM by carrying the CD44v7–8 isoform, which binds
weakly to HA and thus prevents the latter from being degraded by intracellular hyaluronidase.

References


Received on May 13, 1996; accepted on July 24, 1996