Published in final edited form as:

Drug Resist Updat. 2008 June ; 11(3): 110–121. doi:10.1016/j.drug.2008.04.002.

Hyaluronan, CD44 and Emmprin: Partners in cancer cell chemoresistance

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Abstract

Hyaluronan is not only an important structural component of extracellular matrices but also interacts with cells during dynamic cell processes such as occur in cancer. Consequently, interactions of hyaluronan with tumor cells play important cooperative roles in various aspects of malignancy. Hyaluronan binds to several cell surface receptors, including CD44, thus leading to co-regulation of signaling pathways that are important in regulation of multidrug resistance to anticancer drugs, in particular anti-apoptotic pathways induced by activation of receptor tyrosine kinases. Emmprin, a cell surface glycoprotein of the Ig superfamily, stimulates hyaluronan production and downstream signaling consequences. Emmprin and CD44 also interact with various multidrug transporters of the ABC family and monocarboxylate transporters associated with resistance to cancer therapies. Moreover, hyaluronan-CD44 interactions are critical to these properties in the highly malignant, chemotherapy-resistant cancer stem-like cells. Perturbations of the hyaluronan-CD44 interaction at the plasma membrane by various antagonists result in attenuation of receptor tyrosine kinase and transporter activities and inhibition of tumor progression in vivo. These antagonists, especially small hyaluronan oligomers, may be useful in therapeutic strategies aimed at preventing tumor refractoriness or recurrence due to drug-resistant sub-populations within malignant cancers.

Keywords

hyaluronan; CD44; emmprin; CD147; cancer stem cells; multidrug resistance; receptor tyrosine kinases; ABC-family drug transporters; monocarboxylate transporters

1. Introduction

A close association between the extracellular polysaccharide, hyaluronan, and malignant tumor progression has been known for some time, where hyaluronan concentrations are usually higher in malignant tumors than in corresponding benign or normal tissues (Knudson et al., 1989; Toole et al., 2002). Several studies have reported enrichment of hyaluronan in the stroma that surrounds tumors (Bertrand et al., 1992; Koyama et al., 2007; Toole et al., 1979), and other studies have shown that hyaluronan production by stromal cells is stimulated by interactions with tumor cells (Asplund et al., 1993; Edward et al., 2005; Knudson et al., 1984). However,

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hyaluronan synthesis is also increased in malignant tumor cells themselves (Calabro et al., 2002; Kimata et al., 1983; Zhang et al., 1995).

Hyaluronan is distributed ubiquitously in adult vertebrate tissues where it clearly plays a structural role that depends on its unique hydrodynamic properties and its interactions with other extracellular matrix components. However, hyaluronan is also concentrated in regions of high cell division and invasion, e.g. during embryonic morphogenesis, inflammation and wound repair, as well as in cancer. In these contexts, hyaluronan has an instructive, cell signaling function in addition to its structural role (Jiang et al., 2007; Slevin et al., 2007; Toole, 2001; Toole, 2004; Turley et al., 2002). Though hyaluronan signals through interaction with several cell surface receptors, CD44 is the best characterized of these receptors in cancer cells. Variant forms of CD44 are commonly up-regulated in cancers and CD44 has been implicated in numerous aspects of cancer progression (Hill et al., 2006a; Liu and Jiang, 2006; Marhaba and Zoller, 2004; Slevin et al., 2007).

Emmprin (Extracellular Matrix Metalloproteinase Inducer; CD147; basigin), which was originally characterized as an inducer of matrix metalloproteinase synthesis (Biswas et al., 1995), is a multifunctional glycoprotein that is also up-regulated on the surface of many types of cancer cells (Yan et al., 2005). Emmprin has now been shown to stimulate hyaluronan production and many of its signaling effects (Toole, 2004). In addition, we have recently shown that emmprin co-localizes with CD44 in a variety of carcinoma cell lines (unpublished results).

Resistance of cancers to multiple classes of chemotherapeutic agents, i.e. multi-drug resistance, can arise in numerous ways, e.g. by decreased uptake of drugs due to cell and tissue barriers, activation of repair and detoxification mechanisms, altered metabolic phenotype, increased activities of cell survival/anti-apoptotic signaling pathways, or enhanced drug efflux via cell membrane transporters of the ATP-binding cassette (ABC) family (Cheng et al., 2005; Dai and Grant, 2007; Gottesman et al., 2002; Assaraf, 2006; Landis-Piwowar et al., 2006; Li and Dalton, 2006; Moretti et al., 2007; Broxterman and Georgopadakou, 2007; Tredan et al., 2007). A relatively new paradigm with respect to solid tumors is the likely contribution of cancer stem-like cells to chemoresistance. Like normal hematopoietic and other adult stem cells, cancer stem-like cells are enriched in ABC-family drug transporters. As these cells may be responsible for resistance of cancer to various therapies and for recurrence after treatment, their unique properties may comprise a novel therapeutic target (Ailles and Weissman, 2007; Dean et al., 2005; Fojo, 2007; Neuzil et al., 2007; Raaijmakers, 2007).

In recent years, inter-related activities of hyaluronan, CD44 and emmprin have been shown to influence drug resistance at several of these different levels, i.e., through cell survival signaling pathways, drug transporter expression and activity, glycolytic phenotype, and cancer stem-like cell characteristics. Their contributions to multidrug resistance are examined in this review.

2. Hyaluronan, CD44 and emmprin

Hyaluronan is a very large, linear glycosaminoglycan composed of 2,000–25,000 disaccharides of glucuronic acid and N-acetylglucosamine: $[\beta 1,4\text{-GlcUA-}\beta 1,3\text{-GlcNAc-}]_n$, with molecular weights usually ranging from 10^5 to 10^7 Dalton. In addition to its structural role, which is dependent on its unique hydrodynamic properties and its interactions with other extracellular matrix components, hyaluronan has an instructive role in signaling via receptors on the cell surface. These receptors include CD44, Rhamm, TLR1/4, Hare and LYVE-1 (Jiang et al., 2007; Slevin et al., 2007; Toole, 2004; Turley et al., 2002). CD44, the most widely studied hyaluronan receptor, exists in numerous spliced forms due to insertion of several exon products into a single position in its ectodomain (Fig. 1). The standard form, which has none of these additional spliced-in exon products, is the most widely distributed in normal tissues. However, so-called variant forms are expressed by several epithelia and are widely expressed in cancers.

Hyaluronan-receptor interactions mediate at least three important physiological processes, i.e. signal transduction, receptor-mediated hyaluronan internalization, and assembly of pericellular matrices (Knudson et al., 2002; Toole, 2001). Each of these general functions is most likely shared by more than one receptor. For example, CD44 and Rhamm can mediate many aspects of hyaluronan-induced signal transduction (Turley et al., 2002). It is not yet clear which specific signaling functions overlap, although interchangeable CD44 and Rhamm signaling has been demonstrated in some systems (Naor et al., 2007). Also, CD44 and Rhamm exhibit cooperative effects in that Rhamm-CD44 interaction can activate hyaluronan-induced signaling (Hamilton et al., 2007; Maxwell et al., 2008). Although underlying regulatory mechanisms are not well understood, it is clear that hyaluronan-induced signaling is activated during dynamic cell processes, such as occur in cancer, and considerable experimental evidence implicating hyaluronan in tumor progression has now been obtained in cell and animal models. Several approaches have been used, including manipulation of levels of hyaluronan production and perturbation of endogenous hyaluronan-protein interactions (Stern, 2005; Toole, 2004).

In our own studies, we have focused on manipulating constitutive hyaluronan-tumor cell interactions. To inhibit these interactions we commonly use treatment with small hyaluronan oligosaccharides (oligomers), experimental expression of soluble hyaluronan-binding proteins (HABPs), or transfection with siRNA against CD44 (Fig. 2). Soluble hyaluronan-binding proteins competitively displace hyaluronan from its endogenous cell surface receptors. Hyaluronan oligomers compete for endogenous polymeric hyaluronan, thus replacing high affinity, multivalent and cooperative interactions with low affinity, low valency receptor interactions (Lesley et al., 2000; Underhill et al., 1983); oligomers containing 6–18 sugar residues are monovalent in their interaction with CD44, whereas larger polymers are multivalent (Lesley et al., 2000). These oligomers also inhibit hyaluronan production (Misra et al., 2006). Over-expression of soluble CD44 in mouse mammary carcinoma cells or in human melanoma cells has been shown to induce apoptosis or cell cycle arrest *in vivo* and thus inhibit growth, local invasion and metastasis (Ahrens et al., 2001; Peterson et al., 2000; Yu et al., 1997). No significant effects were obtained in these studies if the soluble CD44 was mutated such that hyaluronan binding was reduced. In addition, we have found that treatment with small hyaluronan oligomers retards growth of several tumor types *in vivo* (Ghatak et al., 2002; Zeng et al., 1998). In our most recent studies, we showed that these oligomers induce apoptosis of glioma cells and glioma stem-like cells *in vivo*, leading to inhibition of growth and invasion (Gill et al., 2008).

Emmprin is an integral plasma membrane glycoprotein and member of the Ig superfamily. It is widespread in normal tissues but is highly up-regulated in cancer cells (Yan et al., 2005). Emmprin was originally identified as a factor on the surface of tumor cells that induces matrix metalloproteinase production in fibroblasts and endothelial cells (Biswas et al., 1995; Guo et al., 1997; Tang et al., 2005). It has now been shown that increased production of emmprin also stimulates matrix metalloproteinase production and increased invasiveness in tumor cells (Sun and Hemler, 2001; Yang et al., 2003; Zucker et al., 2001). However, *in vivo*, experimental overexpression of emmprin causes markedly enhanced tumor growth as well as invasion (Zucker et al., 2001).

Other studies in our laboratory have revealed that emmprin stimulates hyaluronan production (Marieb et al., 2004) and co-localizes with CD44 (unpublished results) in a variety of cancer cells. Consequently, many of the downstream effects of increased hyaluronan production are also induced by increased emmprin expression (Ghatak et al., 2005; Marieb et al., 2004; Misra et al., 2003). It is now clear that emmprin interacts with a wide range of binding partners and consequently is multifunctional (Yan et al., 2005). For example, emmprin interacts functionally with three of the monocarboxylate transporters (MCTs), namely MCT1, MCT3 and MCT4 (Kirk et al., 2000; Philp et al., 2003; Wilson et al., 2005; Wilson et al., 2002); a close relative

of emmprin, embigin, is required for MCT2 activity (Wilson et al., 2005). The MCTs transport lactate across the plasma membrane and emmprin-MCT interaction is required for trafficking to and activity of MCTs at the plasma membrane in muscle and retinal cells (Kirk et al., 2000; Philp et al., 2003; Wilson et al., 2005; Wilson et al., 2002). MCT activity is also fundamental to the “Warburg effect” (Warburg, 1956), a glycolytic phenotype that is common to most malignant cancers and associated with resistance to therapies (Gatenby and Gillies, 2004; Koukourakis et al., 2006; Tredan et al., 2007). MCT-empirin interactions have been demonstrated in human breast carcinoma cells (Gallagher et al., 2007; Xu and Hemler, 2005).

3. Promotion of drug resistance by hyaluronan, CD44 and emmprin

The possibility that hyaluronan might influence drug resistance was suggested by earlier findings that hyaluronidase treatment enhances the action of chemotherapeutic agents *in vivo* (Baumgartner et al., 1998), and that hyaluronidase-induced dispersion of drug-resistant, multicellular, tumor cell spheroids reverses their drug resistance (Kerbel et al., 1996; St Croix et al., 1998). The mechanistic action of hyaluronidase on drug resistance was explained in terms of possible effects on cell adhesion barriers (Kerbel et al., 1996) or drug penetration (Baumgartner et al., 1998; Desoize and Jardillier, 2000) rather than hyaluronan-specific effects on signaling pathways. Early studies by our laboratory showed that calcium-independent aggregation of transformed cells can be due to hyaluronan-mediated, multivalent cross-bridging of receptors on adjacent cells (Underhill and Toole, 1981). This observation and the finding that hyaluronan-receptor interactions regulate cell survival signaling pathways known to be important in drug resistance led our group and others to further investigate the possible role of hyaluronan in multi-drug resistance.

Employing a drug-resistant human carcinoma cell line, we demonstrated that disruption of endogenous hyaluronan-induced signaling by treatment with small hyaluronan oligomers suppresses resistance to several anticancer drugs, including doxorubicin, taxol, vincristine, and methotrexate (Misra et al., 2003). Other antagonists of hyaluronan-CD44 signaling had similar effects (Misra et al., 2005). It should be noted that the resistant cell line used in these studies was the MCF-7/Adr human breast cancer cell. However, it has now been shown that this cell line is actually a drug-resistant ovarian carcinoma line, specifically OVCAR-8 (Liscovitch and Ravid, 2007). In addition, we showed that increased hyaluronan production, induced by over-expression of a hyaluronan synthase, caused increased drug resistance in the relatively chemosensitive MCF-7 breast cancer cell line. This increased resistance in the MCF-7 cells was reversed by treatment with hyaluronan oligomers or other antagonists of hyaluronan-CD44 signaling (Misra et al., 2005; Misra et al., 2003). Studies from other laboratories have shown that hyaluronan promotes resistance to cisplatin, methotrexate, doxorubicin and etoposide in head and neck squamous carcinoma cells (Wang and Bourguignon, 2006; Wang et al., 2007), to cisplatin in non-small cell lung cancer cells (Ohashi et al., 2007), and to vincristine in lymphoma cells (Cordo Russo et al., 2008).

In all of the studies described in the previous paragraph, the effects of hyaluronan were CD44-dependent. CD44 is widely expressed on non-transformed and transformed cells but, in many malignant cancers, expression of splice variants of CD44 is induced or increased. Numerous studies have documented the prevalence as well as diagnostic value of CD44 variant isoforms in human cancer, including the expression of alternatively spliced combinations of the v3, v6, and v9 isoforms (Gunthert et al., 1991; Gunthert et al., 1995; Stauder et al., 1996). In addition, it has been demonstrated in pancreatic carcinoma that the CD44v6 isoform can confer metastatic behavior (Gunthert et al., 1991). Moreover, CD44 variant isoforms regulate Ras signaling and consequently induce cell proliferation and invasiveness (Cheng and Sharp, 2006; Cheng et al., 2006). Expression of CD44 splice variants may also play a role in drug

resistance. Antibody-directed activation of variant CD44 in colon carcinoma cell lines has been shown to cause resistance to the drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), whereas identical treatment of a carcinoma line expressing the standard, non-variant CD44 isoform had no such effect (Bates et al., 2001). In line with this finding, transfection of colon carcinoma cells containing low levels of CD44 with the v3–10-containing isoform of CD44 conferred resistance to apoptosis induced by etoposide, whereas transfection with standard CD44 had less pronounced effects (Lakshman et al., 2004).

Hyaluronan has also been found to act as a survival factor against dexamethasone-induced apoptosis in multiple myeloma cells, an effect which may lead to resistance to conventional chemotherapy and accumulation in the bone marrow. However, in this case, hyaluronan acts by concentrating cytokines in the pericellular milieu in a CD44-independent manner (Vincent et al., 2001; Vincent et al., 2003).

As stated above, emmprin induces hyaluronan production. One consequence of this is that increased emmprin expression causes increased drug resistance in a hyaluronan-dependent fashion (Misra et al., 2003). This finding provides an explanation for the positive correlation between drug resistance and emmprin expression in malignant cancer cells (Yang et al., 2003).

4. Regulation of cell survival signaling pathways by hyaluronan, CD44 and emmprin

Elevation of the levels of cell survival/anti-apoptotic pathways, a common occurrence in cancer cells, is a major factor contributing to drug resistance (Cheng et al., 2005; Dai and Grant, 2007). Receptor tyrosine kinases are a class of plasma membrane receptors that bind various regulatory factors, such as EGF, IGF, HGF and PDGF, and activate several intracellular signaling pathways, including the phosphoinositide 3-kinase/AKT cell survival pathway. Aberrant activities of these receptors, especially members of the ERBB family, have been implicated in the progression of numerous types of human cancers. Increased activity of receptor tyrosine kinases can arise from gene amplification, activating mutations or altered regulation, e.g. by cross-talk between these receptors and integrins or other receptors, or by altered autocrine and paracrine stimulation by various regulatory factors. These changes lead in turn to enhanced tumor cell growth, motility, survival, and resistance to therapies (Gschwind et al., 2004; Krause and Van Etten, 2005; Yarden and Sliwkowski, 2001).

Several reports have documented augmentation of receptor tyrosine kinase and downstream signaling pathway activities after treatment of cancer cells with exogenous hyaluronan (Bourguignon et al., 2006; Bourguignon et al., 2007a; Bourguignon et al., 2007b; Tsatas et al., 2002; Wobus et al., 2002). In addition, we have demonstrated that manipulation of constitutive hyaluronan production and interactions in cancer cell themselves has profound effects on these pathways. Specifically, we have shown that constitutively high levels of active, i.e. autophosphorylated, ERBB2 in carcinoma cells are dependent on endogenous hyaluronan-CD44 interaction and that experimentally increased hyaluronan production causes a sustained elevation in ERBB2 phosphorylation in cells that normally exhibit low levels of ERBB2 activity (Ghatak et al., 2005). Furthermore, stimulation of hyaluronan production induces assembly of a constitutive, lipid raft-associated, signaling complex containing phosphorylated ERBB2, CD44, ezrin, phosphoinositide 3-kinase, and the chaperone molecules, HSP90 and CDC37; inhibition of endogenous hyaluronan-receptor interactions causes disassembly of this complex.

Antagonists of hyaluronan interactions used in these studies included hyaluronan oligomers, soluble hyaluronan-binding proteins and siRNA against CD44 (Fig. 2), each of which caused

disassembly of the signaling complex and inactivation of ERBB2 (Ghatak et al., 2005). Recent mechanistic investigations in our lab have shown that hyaluronan oligomers cause rapid internalization of ERBB2 and CD44 accompanied by their disassociation from one another (unpublished results). Based on the previous work of other groups (Bourguignon et al., 2001; Cheng et al., 2006), it is likely that variants of CD44, rather than standard CD44, are involved in these events. In addition, similar influences of constitutive hyaluronan-CD44 interaction may occur with other receptor tyrosine kinases, i.e. EGFR, IGF-1R, PDGFR and c-MET (Misra et al., 2006), and corresponding effects have been shown for downstream anti-apoptotic and proliferation pathways known to be regulated by these receptor kinases. For example, increased hyaluronan production stimulates the phosphoinositide 3-kinase, MAP kinase and COX-2 pathways whereas antagonists of hyaluronan interactions suppress these pathways (Ghatak et al., 2002; Misra et al., 2003; Misra et al., 2008). Interestingly, interactions between CD44 and receptor tyrosine kinases in normal cells may lead to very different outcomes depending on hyaluronan concentration (Li et al., 2007c; Li et al., 2006). Furthermore, since emmprin stimulates hyaluronan production (Marie et al., 2004), it should come as no surprise that it also increases activation of ERBB2 (Ghatak et al., 2005), the activities of cell survival signaling pathways (Misra et al., 2003), and the ability to grow in an anchorage-independent manner (Marie et al., 2004), in a hyaluronan-dependent fashion.

5. Regulation of drug transporter expression and activity by hyaluronan and CD44

As mentioned above, the phosphoinositide 3-kinase/AKT signaling pathway is an antiapoptotic pathway that is up-regulated in most malignant cancer cells and is regulated by several receptor tyrosine kinases. In addition to its pro-malignant and anti-apoptotic activities, this pathway may lead to increased expression of ABC family multidrug transporters, such as P-glycoprotein (MDR1/ABCB1), multi-drug resistance-associated protein-1 (MRP-1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) (Lee et al., 2004; Misra et al., 2005; Mogi et al., 2003). Not surprisingly, several publications have demonstrated a close relationship between malignant cell properties and resistance to therapy; major factors that appear to mediate this relationship are CD44 and emmprin (Colone et al., 2008; Milette-Gonzalez et al., 2005; Raguz et al., 2004; Yang et al., 2003).

We have demonstrated that the hyaluronan-CD44 interaction regulates expression of ABC family drug transporters, P-glycoprotein and BCRP, in carcinoma and glioma cells (Gilg et al., 2008; Misra et al., 2005). Recent data suggests this effect may be due to stabilization of transporter localization at the plasma membrane. CD44 co-localizes in the plasma membrane of cancer cells with P-glycoprotein and BCRP and treatment of these cells with an antagonist of hyaluronan interactions, viz. hyaluronan oligomers, rapidly induces internalization of the transporters and CD44 into the cell (Fig 3)(unpublished results). Others have also shown that hyaluronan and CD44 influence transporter expression and activity (Cordo Russo et al., 2008; Milette-Gonzalez et al., 2005; Ohashi et al., 2007). In a study comparing multi-drug resistant cell lines of breast, oral, and ovarian origin that express elevated levels of P-glycoprotein with their respective P-glycoprotein-negative, drug-sensitive, parental cell lines, a positive correlation was demonstrated between the expression of CD44 and P-glycoprotein. The two proteins were found to co-immunoprecipitate, and drugs or siRNA that interfere with the function of P-glycoprotein were shown to inhibit cell motility and invasion (Milette-Gonzalez et al., 2005), which are properties strongly related to CD44 receptor activity (Hill et al., 2006b; Tzircotis et al., 2005). In a similar way, co-immunoprecipitation and co-localization of P-glycoprotein and CD44 have been demonstrated in drug resistant melanoma cells, and the two molecules were found to cooperate in promoting invasive behavior (Colone et al., 2008). Moreover, ABC transporter inhibitors have been shown to interfere with hyaluronan secretion in fibroblasts (Prehm and Schumacher, 2004). These observations would agree with the finding

that both P-glycoprotein and CD44 need to be active in T-lymphocytes for their proper migration to lymph nodes (Honig et al., 2003).

Confocal microscopic co-localization and fluorescence resonance energy transfer (FRET) studies in NIH3T3 cells have shown that P-glycoprotein is closely associated with CD44 and other components of plasma membrane lipid microdomains, commonly known as lipid rafts (Bacso et al., 2004). It was also shown in this study that P-glycoprotein is anchored to the cytoskeleton. CD44 binds to the actin cytoskeleton through ERM-family proteins (Tsukita et al., 1994) or ankyrin (Singleton and Bourguignon, 2004). Thus, these results suggest that CD44 resides in close molecular vicinity to P-glycoprotein and may be one of the proteins responsible for the cytoskeletal association of this transporter. Furthermore, raft localization of P-glycoprotein seems to be of functional importance since cholesterol depletion results in inhibition of transporter activity (Bacso et al., 2004). It has also been noted that drugs that interfere with P-glycoprotein can also affect localization of CD44 on the cell membrane and promote CD44 capping, and therefore might act via inhibition of actin polymerization (Miletti-Gonzalez et al., 2005). Similarly, we have seen that hyaluronan oligomer-induced internalization of CD44 and transporters is inhibited if the cells are co-treated with an inhibitor of actin polymerization, latrunculin, thus suggesting that the transporters and CD44 are anchored to actin filaments (Fig 3)(unpublished results).

Hyaluronan synthesis and secretion may be directly related to drug transport since recent work indicates that hyaluronan might be secreted through multidrug transporters in vertebrate cells (Prehm and Schumacher, 2004; Schulz et al., 2007). Studies employing a battery of inhibitors as well as siRNA to sort out possible transporters involved in hyaluronan export led to the conclusion that MRP5 is the most likely hyaluronan transporter in human fibroblasts (Prehm and Schumacher, 2004). The MRP5 gene is ubiquitously expressed but MRP5 knockout mice are viable (de Wolf et al., 2007). Since hyaluronan deficiency is incompatible with life in vertebrates (Camenisch et al., 2000), hyaluronan export could not rely entirely upon MRP5. Although this evidence supports a role for drug transporters in hyaluronan secretion, other studies strongly suggest that constitutive export of hyaluronan requires only the hyaluronan synthases themselves (Weigel and Deangelis, 2007). Moreover, definitive direct evidence for hyaluronan export through ABC transporters, rather than regulation by transporter activity, is lacking. Nevertheless it is likely that such export does occur at least under certain circumstances. Indeed, our findings support a close relationship between hyaluronan and drug transporters in that treatment with hyaluronan oligomers inhibits hyaluronan production or export (Misra et al., 2006) and induces rapid internalization of the drug transporters, BCRP and P-glycoprotein (unpublished results).

6. Role of emmprin and hyaluronan in the glycolytic phenotype of cancer cells

Malignant tumors usually exhibit a metabolic phenotype of diminished respiration and enhanced glycolysis – the “Warburg effect”. Increased glycolysis in these cancers is associated with various conditions such as hypoxia, acidosis and mitochondrial defects, which result in enhanced drug resistance and malignancy (Gatenby and Gillies, 2004; Pelicano et al., 2006; Tredan et al., 2007). An outcome of increased glycolysis is lactate production. Lactate is pumped across the plasma membrane via proton-coupled monocarboxylate transporters (MCTs). Functional MCTs are crucial to avoid cytotoxic intracellular accumulation of lactate in cancer cells. For example, it has been shown that inhibition of expression of MCT1 and MCT2 in glioma cells induces cell death (Mathupala et al., 2004). Also lactate efflux at the leading edge of tumor cells acidifies the surrounding microenvironment, which can enhance cell invasion (Gatenby et al., 2006; Martinez-Zaguilan et al., 1996), metastasis (Schlappack et al., 1991) and drug resistance (Pelicano et al., 2006; Tredan et al., 2007).

Emmprin is crucial for the proper function of several monocarboxylate transporters (MCTs). At least 14 members of this family have been cloned and are distinguished by their kinetic properties and tissue distribution (Enerson and Drewes, 2003; Halestrap and Meredith, 2004). MCT1, MCT3 and MCT4 require association with emmprin in the endoplasmic reticulum for trafficking to the plasma membrane and, in the absence of emmprin, MCTs are targeted for degradation (Gallagher et al., 2007; Kirk et al., 2000; Wilson et al., 2005). MCT1 is the most widely expressed member of this family and was recently shown to be elevated in a variety of cancers, including neuroblastoma (Fang et al., 2006) and colorectal carcinomas (Koukourakis et al., 2006). MCT4, which is expressed preferentially in tissues dependent on glycolysis for the metabolism of glucose, is co-localized with emmprin in the plasma membrane of metastatic MDA-MB-231 breast cancer cells. Trafficking of these two proteins to the plasma membrane was shown to be mutually inter-dependent. Moreover, suppressed expression of MCT4 resulted in decreased migratory capacity in these cells, most likely due to inhibition of emmprin function (Gallagher et al., 2007).

Two sets of observations indicate a possible relationship of hyaluronan to the glycolytic phenotype. The first of these is that emmprin, an essential partner in the activity of MCTs, stimulates hyaluronan production (Marieb et al., 2004). Second, lactate stimulates hyaluronan synthesis and expression of CD44 variants in fibroblasts (Stern et al., 2002) and melanoma cells (Rudrabhatla et al., 2006), and lactate response elements are present in several hyaluronan-related genes, e.g. CD44 and the hyaluronidase, HYAL1 (Formby and Stern, 2003). Thus, we have examined the relationship of hyaluronan and CD44 to MCT1 and MCT4 and found that CD44 co-localizes with MCT1 and MCT4 in the plasma membrane of breast cancer cells. Furthermore, treatment of the cells with hyaluronan oligomers leads to internalization of these MCTs and to attenuation of their function, in similar fashion to our findings with RTKs and drug transporters (unpublished results). These results imply that hyaluronan-CD44 interactions stabilize several types of cell surface complexes. Akin to these findings, other investigators have documented a similar relationship between hyaluronan-CD44 interaction and the Na(+)-H(+) exchanger 1 (Bourguignon et al., 2004).

7. Regulation of cancer stem cell properties by hyaluronan and CD44

A rapidly growing body of work provides evidence that tumors contain subpopulations of stem-like cells that are highly malignant and resistant to therapies. These cells are frequently termed: “cancer stem cells”, “cancer progenitor cells” or “tumor-initiating cells”. The malignancy of these cells is illustrated in their capacity to rapidly regenerate a fully grown tumor, which recapitulates the heterogeneous cellular composition of the tumor of origin, when implanted in small numbers in an animal host (Dalerba et al., 2007a; Hill and Perris, 2007; Vescovi et al., 2006). Another striking property of these cells is their resistance to treatment with cytotoxic chemotherapeutic agents or radiation, and consequently they may have a role in tumor recurrence or persistence after therapy (Ailles and Weissman, 2007; Dean et al., 2005; Neuzil et al., 2007).

However, the cancer stem cell hypothesis remains controversial as the precise nature and origin of cancer stem-like cells have yet to be elucidated (Hill and Perris, 2007; Patrawala et al., 2005; Shipitsin et al., 2007; Wang et al., 2008). These cells may comprise the metastatic subpopulation of tumors (Brabletz et al., 2005; Li et al., 2007b) or, stated differently, may merely reflect heterogeneity within tumors with respect to various oncogenic and metastatic properties (Shipitsin et al., 2007). Nevertheless, the presence of highly malignant, therapy-resistant subpopulations within human tumors is well-established and our increased understanding of the properties of these cells is likely to yield more effective therapeutic strategies.

The hyaluronan receptor, CD44, is one of the most common markers used for isolation of cancer stem-like cells, especially from carcinomas (Al-Hajj et al., 2003; Dalerba et al., 2007b; Li et al., 2007a; Prince et al., 2007). Recent studies indicate that CD44 is functionally important in leukemia stem cells (Jin et al., 2006; Krause et al., 2006) and other studies point to a possible role for another hyaluronan-binding protein, Rhamm, in myeloma progenitors (Crainie et al., 1999; Maxwell et al., 2004). Hyaluronan appears to have a role in normal stem cell behavior (Avigdor et al., 2004; Matrosova et al., 2004; Nilsson et al., 2003; Pilarski et al., 1999) and hyaluronan synthases are altered in myeloma progenitors (Adamia et al., 2005; Calabro et al., 2002). Interestingly, another common marker for cancer stem-like cells is EpCAM, also known as ESA (Al-Hajj et al., 2003; Dalerba et al., 2007b; Li et al., 2007a). EpCAM is a binding partner of emmprin (Xu and Hemler, 2005), implying that emmprin may also be associated with cancer stem-like cells. Moreover, it has been observed that breast carcinoma cells that express both CD44 and EpCAM are more malignant than cells expressing CD44 but not EpCAM (Al-Hajj et al., 2003). Despite these observations, very little has been published on the functional significance of hyaluronan, CD44 or emmprin in the properties of cancer stem-like cells from solid tumors.

A method that has been used extensively to enrich cancer stem-like subpopulations is cell sorting on the basis of the so-called “side-population” phenotype (Dean et al., 2005; Hadnagy et al., 2006). This phenotype depends on efflux of the Hoechst 33342 dye by drug transporters, particularly BCRP, which are usually enriched in cancer stem-like cells. Recently we examined the effects of perturbing hyaluronan interactions, using treatment with hyaluronan oligomers, on the malignant and therapy-resistant properties of stem-like cells isolated from the C6 glioma cell line by the side population approach (Gilg et al., 2008). The C6 side population cells exhibited stem-like properties, were highly resistant to the cytotoxic effects of methotrexate, and were very aggressive when implanted *in vivo* using a spinal cord engraftment model that replicates invasive behaviors of human gliomas in the central nervous system (Gilg et al., 2008). As mentioned above, the treatment with hyaluronan oligomers most likely displaces constitutively bound hyaluronan polymer from its receptors, resulting in attenuation of hyaluronan-induced signaling (Ghatak et al., 2002; Ghatak et al., 2005; Misra et al., 2006). We found that these oligomers cause increased apoptosis and decreased proliferation in the tumors, and as a result inhibit growth and invasion of the C6 stem-like subpopulation *in vivo* (Gilg et al., 2008). The C6 stem-like cells showed elevated activation of EGFR and AKT, expression of the BCRP drug transporter and resistance to treatment with methotrexate, when compared with the parental cells. All of these parameters were reduced by treatment with the hyaluronan oligomers (Gilg et al., 2008), indicating the potential importance of hyaluronan in the properties of these cells.

Cancer stem-like cell subpopulations can be enriched using Hoechst dye exclusion, cell surface markers or spheroid formation. However, the efficacy of these different methods varies among different cancer types, and in each case the enriched preparations are heterogeneous. Depending on the method of separation and the cancer type, these cell preparations express various subsets of markers and exhibit varying degrees of malignancy and resistance to drug treatment. For example, in a study of lung cancer the side-population cells were found to exhibit stem cell-like properties such as multidrug resistance, high telomerase activity, and tumor-repopulating capacity, and consistently expressed high levels of ABC transporters, in comparison to non-side population cells (Ho et al., 2007). However, CD44 and other commonly used cancer stem cell markers were present in both side-population and non-side population cells, pointing to lack of exclusivity of the markers to the side-population in these lung cancer cells (Ho et al., 2007). In another study it was found that the side-populations from various carcinoma cells were enriched in stem-like and malignant properties, yet cancer cells lacking BCRP expression were similar in tumorigenicity to BCRP-positive cells (Patrawala et al., 2005). A common marker for identification of cancer stem-like cells is CD133 (Mizrak et al.,

2008; Neuzil et al., 2007) but it has been shown recently that CD133-negative cells can also express stem-like properties (Beier et al., 2007; Wang et al., 2008). Thus, a challenge to understanding the relationship of multidrug resistance to cancer stem-like cells will be isolation of homogenous populations that can be compared and analyzed with respect to transporter expression and function, anti-apoptotic signaling pathways and their specific relationships to hyaluronan, CD44 and emmprin.

8. Conclusions

Hyaluronan, CD44 and emmprin are important co-regulators of a variety of activities crucial to drug resistance. These include receptor tyrosine kinase, ABC transporter and MCT activities (summarized in Fig. 4). It is not yet clear how interactions between hyaluronan, CD44 and emmprin at the cell surface initiate these activities. Some effects appear to be direct, e.g. emmprin-MCT interactions, but some are most likely indirect. Pericellular hyaluronan interacts with other extracellular macromolecules that may serve structural or signaling functions (Evanko et al., 2007; Toole, 2001). These interactions may also be important in activation or modulation of hyaluronan signaling.

Irrespective of the mechanisms involved, it is clear that antagonists of hyaluronan, CD44 and emmprin-induced events are promising candidates for therapeutic strategies aimed at preventing tumor refractoriness or recurrence due to drug-resistant subpopulations within malignant cancers.

Acknowledgements

Recent work from our lab that is described herein was supported by grants to B.P.T. from the National Institutes of Health (CA073839 and CA082867), the Department of Defense (OC050368) and The Charlotte Geyer Foundation.

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Fig. 1. Structure of CD44

The major domains of the standard form of CD44 are shown. These are: a) the hyaluronan-binding domain, which is related to the so-called link modules of other hyaluronan-binding proteins (Day and Prestwich, 2002); b) the membrane proximal domain, which includes the single site into which additional exon products are spliced to form numerous CD44 variants; c) the transmembrane domain; and d) the cytoplasmic domain, to which numerous signaling molecules bind directly or indirectly on activation and engagement of the link module by hyaluronan.

Fig. 2. Antagonists of hyaluronan-CD44 interactions

Other antagonists include blocking antibodies against CD44 (Guo et al., 1994) and inhibitors of hyaluronan synthesis (Simpson et al., 2002). Adapted from (Ghatak et al., 2005; Toole, 2004). HA, hyaluronan; HABP, HA-binding protein.

Fig. 3. Internalization of drug transporters by treatment with hyaluronan oligomers

A. In the absence of treatment, hyaluronan is tethered by CD44 at the plasma membrane whereby it stabilizes actin-linked CD44-transporter complexes in lipid microdomains. Hyaluronan is cleaved by hyaluronidase (most likely Hyal-2) and internalized via CD44 in an orderly manner (Knudson et al., 2002; Tammi et al., 2001). **B.** Oligomers of hyaluronan (o-HA) stimulate CD44 internalization en masse, destabilizing transporter complexes. Inhibitors of actin assembly inhibit this process.

Fig. 4. HA, CD44 and emmprin interactions

A scheme showing possible interactions of hyaluronan, CD44, and emmprin with receptor tyrosine kinases (RTKs), ATP-binding cassette (ABC) multidrug transporters, and monocarboxylate (lactate) transporters (MCT).