Arch Toxicol (2015) 89:3–14
DOI 10.1007/s00204-014-1424-2

REVIEW ARTICLE

Perspectives of CD44 targeting therapies

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Received: 10 November 2014 / Accepted: 24 November 2014 / Published online: 5 December 2014

Abstract CD44 is a family of single-span transmembrane glycoproteins. Members of this family differ in the extracellular domain where ten variant exons are either excluded or included in various combinations. CD44 isoforms participate in many physiological processes including hematopoiesis, regeneration, lymphocyte homing and inflammation. Most importantly, they are involved in pathological processes and in particular in cancer. In several types of tumors, CD44 together with other antigens specifies for cancer stem cell populations. Mechanistically, CD44 proteins act as receptors for hyaluronan, co-receptor for receptor tyrosine kinases (RTKs) or G-protein-coupled receptors or provide a platform for metalloproteinases. For all these reasons, targeting CD44 may be a successful approach in cancer therapy. In this review, we discuss the various possibilities of targeting CD44. Among these are the production of CD44 ectodomains, antibodies, peptides or aptamers. Also inhibition of CD44 expression has been proposed. Finally, the function of CD44 as a hyaluronan receptor was also taken advantage of. We are convinced that the success of these therapies will depend on an increased understanding of the molecular functions of specific CD44 isoforms in particular in cancer stem cells.

Keywords CD44 · Therapy · Hyaluronan · Receptor tyrosine kinases

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CD44 designates a family of single-span trans-membrane proteins that are encoded by a single gene of about 50 kb of length located on chromosome 11 in humans and on chromosome 2 in mice (reviewed in Orian-Rousseau 2010). The CD44 gene is composed of 20 exons (Fig. 1). Ten of these exons (also known as "constant" exons) are expressed in all isoforms. They also account for the N-terminal extracellular part, the trans-membrane region and the intracellular domain of all members. The ten central exons known as "variant" exons are excised or included in various combinations by alternative splicing in the membrane-proximal stem region. They account for the heterogeneity of this protein family. The last two exons encoding the CD44 cytoplasmic domain are also subjected to alternative splicing. The smallest isoform (CD44s) lacking all variant exons in the extracellular domain is ubiquitously expressed, whereas the expression of variant isoforms is confined to only few tissues and takes place only under specific developmental conditions. Most strikingly, CD44 variant isoforms are expressed in a variety of different cancers, particularly in advanced stages (reviewed in Naor et al. 2002; Orian-Rousseau 2010]. The complexity of the CD44 protein family is further enhanced by post-translational modifications such as N- and O-glycosylations, chondroitin sulfations or heparan sulfate additions (for a more detailed description see Orian-Rousseau and Sleeman 2014; Ponta et al. 2003).

CD44 came into focus for the first time in cancer research when it was identified as a homing receptor for migrating thymus progenitor cells (O'Neill 1989) and human lymphocytes (Jalkanen et al. 1987; Pals et al. 1989). Furthermore, CD44 appeared to mediate the binding of lymphocytes or lymphoma cells to endothelial cells most likely only upon activation of the lymphocytes (Lesley and Hyman 1992; Oppenheimer-Marks et al. 1990). These functions are not only instrumental for the fate of

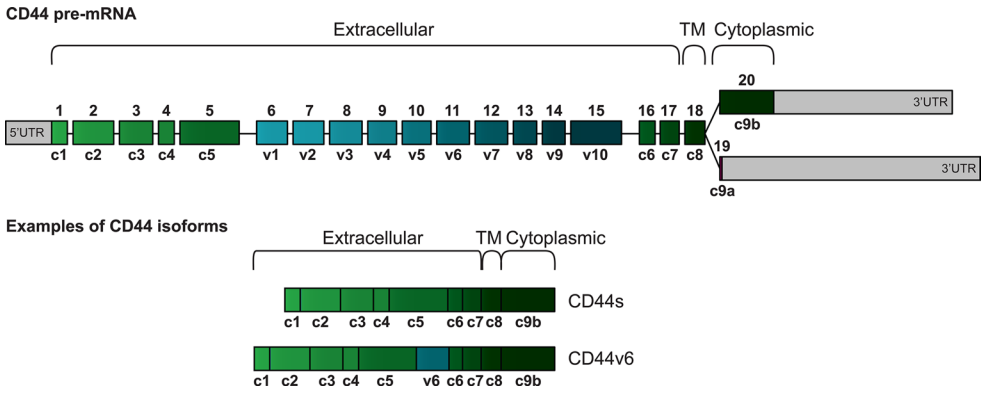

Fig. 1 The CD44 proteins are encoded by one single gene. The CD44 gene comprises 20 exons. Ten of these exons (v1–v10) are alternatively spliced. The CD44s isoform does not contain any variant exon. Variant exons (v6 is shown) are included in the stem region of the protein. In humans, exon v1 contains a stop codon and is not found in any isoform. The last two exons encoding the CD44 cytoplasmic domain are also subjected to alternative splicing (reviewed in Ponta et al. 2003)

lymphocytes but are also required for the hematogenic spreading of tumor cells.

Molecular functions of CD44

CD44 is the main receptor for hyaluronan

The migration of lymphocytes as well as the spreading of tumor cells are controlled by CD44 and require interactions with constituents of the extracellular matrix (ECM). A hallmark in the CD44 research was the identification of CD44 as the principal receptor for hyaluronan (HA) (Aruffo et al. 1990). HA is a linear non-sulfated polysaccharide composed of disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with a MW of 10^6–10^7 kDa. HA is particularly abundant in connective tissue and in the lymph and lymph node matrix. HA does not only provide a cellular support and hydrophilic matrix but also regulates cell–cell adhesion, cell migration as well as growth and differentiation (Laurent and Fraser 1992). Consequently, HA is involved in many physiological processes such as wound healing, inflammation, morphogenesis and in pathological processes such as cancer. Furthermore, upon interaction with the cell surface HA forms a "coat" that can act as a cellular barrier (Gately et al. 1984; McBride and Bard 1979) and eventually can protect tumor cells from an immune attack. Interestingly, several tumor cells produce increased amounts of HA or induce the production of HA by surrounding fibroblasts thereby leading to enhanced metastatic spreading (Knudson et al. 1984; Turley and Tretiak 1985; Zhang et al. 1995).

The binding domain of CD44 for HA is located in the N-terminal extracellular part of the molecule. This domain is called the "link region" for its homology with the HA-binding domain of the cartilage link protein that allows network formation between HA and glycosaminoglycans in the ECM (Laurent and Fraser 1992). It is a globular domain with three conserved cysteine bridges and with two BX_7B sequences where two basic amino acids (B) are separated by seven non-acidic amino acids (Goetinck et al. 1987; Goldstein et al. 1989; Peach et al. 1993; Yang et al. 1994). This binding domain exists in all CD44 isoforms. However, in some cells, the insertion of variant exons in the stem region results in a loss of HA binding, whereas in other cells, the inclusion of variant exons even enhances HA binding (reviewed in Naor et al. 1997; Orian-Rousseau and Sleeman 2014). Several other factors influence the binding of CD44 to HA. Among these are CD44 aggregation, interaction of CD44 with other cell surface proteins and post-translational modifications of the CD44 protein (reviewed in Naor et al. 1997; Orian-Rousseau and Sleeman 2014). Although not all parameters regulating HA binding to CD44 have been unraveled CD44 turned out to be the most important cellular receptor for HA and seems to be involved in the majority of HA-dependent cellular responses. Additionally, several other HA-binding cellular receptors have been identified (e.g., RHAMM, Lyve1, TLR4, ICAM1), which have functions in rather restricted tissues (reviewed in Naor et al. 1997).

The minimal size of HA fragments binding to CD44 corresponds to six disulfide units. This is important since high molecular weight HA (hHA) in the ECM is degraded by hyaluronidases into smaller fragments (sHA) that still can bind to CD44. Interestingly, hHA and sHA often exert opposite effects on several physiological and pathological processes (for a detailed discussion see Orian-Rousseau and Sleeman 2014).

Although HA and CD44s are expressed in nearly all tissues, only few physiological functions requiring their

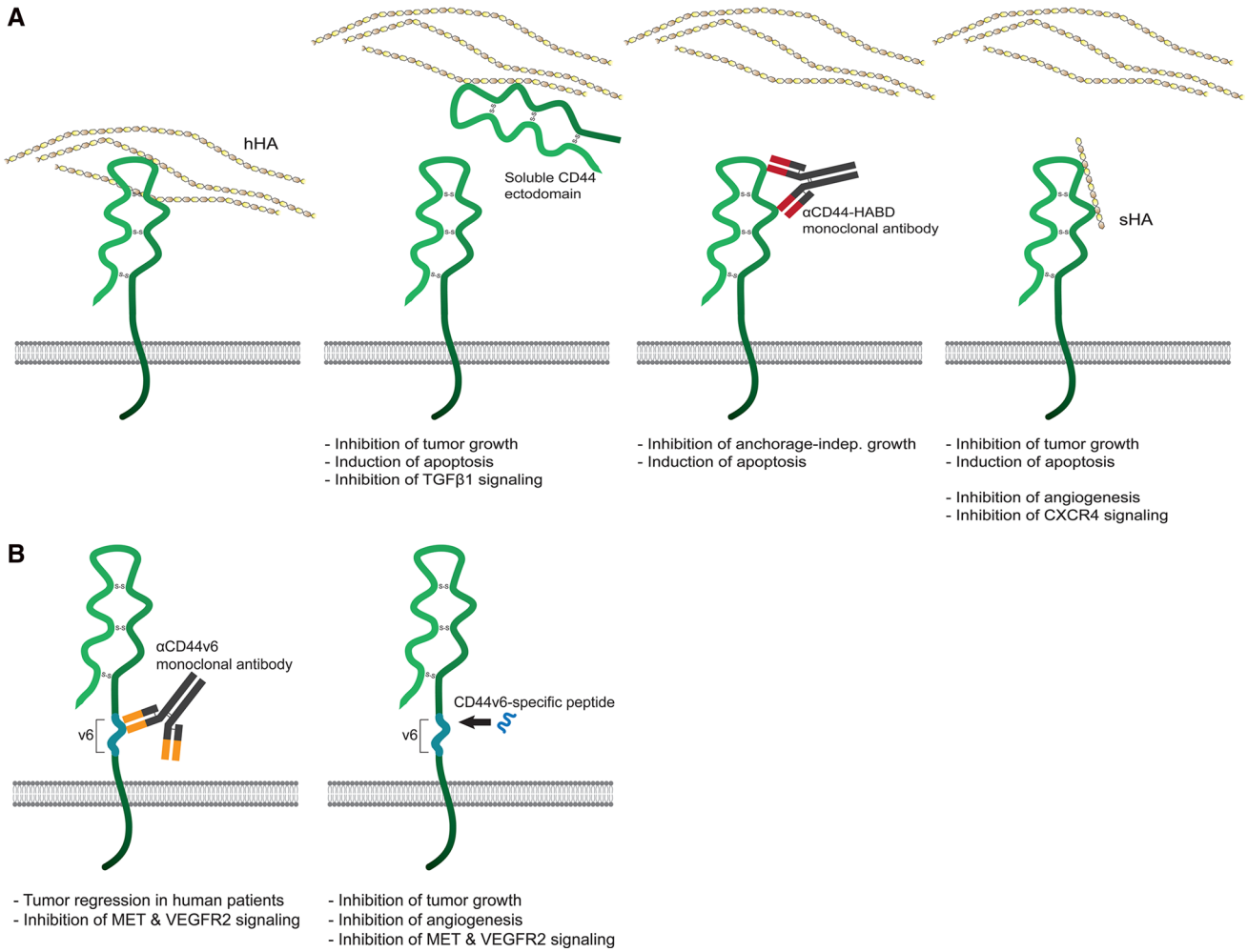

Fig. 2 Most common blocking reagents against CD44 isoforms: **a** CD44 ectodomain [e.g., (Yu et al. 1997), antibodies blocking CD44–HA interaction (Ghatak et al. 2002) or small fragments of HA inhibiting the binding of hHA (Ghatak et al. 2002; Fuchs et al. 2013). **b** Antibodies against CD44v6 (Heider et al. 1996; Schrijvers et al. 1993) or peptides (Matzke et al. 2005)

collaboration have been identified. One of which is the already mentioned homing of lymphocytes, where antibodies against CD44 that block the binding to HA, inhibit the binding of lymphocytes to high endothelial venules, a key step in homing (Jalkanen et al. 1987). In addition, the homing of mesenchymal stem cells to the kidney during acute renal failure is instrumental for the healing process and is dependent on CD44–HA interaction (Herrera et al. 2007). Another one is the rolling of lymphocytes in the blood stream, which is one of the first steps in the extravasation of lymphocytes (and metastasizing tumor cells). This step requires the interaction of leukocytes to the endothelial cells of blood vessels. For some T cells, this interaction can be blocked by CD44-specific antibodies and by treatment with soluble HA (DeGrendele et al. 1996, 1997). Furthermore, CD44 antibodies, which prevent the binding of CD44 to HA inhibit the migration of hematopoietic stem cells to the bone marrow (Avigdor et al. 2004). In angiogenesis, the formation of new blood vessels from existing ones, a phenomenon important in wound healing and tumor growth, the interaction of CD44 and HA also appears to be instrumental (Fuchs et al. 2013) (Fig. 2a).

CD44 isoforms act as co-receptors

A milestone in the research on CD44 was the identification of the CD44v6 isoform as one of the first metastatic determinants in cancer (Gunthert et al. 1991; Hofmann et al. 1991; Rudy et al. 1993). CD44v6-specific antibodies were able to block the metastatic spreading of rat pancreatic tumor cells. Moreover, the transfection of CD44v4–v7 (the v6 exon is contained in the stem region) cDNA but not of CD44s cDNA into non-metastatic tumor cells conferred metastatic propensity to these cells. These findings prompted a huge number of studies aiming at unraveling

the relevance of CD44 isoforms and particular CD44v6 isoforms in all different types and stages of human tumors, and indeed, there is ample of evidence for a correlation between the expression of CD44 isoforms and advanced stages of carcinomas (reviewed in Naor et al. 2002; Orian-Rousseau 2010).

A breakthrough in the understanding of molecular functions of CD44 isoforms in physiological and pathological conditions was the observation that heparan sulfate-modified CD44v3 isoforms are able to bind several heparan sulfate binding growth factors such as FGFs or HB-EGF (Bennett et al. 1995; Jackson et al. 1995). It turned out that such a function is not confined to heparan sulfate-modified CD44 isoforms but can also be provided by other isoforms. Of particular interest for cancer research was the identification of CD44v6 isoforms as co-receptors for the receptor tyrosine kinases (RTKs), Met and VEGFR-2 (Orian-Rousseau et al. 2002; Tremmel et al. 2009). Met and VEGFR-2 activation and subsequent signaling are both dependent on CD44v6 (Orian-Rousseau et al. 2002, 2007). Consequently, the formation of new blood vessels in a human pancreatic xenograft was blocked upon inhibition of CD44v6 (Tremmel et al. 2009). These data point toward a requirement of the co-receptor functions of CD44v6 for these RTKs for tumor progression. In agreement with this assumption is the finding that metastasis of colorectal cancer spheres injected into the murine cecum is dependent on both, CD44v6 and Met (Todaro et al. 2014).

The binding ability of CD44 to ECM components, particularly to HA, and the co-receptor function of CD44 isoforms are features that might explain the contribution of CD44 to tumorigenesis. Furthermore, the functions of CD44 isoforms as co-receptors for several RTKs might also explain why so many different CD44 isoforms exist. Indeed, different isoforms can address different receptors and are specialized for different ligands (reviewed in Orian-Rousseau and Sleeman 2014).

It is worth noting that CD44 proteins collaborate not only with RTKs but are also involved in the CXCL12-CXCR4 axis (Fuchs et al. 2013) and the Wnt signaling pathway (Schmitt et al. 2014). Furthermore, CD44 proteins can contribute to signaling pathways by binding metalloproteinases and thereby facilitating the activation of growth factor pro-forms to the active protein (Yu and Stamenkovic 2000; Yu et al. 2002).

Particularly important in the context of carcinogenesis is the contribution of CD44 to the inhibition of apoptosis (Yu et al. 1997). Several mechanisms have been proposed that account for this function. Among these are the HA-dependent activation of TGFβ1 (Yu and Stamenkovic 2004), the activation of HB-EGF, the activation of osteopontin by CD44v6 containing isoforms and even the inhibition of Fas signaling by CD44 (reviewed in Ponta et al. 2003; Mielgo et al. 2005). A completely different function of CD44 in apoptosis was recently suggested. CD44v6 isoforms account for the formation of a pre-metastatic niche that promotes survival of tumor cells and induces chemoresistance (Jung et al. 2009, 2011).

The involvement of CD44 in the establishment and progression of several cancers makes it a suitable target for cancer therapy. In this review, we present various ways of targeting CD44 isoforms and discuss the future prospects of these therapies.

Anti-CD44 strategies

Hyaluronan-dependent anti-cancer strategies

There is ample evidence that the CD44–HA interaction is involved in tumor progression (reviewed in Misra et al. 2011; Orian-Rousseau and Sleeman 2014). Therefore, the interference with the binding of CD44 expressed on tumor cells to HA using either the soluble CD44 ectodomain as a competitor or antibodies that specifically block the binding of HA to CD44, impaired tumor growth and metastasis. Indeed, the local administration of the ectodomain of CD44 in mice transfected with human melanoma cells inhibited tumor growth, whereas injection of a mutant, non-HA-binding CD44 ectodomain had no effect (Bartolazzi et al. 1994). Similarly, human melanoma cells transfected with an expression construct for the CD44 ectodomain showed retarded tumor growth when compared to cells transfected with a HA-binding mutant (Ahrens et al. 2001). Most strikingly, the expression of a peptide of 42 amino acid of length that contained three BX_7B HA-binding motifs (found in CD44 and other HA-binding proteins) induced apoptosis and inhibited tumor growth of melanoma cells in vivo (Xu et al. 2003).

The expression of the CD44 ectodomain in metastatic murine mammary carcinoma cells also inhibited tumor growth upon the induction of apoptosis and repressed the invasion of tumor cells into the surrounding tissues (Yu et al. 1997) (Fig. 2a). A mutant ectodomain in the HA-binding sequence, however, did not interfere with tumor growth (Peterson et al. 2000). In these mammary carcinoma cells, CD44 recruits the metalloproteinase MMP9 most likely in a HA-dependent manner since MMP9, CD44 and HA are found in clusters (Yu and Stamenkovic 1999). The binding of MMP9 to CD44 allowed the activation of TGFβ1, which resulted in cell survival and metastasis (Yu and Stamenkovic 2004).

Monoclonal CD44-specific antibodies, which interfere with the binding of HA to CD44, have similar effects as the CD44 ectodomain. They led to the inhibition of anchorage-independent growth of murine mammary carcinoma cells

and human colon carcinoma cells and induced apoptosis (Ghatak et al. 2002) (Fig. 2a). Furthermore, HA oligosaccharides had similar effects and inhibited tumor growth in vivo most likely by interfering with the binding of hHA to CD44 (Ghatak et al. 2002) (Fig. 2a). This is an example of apparent opposing effects of hHA and sHA.

The binding of HA to CD44 can lead to the internalization of HA (Culty et al. 1992, 1994). This feature and the unique properties of HA, namely its bio-degradability, its bio-compatibility and non-immunogenicity makes HA a good candidate for drug delivery applications. Several labs have shown that HA can be covalently coupled with drugs can efficiently target CD44-expressing cells (Akima et al. 1996; Luo et al. 2000; Pouyani and Prestwich 1994; Yadav et al. 2008). HA contains multiple functional residues (hydroxyl and carboxylic acid) on the HA backbone, which can be used to form HA-drug conjugates. Upon internalization, the drug is released mainly by enzymatic hydrolysis. Several preclinical studies have shown that the anti-cancer properties are efficiently improved by the covalently coupling of drugs to HA. For example, the coupling of the anti-mitotic chemotherapeutic agent paclitaxel to HA increased its solubility and selectively targeted the CD44-dependent human ovarian, colon and breast cancer cells (Luo and Prestwich 1999).

HA can also be covalently or non-covalently coupled with nanoparticles (NPs). An in-depth description of the potential of HA-based nanocarriers can be found in the reviews by (Choi et al. 2012; Ghosh et al. 2012; Misra et al. 2011). Here, we describe only a few examples. Several versions of HA-coupled nanocarriers loaded with anti-cancer drugs were examined and have demonstrated advantages for cancer treatments in animal models. Their non-modified counterparts showed no such advantages. For example, coupling of high molecular weight HA to lipid-based NPs enhanced their circulation time and improved the specificity of tumor targeting (Mizrahy et al. 2014). Interestingly, coupling of sHA did not show this effect. Several anti-cancer drugs such as epirubicin, doxorubicin, paclitaxel or mitomycin c were incorporated in the inner hydrophobic part of HA-nanocarriers and were tested for their therapeutic efficacy (Eliaz et al. 2004; Eliaz and Szoka 2001; Peer and Margalit 2004). In all cases, the encapsulation of the drugs led to a significant improvement of their efficacy.

Examples of more recently developed NPs with extremely high efficient tumor targeting, optimal release of encapsulated drugs due to fine-tuning of the pH conditions and extremely low cytotoxicity are found in the following papers: Qiu et al. (2014); Song et al. (2014a, b). Some of these combinations have made it to clinical trials. One example is a combination of HA and paclitaxel, a highly hydrophobic anti-cancer drug, referred to as ONCOFID™-P undergoing phase II clinical study in Europe for treatment of refractory bladder cancer (reviewed in Choi et al. 2012).

Antibody-based strategies against CD44

Monoclonal antibodies against CD44v6 in head and neck cancer

Expression studies for several CD44 isoforms revealed that they are particular abundant in advanced stages of carcinoma. This is particularly true for CD44v6 (reviewed in Naor et al. 2002; Orian-Rousseau 2010). Since the expression of CD44v6 is particularly high and homogenous in human head and neck carcinoma (HNSCC), HNSCC was considered promising for treatment with the CD44v6 antibodies (Heider et al. 1996; Schrijvers et al. 1993) (Fig. 2b). Two monoclonal antibodies were used. The first one, designated BIWA, was derived from mice injected with the human CD44v6 part (Heider et al. 1996). The second one, named U36, was obtained from a screen for specific epitopes expressed on human head and neck carcinoma cells. It turned out that U36 is also specific for CD44v6 (Schrijvers et al. 1993; Van Hal et al. 1996). Interestingly, the epitopes recognized by the two mAbs overlap and differ only by two amino acids (Van Hal et al. 1997).

Both mAbs were radiolabelled and showed selective tumor targeting and high tumor uptake in HNSCC patients undergoing surgery (Colnot et al. 2000; de Bree et al. 1995; Stroomer et al. 2000; Van Hal et al. 1996; Verel et al. 2002). Since the BIWA antibody induced an immune response in patients, it was humanized to give BIWA4 (bivatuzumab) that was then used for further clinical studies (Colnot et al. 2003; Stroomer et al. 2000). A radio-immune therapy study (RIT) with ^{186}Re-labeled bivatuzumab in HNSCC patients gave promising anti-tumor effects with consistent stable disease at higher radioactivity dosage (Borjesson et al. 2003; Postema et al. 2003). The U36 antibody was also tested in several RIT studies with promising outcomes especially in adjuvant settings (Colnot et al. 2000, 2002).

Based on these results, the non-radioactive cytotoxic drug mertansine, an anti-microtubule agent, was coupled with bivatuzumab (Sauter et al. 2007). Indeed, bivatuzumab could direct mertansine activity to CD44v6-expressing tumor cells. Pharmacokinetics, immunogenicity and safety of the antibody drug conjugate were evaluated. In a phase I escalation study with 31 HNSCC patients, no immune response was observed with bivatuzumab–mertansine (Sauter et al. 2007), and in a phase I trial, 12 HNSCC patients were treated with the maximal tolerated dose (Riechelmann et al. 2008). Unexpectedly, the binding of the conjugate to skin keratinocytes mediated serious skin toxicity and had a fatal outcome, and therefore, the study was terminated. However, in three patients, a partial response with a

disappearance of tumor infiltration could be observed. The tumor regression lasted for 4–8 months under continued drug treatment (Riechelmann et al. 2008).

In a parallel study, seven HNSCC patients received bivatuzumab–mertansine for 23 weeks. With the highest dose, one patient developed toxic epidermal necrolysis and died. The risk–benefit assessment turned out to be negative mainly for skin-related adverse events although the majority of skin reactions were reversible. Further clinical development was discontinued (Tijink et al. 2006).

Despite the clinical drawbacks with bivatuzumab–mertansine CD44v6 mAbs were further developed for targeting CD44v6 in HNSCC tumors. The U36 antibody was labeled with ^{111}In using the chelator CHXA"-DTPA and the chimeric molecule showed promising results regarding bio-distribution and tumor uptake in HNSCC bearing nude mice (Sandstrom et al. 2008). F(ab′)2 and Fab′ fragments of U36 were even superior to the mAb regarding bio-distribution and accumulation in the tumor (Sandstrom et al. 2012). Although the tumor uptake of ^{125}I-F(ab′)$_2$ was lower as compared to the ^{125}I-labeled mAb U36, a higher tumor-to-blood ratio was observed.

Bivatuzumab was also used in bio-distribution and safety studies in other tumors (breast cancer (Koppe et al. 2004); thyroid cancer (Fortin et al. 2007) and for tumor imaging (Vermeulen et al. 2013)). It was additionally used for the detection of lymph node metastases (Borjesson et al. 2006). Most recently, a fully human Fab fragment obtained from a synthetic Fab library and selected for binding to CD44v6 isoforms was assessed for tumor imaging. A comparison between ^{111}In- and ^{125}I-labeled Fab fragments revealed that both had a high tumor targeting capacity but the ^{111}In-labeled had a higher tumor-to-blood ratio and could discriminate better between high and moderate expression of CD44v6 in HNSCC xenografts (Haylock et al. 2014).

A very interesting and often successful approach is the targeting of tumor antigens by adoptive cell therapy. For this approach, T cells were genetically engineered to express receptors directed against the tumor antigen. CD44v6 was used as such an antigen. The antigen-recognizing determinant of bivatuzumab was fused to the signaling domains of CD28 and CD3ζ. The chimeric genes were introduced by means of retroviral gene transfer into human T cells, which then displayed anti-CD44v6 effector functions. They eliminated CD44v6 positive acute myeloid leukemia and multiple myeloma cells in murine xenografts (Casucci et al. 2013). The drawback of this approach is the off-tumor/on-target toxicity due to the expression of the tumor antigen on normal tissue. For CD44v6, these tissues are mainly keratinocytes and circulating monocytes. The elimination of these monocytes in particular would lead to long-term monocytopenia, a disease that is life threatening. To avoid side effects, the authors have incorporated a suicide gene into the engineered T cells namely, the non-immunogenic, quick acting and inducible iCasp9 (Straathof et al. 2005). iCasp9 refers to a Casp9 gene fused to a dimerization sequence. This dimerization sequence can be addressed by treatment with a small molecule that exclusively drives iCasp9 dimerization and activation in the transfected T cells thereby leading to their elimination.

Other antibodies against CD44

A study with pancreatic cancer patients that underwent surgery demonstrated that patients with higher levels of panCD44 in the pancreatic adenocarcinoma had a worse prognosis for survival when compared to patients with lower levels (Li et al. 2014) suggesting CD44 as a therapeutic target. Indeed, a panCD44 Ab reduced growth, metastasis and post-radiation recurrence of pancreatic xenograft tumors. The antibody also reduced the number of tumor initiating cells (TICs or CSC, cancer stem cells) in cultured pancreatic cancer cells and xenograft tumors. The elimination of theses TICs is most likely due to the down-regulation of stem cell self-renewal markers as well as inhibition of the survival factor STAT. Interestingly, the RTK Met appears to be also a marker of pancreatic cancer stem cells and its inhibition by specific inhibitors or its down-regulation by shRNA also reduced the population of CSCs (Li et al. 2011).

Targeting of CD44 also eradicated human acute myeloid leukemic stem cells (AML-LSCs) (Jin et al. 2006). Administration of a pan CD44-specific monoclonal antibody into immunosuppressed mice transplanted with AML-LSCs drastically decreased the leukemic population. The trafficking of LSCs to the bone marrow niche and its engraftment in this supportive microenvironment were drastically reduced. An interesting point to note concerning the pan CD44 mAb used in this study is that it is an activating Ab that is thought to induce ligation of CD44, thereby reverting the differentiation of immature AML blasts.

Targeting CD44 with a humanized monoclonal antibody resulted in the complete clearance of engrafted human mammary carcinoma cells or human chronic lymphocytic leukemia (CLL) cells in immune-deficient mice (Weigand et al. 2012; Zhang et al. 2013). In CLL cells, but not in normal B cells, the zeta-associated protein of 70 kDa (ZAP-70), a survival factor that inhibits spontaneous or drug-induced apoptosis, seems to be up-regulated and is found in a complex with CD44. The treatment with already low doses of CD44-specific Ab leads to internalization of the complex resulting in down-modulation of ZAP-70 thereby impairing BCR-dependent survival signaling. This might explain the cytotoxic effect of the CD44 mAb. Preclinical evaluation of the ^{89}Zr-labeled mAb in mice and

in cynomolgus monkeys transplanted with CD44 positive human carcinoma cells or CD44 negative tumor cells revealed a selective targeting of the CD44 positive tumors (Vugts et al. 2014).

Other strategies against CD44

Aptamers

Aptamers are small synthetic molecules (either DNA, RNA or peptides) that have high binding affinity and specificity (similar to mAbs) for target proteins thereby inhibiting their functions (Cox and Ellington 2001). DNA aptamers were isolated based on their binding to exon v10 of CD44 by SELEX technology (Iida et al. 2014). In breast cancer cells, CD44v10 proteins appear to form complexes with the surface protein EphA2, which accounts for the migratory ability of the cells (Iida et al. 2014). This complex formation is impaired by the treatment of the breast cancer cells with the v10-specific aptamers, and consequently, the migration of the breast cancer cells is inhibited. This suggests a potential therapeutic use of the aptamers that should now be tested in vivo.

Peptide-based strategies

The understanding of the molecular mechanism of action of CD44v6 in the activation of RTKs such as Met and VEGFR-2 led to the identification of CD44v6 peptides that inhibit both RTKs (Matzke et al. 2005; Tremmel et al. 2009). Mutational analysis of CD44v6 revealed that three amino acids in the exon v6 region are absolutely required for its co-receptor function for Met and VEGFR-2. Peptides with the minimal length of five amino acids containing these critical amino acids interfere with the co-receptor function of CD44v6 and inhibit vascularization of pancreatic tumors (Matzke et al. 2005; Tremmel et al. 2009) (Fig. 2b). These observations are a further hint for the functional relevance of the co-receptor function of CD44v6 in tumor growth and metastasis and are the basis for the development of therapeutic tools for treatment of pancreatic cancers (http://amcure.com/).

Interestingly, a peptide comprising eight amino acids and derived from human urokinase plasminogen activator (A6) acts in an uPA-independent pathway to inhibit migration, invasion and metastasis of cancer cells (Boyd et al. 2003). This peptide turned out to bind specifically to CD44 (Piotrowicz et al. 2011) and is now examined in a trial phase II study for its efficacy in the treatment of human ovarian cancer (Ghamande et al. 2008; Gold et al. 2012). Another CD44 binding peptide, was identified in an indirect way, namely in a screen for overlapping synthetic peptides from the laminin α5 globular domain. One of the peptides that inhibited tumor growth and lung colonization of B16-F10 mouse melanoma cells was shown to target CD44 (Hibino et al. 2004).

The use of peptides was also proposed to fight against CLL (Ugarte-Berzal et al. 2012, 2014). Advanced stages of CLL and poor survival of patients correlate with elevated levels of (pro)MMP9. This (pro)MMP9 is localized at the membrane of CLL cells and forms a complex with the α4β1 integrin and an isoform of CD44 that accounts for cell survival, cell adhesion and transendothelial migration. The hemopexin domain of (pro)MMP9 contains binding sites for CD44 and the α4β1 integrin. Blocking of the binding sites with specific peptides unraveled the independent contribution of CD44 and α4β1 to the pathogenesis of CLL. However, interference with each binding site independently had only partial therapeutic effects (Ugarte-Berzal et al. 2012). Interestingly, one peptide was able to prevent the binding of both, CD44 and α4β1, and is thus a promising candidate for therapeutic approaches (Ugarte-Berzal et al. 2014). A therapeutic approach for CLL using CD44-specific antibodies has been described in chapter II,2,b.

Another peptide of 24 amino acids was obtained from the sequence of the FKBLP protein, a Hsp90 co-chaperone with anti-angiogenic activity. Similar to the protein, the peptide was anti-angiogenic and inhibited tumor cell migration and tumor growth in two human tumor xenograft models (Valentine et al. 2011). Interestingly, this peptide conferred this inhibition only if the cells express CD44 and prevented HA-induced signal transduction by CD44.

As mentioned in a previous chapter, a 42 amino acid peptide containing three BX_7B HA-binding motifs found also in CD44 induced apoptosis of melanoma cells and thereby inhibited tumor growth in vivo (Xu et al. 2003).

Inhibition of CD44 expression

Instead of interfering with the function of CD44 proteins (e.g., by antibody treatment), the inhibition of their expression in tumor cells is an alternative. A powerful method for such an approach is the delivery or the expression of siRNA in tumor cells. In tumor cell lines, the treatment with siRNA or the transfection of expression vectors for shRNA is a standard tool to examine the relevance of proteins in the transformation process. The involvement of CD44 isoforms in tumor growth and metastatic spreading has been confirmed by such approaches. For therapeutic use, the problem of tumor-specific delivery and/or expression has to be solved.

One example is the down-regulation of CD44v6 by siRNA in a tumor-specific manner (Misra et al. 2009). It is based on nanoparticles coated with transferrin (Tf), an iron-transporting protein binding to Tf-receptors (Bellocq

et al. 2003). Such receptors are highly expressed on tumor cells and mediate the tumor-specific targeting of the nanoparticles. Binding of the nanoparticles to Tf-R induces uptake of the particles into dividing and non-dividing tumor cells via endocytosis. The nanoparticles carried a CD44v6-specific shRNA generator plasmid that is silenced by interrupting sequences. These sequences can be eliminated by expression of the Cre recombinase in a promoter-specific manner. In $Apc^{Min/+}$ mice that develop spontaneously colorectal carcinoma with high expression of CD44 variant isoforms, the tissue-specific delivery of siRNA was performed using a colon-specific promoter. The expression of CD44v6-specific shRNA led to the reduction of tumors in these mice (Misra et al. 2009). This approach is highly flexible since the use of specific shRNA and tissue-specific promoters for expression of the Cre recombinase allow its application for several tumor types and target genes.

A direct delivery of CD44-specific siRNA was mediated by a dendrimer-based nanoparticle used for treatment of ovarian cancer (Shah et al. 2013). The system is based on a polypropylenimine dendrimer as a carrier for the cell death inducing drug paclitaxel, a synthetic analog of the luteinizing hormone-releasing hormone for tumor targeting and CD44-specific siRNA. The efficiency of these combinatorial particles for treatment of ovarian carcinoma was demonstrated in vitro on cells isolated from patients and in vivo in murine xenograft models.

A very efficient means to control gene expression is the use of microRNAs (miRNAs). These RNAs bind to specific sequences in the 3'UTR region of RNAs and either repress or enhance their translation. These miRNAs have pleiotropic actions since binding sites for these miRNAs are not only found on one RNA species but on several RNAs that are most often involved in the regulation of common cellular programs. Importantly, several miRNAs are aberrantly expressed in tumors and metastasis (Nicoloso et al. 2009; Sotiropoulou et al. 2009; Ventura and Jacks 2009) and expression profiles of miRNAs even predict the clinical outcome of neoplasias (Calin and Croce 2006). Interestingly, the majority of miRNAs affected in tumors are down-regulated and function as bona fide tumor suppressor genes. This holds also true for several miRNAs that affect CD44 expression. Examples are miR-34a (Liu et al. 2011), miR-328 (Chen et al. 2014), miR-143 (Ma et al. 2013) and miR-199a-3p (Henry et al. 2010).

miRNA34a is one of the best characterized tumor suppressor within the miRNAs. Ectopic expression of miRNA34a induces cell cycle arrest, apoptosis and inhibits cancer proliferation, migration and metastasis in a variety of cancer types (Hermeking 2010). This miRNA regulates several target RNAs involved in cell proliferation, survival and migration among which the cyclin-dependent kinases, the RTK Met, Bcl-2, Myc and CD44. The miRNA34a gene was cloned into an expression plasmid driven by a breast cancer-specific promoter to allow expression in breast cancer cell lines (Li et al. 2012). Transfection of several breast cancer cell lines with this expression vector resulted in growth arrest and apoptosis in vitro. Most importantly, in an orthotopic mouse model of human breast cancer, the injection of liposomal complexes of the miRNA34a expression plasmid resulted in reduced tumor size and extended life span of the animals with only minor side effects (Li et al. 2012). Similarly, the systemic application of miR-34a complexed with a lipid-based delivery agent in orthotopic tumor models of prostate cancer in mice resulted in inhibition of tumor growth and metastasis and an extended life span (Liu et al. 2011).

CD44 as a stem cell marker

Many studies within the last years have identified cells in tumors with stem-like characteristics, so-called CSCs or TICs. These cells have self-renewal capacity; the potential to give rise to several cell types within a tumor; and account for tumor initiation, tumor recurrence, tumor metastasis and the resistance of tumors to chemo-or radiotherapy. These CSCs should be the target of efficient tumor therapies. In many tumors including breast cancer HNSCC and colorectal cancer, CD44 have been identified as a marker on CSCs although in most cases the specific isoforms are not known (reviewed in Trapasso and Allegra 2012; Williams et al. 2013; Woodward and Sulman 2008). The role of CD44 in cancer stemcellness is, however, not yet unraveled. Interestingly, in HNSCC and pancreatic cancer cells, Met has also been identified as a marker of CSCs. Met positive HNSCCs have self-renewal capacity, form spherical colonies, are highly chemo-resistant and their transplantation into immunosuppressed mice leads to metastasis (Sun and Wang 2011). Pancreatic cancer cells that express Met and CD44 have the capability of self-renewal and show the highest tumorigenic potential of all cell populations (Li et al. 2011).

CD44v6 isoforms have been identified as markers of CSCs in colon cancer and account for the metastatic propensity of the tumors (Todaro et al. 2014), suggesting that CD44v6 targeting in colon cancer is a promising therapeutic approach. Indeed, CD44 variant isoforms (the one tested was CD44v4–10) but not CD44s in the intestinal stem cells in the crypts of $Apc^{Min/+}$ mice, that are prone to develop colorectal cancer, account for tumor formation and relapse controlling the balance between cell survival and apoptosis (Zeilstra et al. 2008, 2014). Indeed, a therapeutic approach targeting CD44v6 by means of shRNA in $Apc^{Min/+}$ mice inhibited the development of colorectal cancer and is described in chapter III). In human gastro intestinal cancer

cells, however, a CD44v8–10 variant isoform seems to be characteristic for CSCs rendering the tumor cells resistant to chemo- or radiotherapy by a mechanism that regulates the redox status of the cells (Ishimoto et al. 2011). It is most probable that the various therapeutic approaches that we have discussed in this review target CSCs in the tumors even if this has not been directly proven and are therefore efficient.

Outlook

Since the discovery of CD44 and in particular of CD44v6 isoforms as prognostic markers in a variety of cancers, several approaches have been developed to target them. Although only few approaches have made it so far to clinical trials, the scientific progress in the last years suggests strong perspectives in anti-CD44 therapies. This, on the one hand, is due to the identification of CD44 isoforms (including CD44v6) as functional markers for CSCs in several human tumors and on the other hand to the molecular functions of CD44 isoforms as multidomain platforms integrating extracellular cues with growth factors, cytokines and metalloproteinases. These findings suggest that CD44 is one of the main players in tumor growth and in the most life-threatening steps of cancer, namely metastasis. The detection of CD44 on CSCs was most of the times performed with antibodies that recognized all CD44 isoforms. Since CD44s is expressed ubiquitously in tissues, there is an urgent need to define which CD44-specific isoforms are present on these CSCs. Only then will the specific strategies be directed more selectively against tumor cells.

Acknowledgments Special thanks to David Koschut for the help with the figures. The group of Véronique Orian-Rousseau is supported by the Deutsche Forschungsgemeinschaft and the Mildred Scheel Foundation.

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