

## THE CD44 PROTEIN FAMILY: ROLES IN EMBRYOGENESIS AND TUMOR PROGRESSION

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### 1. ABSTRACT

Members of the CD44 protein family are engaged in various physiological and pathological processes such as lymphocyte activation, lymphocyte homing, wound healing, delayed type hypersensitivity, apoptosis, tumor progression and limb development. Here we summarize observations which lead to a molecular understanding of the function of CD44 in some of these physiological processes. The most striking function for a CD44 variant protein is its presentation of growth factors to high affinity receptors on mesenchymal cells in the developing limb. Furthermore we summarize the experiments that allowed the identification of CD44 variant exon encoded sequences which account for differentiation-specific and inducible splice regulation.

### 2. INTRODUCTION

The major challenge in the CD44 field originates from the fact that CD44 comprises a large family of proteins, the different members of which fulfill quite different functions. Functional differences are based on structural differences due to alternative splicing to which more than 50% of the 20 exons of the CD44 gene are subjected, and to posttranslational modifications. The molecular properties linked to CD44 are not static but subject to changes. For example, the N-terminal domain of CD44 carries a basic amino acid motif which, in principle, has affinity for glycosaminoglycans. Non-activated lymphocytes express CD44s (smallest CD44 splice form)

but do not bind hyaluronate (HA). Activation of lymphocytes with a tumor promoter or Concanavalin A leads to HA binding of lymphocytes (1-4). On the same line of observations, the transfection of a CD44 splice variant into two different cell lines lead to HA binding in one, but not in the other (5).

Examples of molecular properties provided by different CD44 splice forms are manifold (for review see 6). Although the precise functions of individual splice forms in nearly all physiological contexts are not known, transfection experiments and the use of antibodies discriminating between different splice forms have suggested specific functions for some CD44 variants in particular physiological conditions. Most striking is a specific role of splice forms in tumor progression and in metastasis formation in rat pancreas carcinoma (7). The smallest splice form CD44s appears not to be involved in this process. Furthermore, on many human tumor cells expression of larger CD44 splice forms (CD44 variants, CD44v) is upregulated in comparison to their tissue of origin. Another striking example of a particular role of some CD44 splice forms but not of others is found in the activation of the immune system. The immune response leads to upregulation of CD44 variants that are not expressed in resting lymphocytes and dendritic cells. CD44 variant specific antibodies can abrogate the immune response (8,9). It is already evident from the current limited knowledge of the molecular properties of CD44 that a given CD44 protein fulfills more than one molecular function, e.g. in the immune system.

### 3. CD44 FUNCTIONS

#### 3.1. CD44 in lymphocyte rolling

Despite the many papers dealing with biochemical functions of CD44 proteins there are only few papers in which the molecular mechanism of action of CD44 proteins in a physiological context are addressed. One example in this regard is the rolling of a subpopulation of lymphocytes, which is dependent on CD44 (10,11). In an *in vitro* assay the rolling of a subpopulation of lymphocytes as well as of B and T cell lines depended on CD44 and its binding to HA (10). Effective rolling required activation of the lymphocytes which is the step that induces binding of lymphocytes to HA (12). The relevance of this processes for the extravasation of activated T cells into inflammatory sites *in vivo* has been demonstrated in mice using superantigen stimulation (11). Interestingly, in accordance with the role of CD44-HA interaction for lymphocyte extravasation, proinflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$  and LPS specifically induce the synthesis of HA in vascular endothelium (13). The question as to which isoform of CD44 might be engaged in the rolling process has not been so far addressed. Since there are quite contradictory reports on the ability of particular splice forms of CD44 to bind to HA (see e.g. 5,14-16) and the activation of lymphocytes induces the expression of splice variants in addition to CD44s, the expression pattern of CD44 should be carefully studied.

#### 3.2. CD44s in tumorigenesis

The binding of cells to hyaluronate mediated by CD44 has been proposed to be a decisive step in tumor formation (17,18). In both papers transfection with CD44 isoforms capable of binding to HA conferred on cell lines the ability to form experimental and spontaneous metastases into the lungs. Tumor formation was abrogated by the simultaneous intravenous injection of soluble CD44 forms, again only those that are capable of binding to HA. In these systems the isoform responsible for tumorigenicity appeared to be CD44s. In line with these results, in a metastatic murine mammary carcinoma cell line expressing CD44 co-expression of soluble CD44s abrogated the outgrowth of experimental pulmonary metastases (19). The soluble CD44s protein inhibited binding and internalisation of hyaluronan and blocked invasion of cells into a hyaluronan-producing cell monolayer. The transfectants adhered to pulmonary endothelium and penetrated interstitial stroma similarly to parental tumor cells but then underwent apoptosis with high frequency. These data suggest that the CD44s-mediated interaction of tumor cells with HA might be required for tumor cell survival in invaded tissue. A role for CD44 in protection against apoptosis has also been described for lymphocytes (20). Immature thymocytes and peripheral T lymphocytes can be triggered to apoptose by treatment with mAbs directed against CD3 or with glucocorticoid hormone. Apoptosis can be prevented if CD44 on the cells is stimulated by HA or by certain anti CD44 monoclonal antibodies (mAbs). Most likely, the target molecule conferring the protection is CD44s.

#### 3.3. CD44v in metastasis formation

As mentioned above, we have identified splice variants of CD44 containing variant exon v6 and v7 sequences (alone or together with other variant sequences) on a rat pancreatic carcinoma cell line with metastatic capacity but not on its non-metastatic counterpart (7). The expression of each one of the CD44 proteins in the non-metastatic tumor cell sufficed to establish the metastatic potential (7,21). The comparison of HA binding by the two parental cell lines and by a variety of transfectants containing expression constructs encoding various CD44 isoforms revealed that only the metastasizing parental line or transfectants expressing CD44 variant sequences of exon v6 and v7 together showed strong affinity to HA (5,22). The binding to HA through CD44s, which is expressed on both the metastasizing and non-metastasizing cell lines, is rather weak. These results suggested that the HA binding mediated by the CD44 variants could account for metastatic spreading. To prove this assumption, cell lines that were transfected with CD44 variants and thereby acquire the ability to bind to HA and to metastasize were further transfected with a human hyaluronidase expression clone (23). The expression of the surface-localized hyaluronidase in fact abrogated HA binding. Interestingly, neither tumor growth nor metastatic spreading was influenced by the expression of the hyaluronidase. This suggests that, at least in this system, tumor formation and metastatic spreading are independent of HA binding, and that the molecular functions of CD44 variants mediating metastasis formation are independent from HA binding. In line with this result is the observation that a CD44 exon v6-specific antibody that blocks metastasis formation in this tumor system (24,25) did not interfere with HA binding (23). Furthermore the HA binding ability of several rat tumor cell lines did not correlate with their metastatic capacity (23).

#### 3.4. CD44v on lymphocytes

In order to investigate CD44v properties important in the formation of metastases, we resorted to studying defined physiological roles. This was necessitated by the fact that the metastatic process is enormously complex. Since tumor cells make use of molecules (and their functions) that are also expressed under normal physiological conditions, either in restricted tissues in the adult or during embryogenesis, one might expect that the study of their function under physiological conditions might lead to an understanding of the molecular mechanism important in both the physiological condition and in cancer. In contrast to the ubiquitous expression of CD44s, CD44 variants are expressed in only few tissues (26). For example, lymphocytes upon activation express transiently CD44 variants (8). In an attempt to understand the function of this transient upregulation of CD44 variants on lymphocytes we have created transgenic mice overexpressing the CD44v4-v7 variant (a variant that was shown to induce tumor metastasis formation) under the control of the Thy-1 promoter. Consequently, the transgenic animals expressed the CD44v4-v7 variant constitutively and more or less exclusively on T

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lymphocytes (27). Unfortunately, these transgenic animals exhibited only a very mild phenotype, in that their immune response was accelerated as compared to control (27). It is however unlikely that this acceleration suffices to explain the rather dramatic effect of the antibody in suppressing the immune response.

### 3.5. CD44v in dendritic cells

Another rather interesting observation arose when the behavior of dendritic cells were examined. Dendritic cells of the skin for instance carry a specific expression pattern of CD44 variant exon epitopes on their surface. Most epitopes appear on the surface after activation (antigen contact) and is maintained until their arrival in lymph nodes. I.v. injected anti v6 or anti v4 antibodies severely reduced the hapten-specific primary immune response (9). Although these expression patterns in lymphocytes and dendritic cells do not unravel the molecular function of CD44 proteins, a plausible proposal can be derived from an observation in a fibroblast cell line. Its migration through matrigel depends on the expression of c-fos which upregulates CD44 and was prevented by antisense CD44 (28). One may speculate about a link of CD44 expression to invasive and migratory behavior, e.g. of dendritic cells.

### 3.6. CD44v in keratinocytes

The heterogeneity of CD44 more and more supports the notion that the proteins exert several molecular properties. Studies on skin have illustrated this point. Skin tissue strongly expresses CD44 variants (29). More specifically keratinocytes express predominantly a CD44 variant containing all variant exon sequences. Binding studies of keratinocytes to carcinoma cell lines expressing different CD44 isoforms revealed that keratinocytes prefer to bind to cells expressing CD44 variants but not CD44s (29). This binding could be inhibited by an excess of hyaluronan or by digestion with hyaluronidase. More specific information on the function of CD44 proteins on keratinocytes was obtained by selective suppression of CD44 expression in keratinocytes of mice. A CD44 antisense sequence under the control of the keratin-5 promoter was introduced into mice as a transgene (30). This transgene selectively abrogated CD44 expression in keratinocytes and corneal epithelium. Lack of CD44 expression led to abnormal hyaluronate accumulation in the superficial dermis and corneal stroma, to morphological alterations of basal keratinocytes and cornea and to reduced keratinocyte proliferation in response to mitogen and wound repair. The transgenic animals have an impaired local inflammatory response and retarded wound healing, a decrease in skin elasticity, delayed hair regrowth, and the epidermis does not respond by hyperplastic changes to carcinogen treatment. Although we do not know whether the phenotype is a direct consequence of CD44 abrogation or more downstream of CD44, the most straightforward suggestion would involve CD44 proteins in keratinocyte proliferation in response to extracellular stimuli, and in the maintenance of correct hyaluronan distribution.

### 3.7. CD44 in embryogenesis

From the onset of embryogenesis, CD44 is expressed in both embryonic and extraembryonic tissue (31). Similar to the situation in adult organisms, the expression of CD44 variants in embryogenesis is restricted to few tissues, predominantly to instructive epithelia which control tissue outgrowth of mesenchymal origin (32-35). One such structure is the apical ectodermal ridge (AER), a specialised end-differentiated ectoderm located at the distal tips of developing limb buds. The AER is critical for the maintenance of proliferation in the underlying mesenchyme (36-38). Since the AER expresses large quantities of CD44 variants and the function of the AER seems straightforward we decided to study the functional role of CD44 variants in the AER.

The first clue with respect to relevance of the CD44 variant proteins for function of the AER was obtained by transplantation experiments. When the AER was removed from the embryo just prior to the onset of limb budding, then soaked with a CD44 v6 or v3 specific antibody and subsequently grafted back onto the embryo, bud formation was strongly inhibited as compared to either untreated limbs or AER soaked with isotype-matched antibodies (34,35). Interestingly, the function of the AER to induce limb bud formation can be substituted by authentic growth factors as heparan-coated beads carrying either FGF2, FGF4 or FGF8 either induce the ectopic development of limbs when inserted into the flank of chick embryos, or sustain limb development when placed on limb buds lacking an AER (39-46). These growth factors are in fact physiologically expressed in the AER (47-49). A characteristic feature of these growth factors is that they require heparanised proteins as low affinity receptors prior to activation of their specific high affinity receptors (50-52). Note that heparan-coated beads are used in the implantation experiments described above.

Since some of the CD44 splice variants show enhanced binding to heparan sulfate (22) and others containing exon v3 sequences can be modified by heparan sulfate (53,54) both of which could serve to sequester heparin-binding growth factors, we wondered whether CD44 protein in the AER would act as low affinity receptors for FGFs. This in fact seems to be the case (35). The major growth factor in the AER, FGF8, colocalizes with CD44. Furthermore, one of the CD44 proteins in the AER contains exon v3 sequences and is therefore heparan sulfate modified. Moreover, the ability of the AER to enhance proliferation of primary limb mesenchymal cells in culture could be substituted for by the addition of FGF8 plus a cell line that expresses a CD44 exon v3 containing protein, but not by cells expressing CD44s or CD44v lacking v3 sequences. FGF8 alone and transfected cells alone did not suffice for the induction of proliferation of the mesenchymal cells. FGF8 binds to these transfectants in a heparan sulfate dependent manner. The presentation of FGF to mesenchymal cells, but not its binding, is inhibited by a CD44 v3 and v6 specific antibody. These experiments describe the first *in vivo* evidence for the limiting function of a cell-bound heparan sulfate proteoglycan (CD44v). It is also the first example in which the presentation of a growth factor on one tissue, the AER, leads to activation of a high affinity receptor on another tissue, the mesenchyme.

### 3.8. CD44 growth factor presentation in tumorigenesis: a hypothesis

The CD44 v6 specific antibody used for the inhibition of AER dependent limb outgrowth and for the inhibition of presentation of FGF to mesenchymal cells has previously been used to block tumor metastasis formation in vivo (25) and to interfere with the activation of immune cells (8). Whether in all these systems the common biochemical function of CD44 variant proteins is the presentation of growth factors as has been shown for limb outgrowth remains to be proven. Binding of heparan sulfate binding growth factors to heparan sulfate modified CD44 proteins could also be the mode of action in other tissues. For instance, the heparin binding growth factor HB-EGF may require CD44 variants expressed on keratinocytes for its activity is maintaining proliferation of keratinocytes in normal and pathological situations. Furthermore, the macrophage inflammatory factor 1beta (MIP-1beta) has been shown to bind to CD44 (55) and it has been suggested that MIP-1beta is sequestered by epithelial cells through heparin-sulfate modified CD44 proteins which may present the cytokine to T-cells and thereby induce their extravasation (55). This concept of growth factor presentation could be further expanded to CD44 variants that are by themselves not heparan-sulfate-modified. Such variants acquire the ability to bind to glycosaminoglycan modifications that the CD44s molecule cannot bind (22). CD44 variants are upregulated in lymphocytes upon activation and on tumor cells during tumor progression (reviewed in 6). It is thus tempting to speculate that tumor cells make use of CD44 for growth factor action as one of perhaps several choices. These upregulated variants could sequester cytokines or growth factors necessary for lymphocyte function or tumor cell colony formation in organs into which they have invaded.

### 3.9. CD44 variant splicing mediated by a composite splice element

The search for specific molecular functions of CD44 splice variants is one major area of CD44 research. A different question asks how alternative splicing of pre-mRNA is regulated. CD44 pre-mRNA is to our knowledge the champion in alternative splicing. In tumorigenesis one could imagine that mutations in the gene might account for changes in the splice pattern - although it is hard to understand why in most tumors not only one new splice variant but several different ones are expressed simultaneously. Under physiological conditions, such as differentiation in keratinocytes or the reversible induction of alternative splicing e.g. by activation of lymphocytes, the basis for alternative splicing has to be a regulatory change in the splice mechanism.

To get an impression whether inclusion of CD44 variant exons is co-dominant or even dominant over excision, two cell lines expressing different splice patterns were fused and examined for changes in the splice pattern in the heterokaryons (56). To avoid chromosomal loss the analysis was performed in transient heterokaryons prior to fusion of the nuclei. To be able to distinguish events

occurring in one nucleus (e.g. the one that expressed only CD44s prior to fusion) cell lines originating from different species (human and rat) were used. This also allowed the separation of interspecies heterokaryons by selection with human and rat CD44 specific antibodies. The analysis of such transient heterokaryons revealed that the ability of a human cell line to include CD44 variant exons was imposed on a rat nucleus in the heterokaryon that by itself expressed only CD44s. This result indicates the existence of trans-acting factors that induce the inclusion of CD44 variant exons in a dominant way. The same result was obtained with several tumor cell lines. Interestingly the splice pattern of one tumor line, which was a candidate to carry cis-acting mutations, could neither be changed in its splice pattern nor could it transmit its splice specificity to other nuclei (56). Taken together these results make it appear feasible to clone factors of the splice regulatory cascade upon cDNA transfection into appropriate cells and examination for changes in splicing.

An independent approach for the identification of components of splice regulation is the definition of the RNA elements that determine the inclusion or excision of exons. Once these sequences had been identified they can be used in binding assays, or in three hybrid assays in yeast (57), to identify factors regulating the splicing. The variant region of CD44 comprises about 25 kb of DNA sequences (58,59) containing 10 variable exons all of which are subjected to alternative splicing. This complexity was reduced by subcloning one variant exons (v5) that is frequently included in tumor progression and upon lymphocyte activation together with surrounding intron sequences into an exon trap vector (60). Introduction of this construct into cell lines revealed that in cell lines that express CD44v the v5 sequences of the exon trap vector are included into RNA whereas in cell lines that express only CD44s the v5 sequences are excised from the RNA. Furthermore, in a T cell line in which the splice pattern for CD44 can be switched from CD44s expression to CD44v expression by activation with CD3 mAbs, or phorbol ester treatment, or activation of Ras signalling, splicing of v5 within transcripts derived from the exon trap vector proceeded as expected. Deletion mutants revealed that the sequences responsible for regulated exon v5 inclusion or excision are exclusively located in the exon. The 117 bp region of the exon can be subdivided into three sequences. The 40 bp at the 3' end are required for recognition of the exon as being an exon. The splice donor and acceptor sites are „weak“ sites and they require accessory recognition proteins (61,62). The 40 bp at the 5' end of the v5 exon are silencer sequences, as binding of factors to these sequences repress inclusion of exon v5 sequences and the function of these factors has to be abrogated for exon inclusion to occur. Finally, the sequences between the 5' motif and the 3' motif are required to „bridge“ the repression to the recognition. Removal of these central sequences still results in an regulated exon, but substitution by unrelated sequences results in constitutive exon inclusion (60). From these results it seems that the silencer sequences in the exon are the decisive ones that regulate exon inclusion in a cell type-specific manner and under conditions of induced splicing.

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### 4. PERSPECTIVES

Members of the CD44 protein family containing variant exon sequences are involved in tumor progression and metastasis formation and are thus targets for diagnosis and therapy. Understanding of their molecular function and the regulation of alternative splicing that accounts for variant exon inclusion is pivotal for the development of effective therapeutic regimens. The mechanism of presentation of growth factor by a CD44 variant located on the ectodermal cells in the limb to high affinity receptors on mesenchymal cells to keep them in proliferation is the first example of a molecular action of a CD44 variant *in vivo*. Inhibition of limb outgrowth by CD44 variant specific antibodies and the retardation of tumor metastasis formation by the same antibodies suggest that the mode of action of CD44 variants in tumor progression might also involve a growth factor presentation mechanism.

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