MOLECULAR MECHANISMS REGULATING THE LIGAND BINDING FUNCTION OF CD44

by

ROLAND K. CHIU
B.Sc. (Hon), Simon Fraser University, 1991
M.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology and Laboratory Sciences)

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The University of British Columbia
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Vancouver, Canada

Date March 19/99

DE-6 (2/88)

ABSTRACT

CD44 is a broadly distributed cell surface glycoprotein that has been shown to play an important role in many adhesion-dependent cellular processes including wound healing, lymphocyte and progenitor cell homing, and tumour metastasis. Defining the precise molecular mechanisms that regulate the ligand-binding function of CD44 is a critical step to the understanding of its role in these diverse processes.

Binding of the principal ligand for CD44, hyaluronan, is a highly regulated process. Although all cells that express CD44 on their surface, present the same conserved amino terminal hyaluronan recognition motif, most do not bind this ligand constitutively. To further explore this fact, several cell lines with differing hyaluronan binding abilities were examined. These studies have demonstrated that changes in avidity achieved through increased transcription and/or aggregation in the plane of the membrane play a critical role in regulating the hyaluronan binding activity of the molecule.

Correlations have been noted between the expression of certain alternatively spliced CD44 isoforms and the metastatic propensity of various histologically distinct tumour cell types. The precise mechanism by which particular CD44 isoforms contribute to the metastatic process is, however, unclear. The studies presented in this Thesis demonstrate that exon v10 containing CD44 isoforms promote cell-cell aggregation through the recognition of chondroitin sulfate presented by CD44 itself. These data help explain the differential involvement of v10 containing CD44 isoforms in tumour metastasis.

Soluble CD44 proteins generated by proteolytic cleavage or aberrant intron retention have been shown to antagonize the ligand binding activity of the corresponding cell surface receptor, inducing apoptosis and inhibiting tumour growth. Interestingly, such findings appear to contradict recent studies demonstrating a correlation between the presence of high levels of soluble CD44 in the serum of cancer patients, and poor prognosis. In this Thesis, a novel naturally occurring soluble CD44 isoform generated by alternative splicing of the "constant exons", was cloned and analyzed. This molecule, designated CD44RC, markedly enhances the hyaluronan binding function of cell surface CD44. CD44RC induces the aggregation of cell surface CD44 via a mechanism that involves the recognition of chondroitin sulfate side chains.

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ABBREVIATIONS

ATCC	American Type Culture Collection
CCII	Cool Calf II
CS'ase	chondroitinase ABC
DMEM	Dulbecco's minimum essential medium
ECM	extracellular matrix
ECMRIII	extracellular matrix receptor III
ERM	ezrin, radixin and moesin family
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FITC-HA	fluorescein isothiocyanate conjugated hyaluronan
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
gp-85	glycoprotein-85
HA'ase	hyaluronan lyase
HBSS	Hank's balanced salt solution
H-CAM	homing cell adhesion molecule
HEV	high endothelial venule
ICAM	intercellular cell adhesion molecule
IFN-γ	interferon-gamma
IL-2Rα	interleukin-2 receptor α
IMDM	Iscove's modified Dulbecco's media
kDa	kiloDalton
mAbs	monoclonal antibodies
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
pgp-1	phagocytic glycoprotein-1
PHA	phytohemagglutinin
PI	propidium iodide
PKC	protein kinase C
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
RHAMM	receptor for hyaluronan-mediated motility
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sLe^x	sialyl Lewis^x
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
[^{3}H]TdR	[methyl-^{3}H]-thymidine deoxyribose

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Graeme J. Dougherty for all his support and encouragement throughout my stay in his lab.

I would also like to thank the members of my advisory committee, Drs. Janet Chantler, Mladen Korbelik, Fumio Takei and Hermann Ziltener for all their helpful and guidance during the course of my project.

And finally, a big thanks to my wife Grit and two sons Nicholas and Thomas, for all their love, support and constant, unwavering patience and tolerance.

CHAPTER I

INTRODUCTION

Adhesive interactions play an essential role in regulating the proliferation, differentiation, migration and functional activity of normal hemopoietic cells, and alterations in the expression, and/or appropriate functioning of the cell surface molecules that mediate such interactions, have long been suggested to contribute to the development and/or pathogenesis of hemopoietic malignancies. Among the molecules thought to be involved in such processes, particular interest has focused on the polymorphic cell surface glycoprotein CD44. Monoclonal antibodies (mAbs) directed against this molecule have been shown to block lymphopoiesis and myelopoiesis in murine long term cultures (Miyake *et al.*, 1990a; Miyake *et al.*, 1990b), and to inhibit both the attachment of hemopoietic cells to vascular endothelial cells *in vitro* (Jalkanen *et al.*, 1987; Stoolman, 1989; Yednock and Rosen, 1989), and their migration into sites of inflammation *in vivo* (Camp *et al.*, 1993). Crosslinking of CD44 has also been shown to transduce signals that may directly or indirectly alter the proliferation and/or activity of various hemopoietic cell types (Lesley *et al.*, 1993). Presumably reflecting these diverse functional roles, correlations have been found between the expression of CD44, or particular CD44 isoforms, and the metastatic propensity of certain hemopoietic and non-hemopoietic malignancies (Herrlich *et al.*, 1993).

At present, the molecular mechanisms that regulate the adhesive function and ligand-binding specificity of CD44 remain largely undefined. Only through a better understanding

of these processes will it be possible to determine the precise role played by this molecule in controlling the behavior of normal and malignant hemopoietic cells.

1.1 Convergent studies of CD44

CD44 was originally defined by Dalchau and colleagues (Dalchau *et al.*, 1980) as a broadly distributed human cell surface glycoprotein reactive with the mAb F10-44-2. Many other independently characterized mAbs are now known to also recognize CD44 and have been instrumental in demonstrating an important role for this molecule in various adhesion-dependent cellular processes including lymphocyte and progenitor cell homing, tumour metastasis, lymphocyte and macrophage activation, and hemopoiesis (Haynes *et al.*, 1989; Herrlich *et al.*, 1993). Reflecting the particular assay system originally employed to identify the molecule, CD44 has been given numerous designations including $gp90^{Hermes}$, extracellular matrix receptor III (ECMRIII), homing cell adhesion molecule (H-CAM), phagocytic glycoprotein-1 (pgp-1), glycoprotein 85 (gp-85), Ly-24, hyaluronate receptor, HUTCH-1, and In (Lu)-related p80 glycoprotein. Comparison of the cellular reactivity of the various mAbs used in these studies allowed the CD44 cluster designation to be assigned at the Third International Workshop on Leukocyte Typing held in Oxford, England in 1987 (Cobbold, 1987).

1.2 Structure of CD44

CD44 is very polymorphic, and species ranging in size from 80 to 250 kDa have been detected on various normal and transformed cell types (reviewed in Lesley *et al.*, 1993). CD44H, the most prevalent form expressed on the majority of resting hemopoietic cells migrates at 85 to 95 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). cDNA clones encoding this molecule were first isolated by two independent groups (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989). Stamenkovic and colleagues (Stamenkovic *et al.*, 1989) utilized a eukaryotic expression cloning strategy in which COS cells transfected with pCDM8-based cDNA libraries prepared from the histiocytic lymphoma cell line U937, the B lymphoblastoid line JY, the Burkitt's lymphoma line Raji, and the myeloid leukemia line KG1 were screened for reactivity with the anti-CD44 mAb J173 (Pesando *et al.*, 1986). A 1354 nucleotide cDNA terminating in a short poly(A) tail was isolated. The predicted sequence contained a single long open reading frame of 341 amino acids corresponding with a typical type I integral membrane protein (Figure 1). The extracellular domain of 248 residues is followed by 21 mostly hydrophobic amino acids, corresponding with the predicted membrane-spanning domain. The intracellular domain consisting of 72 predominantly hydrophilic residues, has a calculated isoelectric point of 8.17 (Yonemura *et al.*, 1998).

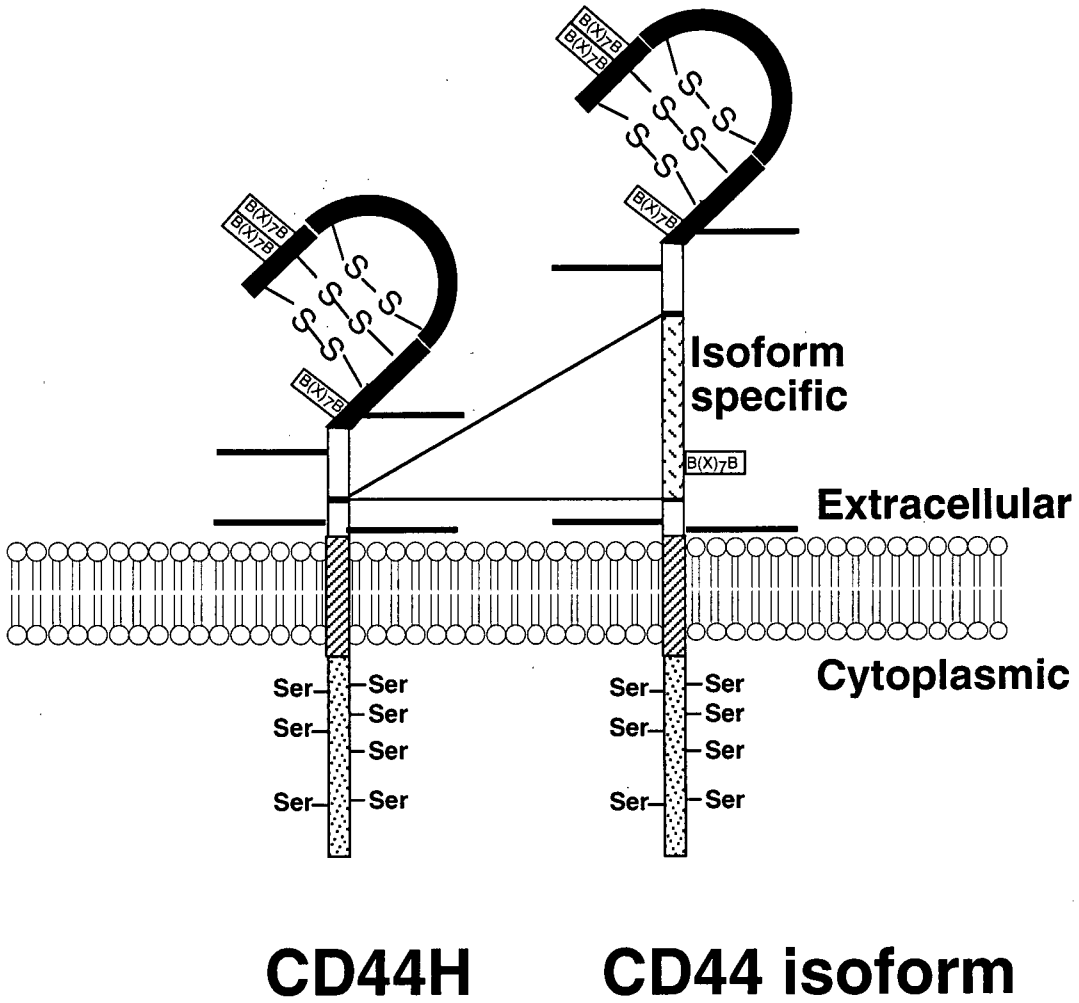


Figure 1: Structure of the CD44 protein. CD44 species range in size from 80-250 kDa. The major form expressed by most resting hemopoietic cells (CD44H; left) has an apparent molecular mass of 80-90 kDa. Higher molecular mass isoforms are generated by the alternative splicing of at least 10 exons producing additional isoform-specific sequences of varying lengths that are inserted into a single site within the extracellular domain proximal to the membrane spanning domain (right). The extracellular domain contains 6 potential sites of N-linked glycosylation, numerous sites of potential O-linked glycosylation, and 4 serine-glycine motifs (dark bar), that may serve as sites of chondroitin sulfate attachment. The cytoplasmic tail contains 7 serine residues (Ser) that may be important for cell signaling. The N-terminus contains 6 cysteine residues (S) that are important in forming the tertiary structure thereby presenting a tandemly repeated domain implicated in hyaluronan binding [$B(X)_7B$].

Goldstein and coworkers (Goldstein *et al.*, 1989) also reported the isolation of the CD44 cDNA. The human B lymphoblastoid cell line, KOA was used as the source of poly(A)-selected RNA for the production of a cDNA library in the λgt11 expression vector. Recombinant plaques were screened using a polyclonal anti-gp90^{Hermes} serum (Jalkanen *et al.*, 1987). The predicted amino acid sequence for this clone contained an open reading frame that would encode a peptide of 293 residues. This clone was identical to the CD44 clone isolated by Stamenkovic and colleagues except it contained a truncated cytoplasmic domain consisting of only 3 amino acids (Stamenkovic *et al.*, 1989). Subsequent studies have suggested that this latter cDNA correspond to a rare transcript that is generated in some cell lines by alternative splicing (see below).

The predicted core protein of CD44 is expected to be approximately 37 kDa and can be subdivided into several domains (Zhou *et al.*, 1989) (Figure 1). The CD44 protein has been demonstrated to be acidic, with estimates of isoelectric point ranging from 4.2 to 5.8 (Jalkanen *et al.*, 1988; Kalomiris and Bourguignon, 1988; Picker *et al.*, 1989; Culty *et al.*, 1990).

1.2.1 Extracellular domain

The extracellular domain of CD44 contains several interesting features. A stretch of amino acid residues between 12 and 101 are predominantly hydrophobic and shows 89% sequence similarity between mouse and human (Zhou *et al.*, 1989). Six cysteine residues are located in the N-terminus region, which may be important in the generation of a functional

tertiary structure (Zhou *et al.*, 1989). The extracellular domain is extensively modified by O- and N-linked glycosylation (Goldstein *et al.*, 1989). Six potential N-linked glycosylation sites of Asn-X-Ser/Thr, are observed (Goldstein *et al.*, 1989). The region proximal to the membrane-spanning domain positioned between amino acids 102 to 248 demonstrates only approximately 45% sequence identity between human and mouse (Zhou *et al.*, 1989). This region contains four serine-glycine dipeptides in the human CD44 and three in the mouse, that are potential sites for chondroitin sulfate attachment (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989; Zhou *et al.*, 1989). Heparan and keratin sulfate have also been demonstrated to modify the extracellular domain of CD44 (Brown *et al.*, 1991). The location for the insertion of sequences generating higher molecular mass CD44 isoforms are found proximal to the transmembrane domain between amino acids 202 and 203 of the mature CD44 protein, and will be discussed below.

1.2.2 Transmembrane domain

The 21 amino acid hydrophobic transmembrane region demonstrates 100% sequence identity between human and mouse (Zhou *et al.*, 1989). Interestingly, this domain contains a cysteine residue, which is conserved in all known mammalian CD44. Liu and colleagues (Liu and Sy, 1996) demonstrated that a site-specific CD44 mutant, in which this cysteine residue was converted to an alanine, was non-functional as compared to wild type CD44 when stably expressed in the CD44-negative cell line Jurkat. These results provide evidence that the transmembrane domain of CD44, more specifically the cysteine residue in the transmembrane domain, is important for regulating CD44 function (Liu and Sy, 1996). The

significance of this cysteine suggests that hyaluronan binding by CD44 is facilitated by homotypic dimerization with another CD44 molecule or heterotypic aggregation with an unrelated molecule.

1.2.3 Cytoplasmic domain

The 72 amino acid cytoplasmic domain is highly conserved, demonstrating 80 to 90% sequence identity among species studied to date (Stamenkovic *et al.*, 1989; Screaton *et al.*, 1992). Seven serine residues exist in the intracellular domain of human CD44. Five of these residues are conserved in, mouse, baboon, cow, and hamster (Idzerda *et al.*, 1989; Stamenkovic *et al.*, 1989; Zhou *et al.*, 1989; Aruffo *et al.*, 1990; Bosworth *et al.*, 1991) while four are conserved in the rat (Gunthert *et al.*, 1991). The serine residue at position 296 is not phosphorylated in intact epithelial cells (Neame and Isacke, 1992) although it is a potential substrate for cAMP- and cGMP-dependent protein kinases (Wolffe *et al.*, 1990). Neame and Isacke have demonstrated that both Ser^{303} and Ser^{305} may be phosphorylated and that mutation of either residue disrupts the ability of CD44 to be phosphorylated in epithelial cells (Neame and Isacke, 1992). Furthermore, Camp and colleagues (Camp *et al.*, 1993) have shown that a CD44 mutant, in which Ser^{305} was changed to an alanine residue, was not phosphorylated when transiently expressed in COS cells. A concurrent study using a CD44 mutated at the serine residue to an alanine at position 303 merely reduced phosphorylation. These data suggest that both Ser-303 and Ser-305 may be important for the phosphorylation of the cytoplasmic tail of CD44. Furthermore, consensus phosphorylation sites for protein kinase A and C, and cAMP- and cGMP-dependent protein kinases, are found in the

cytoplasmic tail. The effect of phosphorylation on this molecule is, however, not well defined.

Several studies have been suggested that in at least some cell lines a proportion of CD44 molecules may be resistant to detergent solublization, lending support to the belief that CD44 is associated with the cytoskeleton. Further studies have indicated that the cytoplasmic tail of CD44 can associate with various cytoskeletal elements including actin and ankyrin (Lacy and Underhill, 1987; Kalomiris and Bourguignon, 1988; Lokeshwar and Bourguignon, 1991; Bourguignon *et al.*, 1991; Lokeshwar and Bourguignon, 1992; Bourguignon *et al.*, 1992; Bourguignon *et al.*, 1993; Lokeshwar *et al.*, 1994). This association appears to be regulated by a number of modifications including PKC-mediated phosphorylation (Kalomiris and Bourguignon, 1989; Bourguignon *et al.*, 1992), palmitoylation (Bourguignon *et al.*, 1991), and GTP binding (Lokeshwar and Bourguignon, 1992; Galluzzo *et al.*, 1995).

The cytoplasmic domain of CD44 can also associate with the cytoskeleton by binding to the ERM (ezrin, radixin and moesin) family of cytoplasmic linker proteins. Immunoprecipitation studies using BHK cells have shown that CD44 directly binds to all of the ERM family members (Tsukita *et al.*, 1994). Hirao and colleagues have confirmed this result *in vitro*, by demonstrating that a GST/cytoplasmic CD44 fusion protein bound to ERM proteins (Hirao *et al.*, 1996). The ERM family members may play an important role in regulating the ligand binding capacity of CD44 as they have been implicated in altering both the distribution and ligand binding function of another adhesion protein, ICAM-2 (Helander

et al., 1996). The importance of cytoskeletal interactions in regulating the function of CD44 will be discussed further in Chapter II.

1.3 CD44 Heterogeneity

Immunoprecipitation and Western blot analysis have suggested that multiple isoforms of CD44 exist (Kansas *et al.*, 1989). CD44, in fact, appears to be a family of molecules generated by both post-translational modification and differential utilization of alternatively spliced exons. The repertoire of isoforms expressed on cells varies with cell type and proliferation status.

1.3.1 Post-translational modifications

There is compelling evidence that higher molecular mass CD44 isoforms arise by post-translational modification such as chondroitin sulfate attachment or glycosylation of the extracellular domain. For example, chondroitin sulfate modification increases the molecular weight of CD44 from 85-95 kDa to 180-200 kDa on some lymphocytes (Jalkanen *et al.*, 1988). Furthermore, CD44 is extensively glycosylated, as more than half of the apparent molecular mass is accounted for by N- and O-linked carbohydrate addition. However, the production of higher molecular mass CD44 species cannot be attributed solely to post-translational modifications of a common polypeptide core.

1.3.2 Alternative splicing

Numerous CD44 variants have been reported to be generated by the insertion of peptide sequences of varying lengths into a single site within the extracellular domain proximal to the membrane spanning domain (Stamenkovic *et al.*, 1991; Dougherty *et al.*, 1991; Gunthert *et al.*, 1991; He *et al.*, 1992; Screaton *et al.*, 1992) (Figure 1). Furthermore, these inserted amino acid sequences are produced by the alternative splicing of a contiguous series of 10 exons present within a single copy CD44 gene (Figure 2), which is located on the short arm of chromosome 11 in humans and on chromosome 2 in mice (Colombatti *et al.*, 1982). The most abundant CD44 isoform does not contain any additional inserted peptides and is denoted the standard form, CD44s or CD44H. The designation CD44H will be used in this thesis.

1.3.3 Genomic organization

The genomic organization of both the human and mouse CD44 has been determined (Cooper *et al.*, 1992; Screaton *et al.*, 1992). To date, 12 of the 20 exons that make up the CD44 gene can be alternatively spliced. Ten exons within the extracellular domain and 2 exons within the cytoplasmic domain, are alternatively utilized (Screaton *et al.*, 1992) (Figure 2). Moreover, one example has been found in which a "constant" exon (exon 16) was deleted (Gunthert *et al.*, 1991). Thus, it is clear that a great number of different CD44 isoforms could potentially be generated by this alternative splicing mechanism. Whether

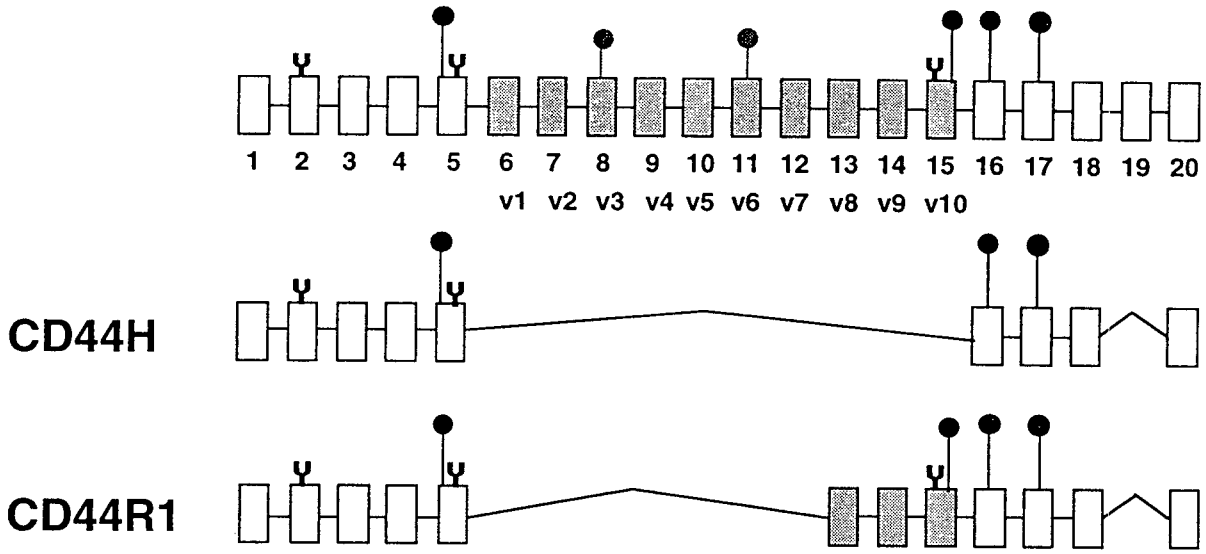


Figure 2: The genomic organization of the CD44 gene. To date, the human genomic CD44 structure is known to consist of 20 exons. Exon 1 encodes the leader peptide whereas exon 2 and 3 represent the putative hyaluronan binding domain (Y). Exons 6 through 15 correspond with the variant exons 1 to 10 (v1-v10). These exons can be alternatively spliced to generate higher molecular mass CD44 isoforms. The isoform CD44R1 containing variant exons 8, 9, and 10 is also shown. Exons 18 encodes the transmembrane and exon 20 the cytoplasmic domains. Sites of potential chondroitin sulfate addition are marked with a (ϙ).

every possible splice variant is translated into a mature protein is unclear, however, numerous species have been confirmed by immunoprecipitation and/or Western blot analysis (Dougherty *et al.*, 1991; Gunthert *et al.*, 1991; Stamenkovic *et al.*, 1991; He *et al.*, 1992; Screaton *et al.*, 1992).

Alternative splicing may be used as a mechanism to generate soluble CD44 molecules by introducing new enzymatic cleavage sites (Dougherty *et al.*, 1991; He *et al.*, 1992). Arginine dipeptides, a potential protease cleavage site, are found in isoform specific sequence encoded by variant exon 10 in the human CD44 (Dougherty *et al.*, 1991). In mice, one similar site is located proximal to the transmembrane domain in all known splice variants, and two sites in high molecular mass variants (He *et al.*, 1992). Whether these protease enzyme targets are utilized has not been determined.

1.4 CD44 and the Extracellular Matrix

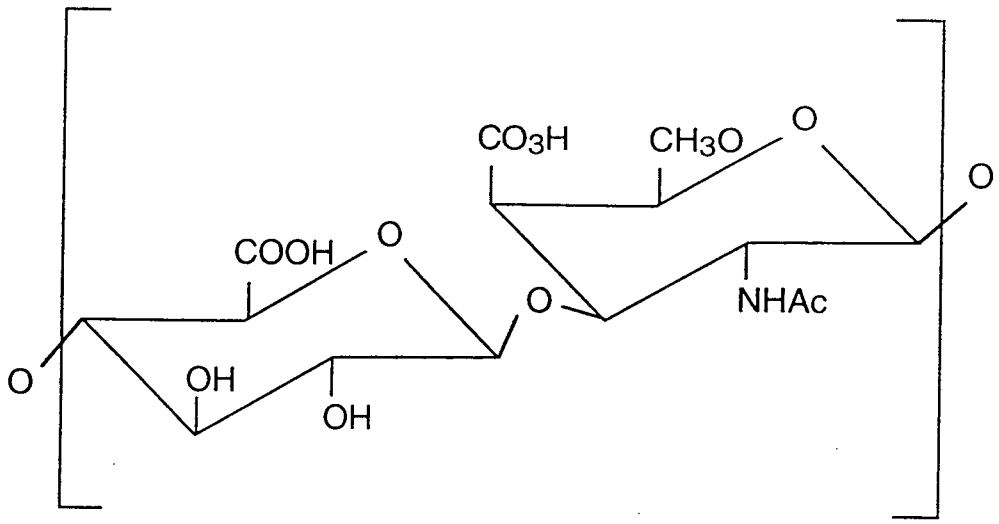
1.4.1 General features of the extracellular matrix

Most cells in multicellular organisms are in contact with a complex network of interacting, extracellular macromolecules that constitute the extracellular matrix (ECM). The components of the ECM were originally thought to function mainly as a relatively inert scaffolding that stabilized the physical structure of tissues. More recently, it has been realized that the ECM plays a far more active and complex role in regulating the function of cells that encounter ECM structures. The ECM has been implicated in a variety of cellular

functions including cell adhesion, migration, proliferation, and differentiation (reviewed in Toole, 1990).

The macromolecules that make up the ECM are secreted by a variety of cells, including fibroblasts and chondroblasts in cartilage and osteoblasts in bone. Two of the main constituents of the ECM are collagenous proteins and proteoglycans (Lindahl and Hook, 1978). The latter are usually several polysaccharide glycosaminoglycans generally covalently linked to a protein core.

A major component of the ECM in some tissues is the glycosaminoglycan hyaluronan. Hyaluronan exists as a single, very long carbohydrate chain of sugar residues in a regular, repeating sequence of D-glucuronic acid (1-β-3) and N-acetyl-D-glucosamine (1-β-4) (Toole, 1990; Laurent and Fraser, 1992) (Figure 3). This polymer can have a molecular weight of 400 to 8 million daltons. In solution, hyaluronan behaves as a random coil (Laurent and Fraser, 1992). A large quantity of solvent is trapped within the coil and the molecule can be considered to be a highly hydrated sphere (Laurent and Fraser, 1992). Hyaluronan is a highly negatively charged molecule, due to the presence of carboxyl groups. Hyaluronan, however, differs from the typical glycosaminoglycans with respect to three important features. First, the others tend to contain a number of different disaccharide units in a complex arrangement, whereas hyaluronan contains only 2 different disaccharides linked in a linear arrangement.



D-glucuronic acid **N-acetyl-D-glucosamine**

Figure 3: The structure of hyaluronan. Hyaluronan is a simple glycosaminoglycan consisting of alternating residues of D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine. It has a molecular weight of up to several million, and in solution forms an extended random coil that can trap large quantities of solvent.

Furthermore, typical glycosaminoglycans have fewer than 300 sugar residues. Finally, hyaluronan appears not to be covalently linked to a protein core, although it is frequently found attached to the surface of mesenchymal cells via appropriate hyaluronan-binding proteins (Knudson *et al.*, 1993).

Lymphoma cells and macrophages can be induced to homotypically aggregate following the addition of hyaluronan even at low concentrations (Toole, 1990). Divalent cation-independent cellular aggregation can be inhibited by treatment with hyaluronidase or large quantities of soluble hyaluronan. Cell lines that display hyaluronan-dependent homotypic aggregation appear to do so by crosslinking of the hyaluronan receptors on adjacent cells with hyaluronan (Toole, 1990). The aggregation of lymphocytes that is mediated by hyaluronan can be blocked with antibodies directed against CD44 (Lesley *et al.*, 1990). The work of Lesley and coworkers suggested that CD44 mediated both hyaluronan-dependent self-aggregation and the binding of soluble hyaluronan to some lymphoid cell lines (Lesley *et al.*, 1990). Studies by St. John and colleagues further supported this idea (St. John *et al.*, 1990). This latter group demonstrated that a fibroblast cell line transfected with a CD44 cDNA could be induced to homotypically aggregate. These studies support the involvement of hyaluronan in a variety of cell functions. The role that hyaluronan plays in these functions is itself regulated by at least three aspects: the size and concentration of hyaluronan and the affected cell type (reviewed in Toole, 1990).

In addition to acting as "structural scaffolding" (Knudson, 1993), hyaluronan may function as a cellular signaling molecule. Bourguignon *et al* have determined that hyaluronan

can induce lymphocyte signal transduction and CD44-cytoskeleton interaction (Bourguignon *et al.*, 1993). Moreover, Hall *et al* demonstrated a role for hyaluronan in focal adhesion turnover and transient tyrosine kinase activity (Hall *et al.*, 1994).

The size of hyaluronan appears to play an important role in regulating cell behavior. In normal synovial fluid, hyaluronan is generally observed to be greater than 1000 kDa (Laurent and Fraser, 1992). However, hyaluronan tends to be more heterogeneous, with a preference for lower molecular weight forms, under inflammatory conditions (Saari *et al.*, 1991). The accumulation of lower molecular weight forms of hyaluronan has been postulated to occur by a variety of mechanisms including depolymerization by reactive oxygen species (McNeil *et al.*, 1985), enzymatic cleavage (Roden *et al.*, 1989) and *de novo* synthesis of lower molecular weight species (Prehm, 1989; Sampson *et al.*, 1992). High molecular weight hyaluronan inhibits phagocytosis (Forrester and Balazs, 1980), and inhibits cell growth (West and Kumar, 1989). Interestingly, low molecular weight hyaluronan (3 to 16 disaccharides) has been observed to stimulate cell proliferation (West *et al.*, 1985; West and Kumar, 1989). Furthermore, low molecular weight hyaluronan, but not high, have been demonstrated to induce in murine macrophages, the expression of inflammatory genes including macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, cytokine responsive gene-2, and monocyte chemoattractant protein-1 (McKee *et al.*, 1996).

It has been suggested that hyaluronan is a highly metabolically active molecule for which the cell focuses considerable attention on the processes of synthesis and catabolism (Weigel *et al.*, 1997). This is exemplified by the tissue half-life of hyaluronan which ranges

from 1 to 3 weeks in cartilage and less than 1 day in epidermis (Tammi *et al.*, 1991). The synthesis of hyaluronan appears to be regulated by various growth factors (Heldin *et al.*, 1989). Hyaluronan production takes place at the inner face of the plasma membrane. The newly generated hyaluronan remains bound to the hyaluronan synthase during production and then extruded through the plasma membrane (Ng and Schwartz, 1989). Many cellular growth factors including platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β (TGF-β), appear to influence hyaluronan synthesis at least in fibroblasts (Heldin *et al.*, 1989). Furthermore, cells have been observed to be arrested in mitosis if hyaluronan synthesis is inhibited (Toole, 1990).

Hyaluronan synthase was first cloned from Group A *Streptococcus pyogenes* by transposon mutagenesis in which an acapsular mutant was generated by transposon insertion into the hyaluronan synthesis operon (DeAngelis *et al.*, 1993a; DeAngelis *et al.*, 1993b). This was the first molecular description of an enzyme shown to synthesize a glycosaminoglycan. The clone is predicted to encode a 395 amino acid integral membrane protein with four membrane-associated helices and a calculated molecular weight of 45 kDa (DeAngelis *et al.*, 1993). In addition to the Group A streptococcal hyaluronan synthase, the cDNA encoding this enzyme has been cloned from Group C *streptococcus equisimilis* (Kumari and Weigel, 1997), Xenopus laevis (Meyer and Kreil, 1996), Type A Pasteurella multocida, a prevalent animal pathogen (DeAngelis *et al.*, 1998), mouse (Fulop *et al.*, 1997; Itano and Kimata, 1996; Spicer *et al.*, 1996; Spicer *et al.*, 1997), and human (Itano and Kimata, 1996; Shyjan *et al.*, 1996; Watanabe and Yamaguchi, 1996). The encoded synthases demonstrate 30% identity at the protein level between prokaryotes and eukaryotes (Weigel

et al., 1997). Interestingly, multiple mammalian cDNAs encode for different hyaluronan synthases suggesting a multiple gene family regulating the presence of hyaluronan (Weigel *et al.*, 1997). This in turn supports the idea that the glycosaminoglycan is important not only for structure within tissues but also for the influencing important cellular processes.

1.4.2 CD44 adheres to hyaluronan

There is now clear evidence that CD44 can function as a receptor for hyaluronan, whether expressed on the cell surface or as a soluble molecule fused in frame to the constant domains of human IgG1 (Aruffo *et al.*, 1990; Miyake *et al.*, 1990) or the enzyme alkaline phosphatase (Dougherty *et al.*, 1994).

1.4.3 Sequence of CD44 suggests a potential role in hyaluronan recognition

A region of the extracellular domain of CD44, residues 12-101 in humans, demonstrates greater than 85% sequence identity between mouse and human (Stamenkovic *et al.*, 1989). Sequence comparisons indicate that this region includes a tandemly repeated domain with low but significant homology (approximately 30% sequence identity) to the second (B) subdomain of cartilage proteoglycan core and link proteins (Doege *et al.*, 1991; Goldstein *et al.*, 1989; Idzerda *et al.*, 1989; Stamenkovic *et al.*, 1989; Wolffe *et al.*, 1990). Further comparisons demonstrated that this region is present within a number of other proteins that recognize and bind hyaluronan. Some of these proteins are aggrecan, versican, and a recently described molecule designated TSG-6 that is induced in fibroblasts by tumour

necrosis factor-α (TNF-α) or IL-1β, and in peripheral blood mononuclear cells by mitogen stimulation (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989; Lee *et al.*, 1993). More limited sequence homology is also observed to RHAMM (receptor for hyaluronan-mediated motility), a 58 kDa surface receptor that plays an important role in hyaluronan-mediated cellular migration events (Hardwick *et al.*, 1992). This domain containing 6 cysteines residues that may potentially be linked by disulfide bonds to form a single globular domain (Goldstein *et al.*, 1989).

Yang and colleagues determined that only two hyaluronan-binding domains exist in the hyaluronan receptor, RHAMM (Yang *et al.*, 1994). Furthermore, these two binding domains contributed approximately equally to the hyaluronan binding ability of RHAMM. The first binding domain contained two sets of two basic amino acids; each spaced seven residues apart. Furthermore, mutations of these basic residues decreased the ability of RHAMM to bind to hyaluronan (Yang *et al.*, 1994). Mutational analysis of the second binding domain implicated Lys^{423} and Arg^{431}, spaced seven amino acids apart, as critical for hyaluronan binding (Yang *et al.*, 1994). This group suggested that the minimal requirement for hyaluronan binding activity is two basic residues flanking a seven amino acid stretch [$B(X_7)B$], a motif found in all hyaluronan binding proteins to date including CD44 and link protein (Yang *et al.*, 1994). Site-directed mutations of these motifs in CD44 abolished hyaluronan-binding activity (Yang *et al.*, 1994).

1.4.4 Evidence for hyaluronan as a ligand for CD44

Many independent research groups have demonstrated by transfection studies that CD44 can function as a receptor for hyaluronan. In one study, the expression of human CD44H in the CD44-negative human Burkitt B cell lymphoma line Namalwa, allowed the transfectants to bind to lymph node high endothelial cells (Aruffo *et al.*, 1990). This adhesion event was inhibited by polyclonal antiserum specific for CD44, the addition of excess soluble hyaluronan or pretreatment of the target cells with the enzyme hyaluronidase. Furthermore, hamster CD44 transfected into COS cells reacted with an antibody directed against the hamster fibroblast hyaluronan receptor (Aruffo *et al.*, 1990).

Lesley and Hyman studied the alteration of adhesion of a murine T cell hybridoma line designated AKR1 when transfected with a murine CD44 cDNA (Lesley and Hyman, 1992). These transfectants were now able to bind immobilized hyaluronan and fluorescein-conjugated hyaluronan from solution. Unconjugated hyaluronan or pretreatment with a mAb directed against CD44 inhibited this binding.

To further study the adhesion of CD44 to hyaluronan, a chimeric protein in which the extracellular domain of human CD44 was fused with the hinge domains (C_H2 and C_H3) of human IgG_1, was generated (Aruffo *et al.*, 1990). The soluble CD44-immunoglobulin fusion protein adhered to lymph node high endothelial cells when expressed in COS cells. This interaction was abolished in the presence of hyaluronan but not other glycosaminoglycans. Furthermore, the binding of the CD44-immunoglobulin fusion protein was completely

inhibited by the pretreatment of the high endothelial cells with hyaluronidase and chondroitinase AC, partially inhibited with chondroitinase ABC but not keratanase, heparinase or heparitinase (Aruffo *et al.*, 1990).

CD44 appears to participate in the uptake and degradation of hyaluronan (Culty *et al.*, 1992). Culty *et al.* demonstrated that incubation of hyaluronan with either transformed fibroblasts (SV-3T3 cells) or alveolar macrophages, anti-CD44 mAb inhibitable degradation of hyaluronan was observed (Culty *et al.*, 1992). Similar antibody blocking studies revealed that the macrophages could internalize fluorescein-tagged hyaluronan. Furthermore, the subsequent degradation of hyaluronan was inhibited by chloroquine and NH_4Cl, agents that block the acidification of lysosomes (Culty *et al.*, 1992). These observations suggest that CD44 mediates the recognition and uptake of hyaluronan, whereas degradation of the glycosaminoglycan is accomplished by acid hydrolases in the lysosome.

1.4.5 Features of CD44 important for hyaluronan binding

Studies on cartilage link protein have suggested that the interaction with hyaluronan is largely ionic in nature. Negatively charged carboxyl groups on hyaluronan appear to mediate binding with clusters of positively charged basic residues in CD44 (Jackson *et al.*, 1991). Using deletion analysis, Peach and colleagues have localized the hyaluronan-binding domain of CD44 to the first 186 amino acid residues of the molecule (Peach *et al.*, 1993). Unlike the remainder of the extracellular domain, this region is highly conserved among different mammalian species (>90% identity between mouse and man) (Nottenburg *et al.*,

1989; Wolffe *et al.*, 1990). The fact that this amino-terminal region is involved in binding of hyaluronan has been inferred from the sequence identity this domain shares with regions contained in other hyaluronan binding proteins as described above. Importantly, the region defined by Peach and coworkers contains two short stretches of 13 amino acids that include 3 and 4 positively charged arginine or lysine residues, respectively (Peach *et al.*, 1993). Although only the first of these basic amino acid clusters appears to be present in other hyaluronan-binding proteins (Goldstein *et al.*, 1989; Lee *et al.*, 1993; Stamenkovic *et al.*, 1989) site-directed mutagenesis studies suggest that both contribute to the hyaluronan-binding ability of CD44, with the arginine residue at position 41 being particularly important (Peach *et al.*, 1993). Some studies have suggested that only residues 18-30 and 88-112 are likely to be exposed to ligands for binding (reviewed in Lesley *et al.*, 1993). Furthermore, an antibody generated against a peptide corresponding with residues 18-30 was not able to interfere with the binding of CD44 to hyaluronan, thus suggesting that these residues are not critical for hyaluronan interaction (Lesley *et al.*, 1993).

1.4.6 Hyaluronan binding domain structure

Kohda *et al* attempted to better define the hyaluronan binding domain by resolving the 3-dimensional structure of the so-called "Link module" by nuclear magnetic resonance (Kohda *et al.*, 1996). The "Link module" is a 100 amino acid polypeptide domain with four characteristic disulfide-bonded cysteines (Neame and Barry, 1993) and found on all hyaluronan binding proteins to date, including CD44, RHAMM, and TSG-6. The 3-dimensional structure of the "Link module" from TSG-6 (amino acids 1-98) was

determined (Kohda *et al.*, 1996). This module was found to consist of two alpha helices and two antiparallel beta sheets arranged around a large hydrophobic core (Kohda *et al.*, 1996). Surprisingly, the "Link module" showed structural similarities with the C-type lectin domain. In fact, the predicted hyaluronan binding site in the "Link module" is found in an analogous position to the carbohydrate-binding pocket in E-selectin suggesting a potential reason for the comparable roles for CD44 and the selectins in leukocyte extravasation at the sites of inflammation.

Using the Link module to predict functionally important amino acid residues, Bajorath *et al* demonstrated by site-directed mutagenesis studies that eight residues were important for hyaluronan binding (Bajorath et al, 1998). The amino acid residues found to be critically important were two pairs of Arg/Tyr dipeptides at positions 21 and 58 found at the center of the predicted binding site and Lys^{18}, Lys^{48}, Asn^{80}, Asn^{81} and Tyr^{85} which would surround the proposed binding site. As this finding does not include all the residues demonstrated by Yang *et al* (Yang *et al,* 1994) to be critical, the importance of the $B[X_7]B$ motif is in question.

1.4.7 Other ligands for CD44

CD44 has been reported to recognize and bind a number of ligands in addition to hyaluronan including fibronectin, collagen types I and IV, laminin, chondroitin-4-sulfate, and a 60 kDa cell surface glycoprotein expressed by mucosal vascular endothelial cells termed mucosal vascular addressin (Picker *et al.*, 1989). Studies have suggested, however, that the

adhesion of CD44 to fibronectin, collagen and laminin may be of relatively low affinity and mediated via the recognition of covalently attached chondroitin sulfate side chains (Faassen *et al.*, 1992). CD44 can also bind chondroitin sulfate moieties presented by the invariant chain of MHC class II (Naujokas *et al.*, 1993), the cytokine osteopontin (Weber *et al.*, 1996) and the hemopoietic cell-specific proteoglycan serglycin (Toyama-Sorimachi *et al.*, 1995).

1.5 Expression and Function of CD44

1.5.1 Expression of CD44

Although CD44 was originally implicated in lymphocyte homing, its expression is not restricted to T and B lymphocytes. CD44 has been observed in a wide variety of tissues including the astrocytes and glial cells of the central nervous system, lung, epidermis, liver, and pancreas (Picker *et al.*, 1989) as well as cell types such as ovarian carcinomas (Pals *et al.*, 1989), monocytes, granulocytes, fibroblasts, keratinocytes, erythrocytes and brain tissue (Dalchau *et al.*, 1980; Haynes *et al.*, 1983). Although expression levels are extremely variable, most hemopoietic cells of mouse and man express CD44 (Trowbridge *et al.*, 1982; Kansas *et al.*, 1989; Kansas *et al.*, 1990). In the murine system, CD44 has been demonstrated to be expressed in every hemopoietic lineage (Spangrude, 1989; Trowbridge *et al.*, 1982).

The expression of CD44 in hemopoietic cells appears to be dependent upon both differentiation stage and activation status. Murine prothymocytes capable of homing to and

populating the thymus, express CD44 (Trowbridge *et al.*, 1982; Spangrude, 1989). CD44 expression is lost during T cell development upon the expression of the α chain of the interleukin-2 receptor complex (IL-2Rα) (Lesley *et al.*, 1993). CD44 reappears on more mature CD4 and CD8 single-positive thymocytes (Lynch and Ceredig, 1989). In fact, murine CD44 is expressed on bone marrow prothymocytes, on 80-90% of day 13-14 fetal thymocytes, and on only about 5% of adult thymocytes (Trowbridge *et al.*, 1985). The pattern of CD44 expression in human fetal thymus is similar to that of the murine thymus (Horst *et al.*, 1990). Approximately 60% of immature $CD4^-$ $CD8^-$ $CD3^-$ human thymocytes are strongly CD44+ve (Denning *et al.*, 1989). Furthermore, most lymphocytes in the human peripheral blood express CD44 (de los Toyos *et al.*, 1989; Horst *et al.*, 1990; Kansas *et al.*, 1989).

The expression of CD44 also appears to be elevated in both memory and activated T cells. Budd and colleagues demonstrated that memory cytotoxic T cell precursors elicited in response to different antigens were CD44+ve as well as CD8+ve (Budd *et al.*, 1987a; Budd *et al.*, 1987b). This study was performed using the C57BL/6 strain of mice that express low numbers of T cells expressing CD44 in the thymus and periphery. Although CD44 appears to be a good marker for memory T cells, the use of this antigen is restricted to mouse strains that express low levels of CD44+ve cells in mature thymus and peripheral T cell populations (Lynch and Ceredig, 1989). In the C57BL/6 mice, the expression of CD44 was observed to be elevated in helper memory T cells, defined by markers such as low CD45RB and low Mel-14 expression (Butterfield *et al.*, 1989; Swain *et al.*, 1990). Human T cells activated *in vitro* were observed to express more CD44 (de los Toyos *et al.*, 1989;

Oppenheimer-Marks *et al.*, 1990; Haegel and Ceredig, 1991). Haegel and Ceredig demonstrated that stimulating T cells with either mitogens or antigens increased surface expression of CD44 (Haegel and Ceredig, 1991).

The expression pattern of CD44 during B cell development is similar to that of maturing T cells. CD44 expression on CD10-positive immature B cells isolated from human bone marrow was observed to be low. Subsequently, the level of CD44 is upregulated on CD20 positive mature B cells (Kansas and Dailey, 1989). Furthermore, the level of CD44 on B cells is influenced by activation states. B cells activated with either LPS or anti-IgD-dextran demonstrated an upregulation of the CD44 expression (Hathcock *et al.*, 1993).

The alternative splicing of CD44 is a regulated process in both malignant and normal cells. Hemopoietic cells express mainly CD44H whereas epithelial cells preferentially express CD44R1. Activated T lymphocytes and other leukocytes transiently upregulate CD44 isoforms expressing variant exons (Naor *et al.*, 1997). Splicing of CD44 also differs in malignant cells as will be discussed below.

1.5.2 Function of CD44

Several independent lines of investigation have implicated the adhesion protein CD44 in numerous functional roles in a variety of cell types (reviewed in Lesley *et al.*, 1993). The adhesion-dependent cellular functions that involve CD44 include T cell activation, cell

adhesion, lymphocyte recirculation, cell migration, hemopoeisis, and tumour metastasis. The role of CD44 in a selection of these processes will be discussed.

Mice deficient in CD44 expression generated by a targeted disruption in CD44 exon 2 has recently been described (Schmits *et al.*, 1997). As this molecule has been implicated in such a myriad of important cellular processes, the finding that these CD44-/- mice were born in Mendelian ratio without any obvious abnormalities was unexpected.

1.5.2.1 The role of CD44 in lymphocyte homing

Lymphocytes circulate throughout the body in the constant process of immune surveillance. These immune cells migrate though the bloodstream, move into lymphoid organs and other tissues, and then enter the lymphatics to return to the circulatory system (Yednock and Rosen, 1989; Shimizu *et al.*, 1992). This process of lymphocyte homing and recirculation allows the full repertoire of antigenic specificities to be constantly presented throughout the body.

The tissues of the immune system can be functionally subdivided into primary, secondary, and tertiary lymphoid organs (reviewed in Picker and Butcher, 1992). Production of functional lymphocytes occurs in the primary tissues including the bone marrow and thymus. The antigen-mediated proliferation and differentiation of antigen-specific lymphocytes occurs in the secondary tissues to which the lymph node, Peyer's patch, and spleen belong. The tertiary lymphoid tissues consist of all other tissues of the body and

represent sites for antigen restimulation of memory lymphocytes and effector precursor cells.

Lymphocytes exit the bloodstream and migrate into secondary lymphoid tissues via adhesion to a specialized endothelium designated high endothelial venules (HEV) (reviewed in Woodruff *et al.*, 1987). Lymphocytes have the ability to distinguish between HEV of the lymph node and Peyer's patch (Butcher *et al.*, 1980; Gallatin *et al.*, 1983). The ability of circulating lymphocytes to specifically recognize and bind to HEV can be measured using an *in vitro* assay system developed by Stamper and Woodruff (Stamper and Woodruff, 1976). This assay strategy used frozen sections of lymphoid tissues to which lymphocyte populations were added. The Mel-14 mAb was defined using this assay system (Gallatin *et al.*, 1983). This mAb, which was later determined to recognize murine L-selectin (Lasky *et al.*, 1989), was able to block murine lymphocyte and lymphoid cell line adhesion to HEV. A cell surface marker on human lymphocytes designated Hermes antigen was cross-reactive with the Mel-14 mAb and thus named "lymphocyte homing receptor". Subsequently, mAbs generated against the Hermes antigen were found to recognize human CD44 (Gallatin *et al.*, 1989; Picker *et al.*, 1989; St. John *et al.*, 1990). Jalkanen and colleagues developed the Hermes series of antibodies (Hermes-1, -2, and -3) (Jalkanen *et al.*, 1986). Of the three antibodies that recognize CD44, only one (Hermes-3) can block human lymphocyte adhesion to HEV frozen sections (Jalkanen *et al.*, 1987). This antibody was not, however, effective in blocking the hyaluronan-dependent adhesion of a B cell line transfected with human CD44 to cultured rat endothelium (Stamenkovic *et al.*, 1991). Furthermore, the adhesion of murine lymphocytes to HEV is not susceptible to hyaluronidase treatment or to

mAbs known to inhibit hyaluronan binding by CD44 (Culty *et al.*, 1990). These observations support the involvement of CD44 in lymphocyte homing but CD44-hyaluronan interaction may not participate in lymphocyte adhesion to HEV.

1.5.2.2 The role of CD44 in hemopoeisis

Miyake and colleagues demonstrated that mAbs directed against CD44 were able to completely block B cell lymphohemopoeisis in long-term bone marrow cultures (Miyake *et al.*, 1990). These antibodies were originally isolated for the ability to inhibit a B cell line to adhere to a cloned stromal cell line. Further analysis demonstrated that CD44 on the B cell line recognized hyaluronan on the surface of the stromal cells (Miyake *et al.*, 1990). Although the mechanism by which CD44 affects hemopoeisis is not well defined, this group has demonstrated an important role for this adhesion protein in this particular function.

More recently, Sugimoto and coworkers (Sugimoto *et al.*, 1994) studied the effect of anti-CD44 antibody (KM81) on the adhesion of erythroid leukemic cells (ELM-I-1) with hemopoietic supportive cells (MS-5). CD44 is expressed on both ELM-I-1 and MS-5 cells. After differentiation, the expression level of CD44 on the ELM-I-1 cells was reduced, and no detectable CD44 expression was observed on erythrocytes (Sugimoto *et al.*, 1994). Although KM81 inhibited the adhesion between ELM-I-1 and MS-5 cells, neither hyaluronidase nor hyaluronate treatment had any effect (Sugimoto *et al.*, 1994). Thus, CD44 appears to be important for this adhesive event, however the ligand for CD44 does not seem to be hyaluronan.

1.5.2.3 The role of CD44 in T cell activation

Several groups have demonstrated that the addition of anti-CD44 antibodies augments both CD2- and CD3-mediated T cell activation (Denning *et al.*, 1990; Huet *et al.*, 1989; Pierres *et al.*, 1992; Rothman *et al.*, 1991; Seth *et al.*, 1991; Shimizu *et al.*, 1989; Tan *et al.*, 1993). Seth and coworkers have demonstrated that the CD44-specific antibody 9F3 could trigger the lytic activity of cytotoxic T lymphocytes (Seth *et al.*, 1991). The antibody H90 developed by Huet and coworker was able to inhibit the adhesion of lymphocytes to HEV (Huet *et al.*, 1989). Furthermore, H90 could enhance [^{3}H]TdR incorporation of peripheral blood lymphocytes when a primary stimulus of CD2 mAb or CD3 mAb linked to plastic culture plates was used. H90 however had no effect on [^{3}H]TdR incorporation when peripheral blood lymphocytes were stimulated with lectins, allogeneic cells, or CD3 mAb in the soluble phase. Denning and colleagues have demonstrated that the addition of antibodies against CD44 to purified T cells resulted in a 25-fold increase of anti-CD2-mediated T cell IL-2 secretion (Denning *et al.*, 1989). The interaction of antibodies with CD44 was believed to mimic the possible effects of ligand binding to CD44 on stimulation through other receptor-ligand interactions such as T cell receptor binding to antigen or triggering via CD2 or CD3 surface molecules. The primary stimulus in the described studies however is often suboptimal. A contrasting study demonstrated that the anti-CD44 mAb 212.3 could completely inhibit T cell proliferation stimulated by the CD3-specific antibody OKT3 (Rothman *et al.*, 1991). The study further showed that this inhibition of CD3-mediated T cell activation was not caused by a reduction of cell viability. It is, however, associated

with an inhibition of IL-2 production and receptor expression, and a reduction of OKT3-mediated increase in intracellular Ca^{2+} levels. This antibody was not able to inhibit T cell activation resulting from stimulation by the T cell mitogen phytohemagglutinin or pokeweed mitogen. A more recent study demonstrated that intracellular cAMP is rapidly increased following treatment with this anti-CD44 antibody (Rothman *et al.*, 1993). This elevation of cAMP is not dependent on activation state and is not observed with non-inhibitory mAbs against CD44. Paul-Eugene and colleagues also demonstrated an upregulation of cAMP in 20 to 30 minutes using anti-CD23 mAb to stimulate CD23 on CD23+ve monocytes (Paul-Eugene *et al.*, 1992). Rothman and colleagues suggested that CD44 may be directly coupled to adenylate cyclase as the elevation of cAMP required only 1 to 2 min in T cells (Rothman *et al.*, 1993). Lokeshwar and Bourguignon characterized CD44 as a GTP-binding protein with GTPase activity in *in vitro* assays (Lokeshwar and Bourguignon, 1992). Interestingly, the binding of GTP significantly enhanced the interaction of purified CD44 with ankyrin (Lokeshwar and Bourguignon, 1992). Perhaps the binding of hyaluronan to CD44 on the cell surface would allow for GTP binding to the cytoplasmic domain leading to attachment to cytoskeletal elements.

The proliferation of peripheral blood T cells induced by CD2 antibodies appears to by dependent upon monocytes (Denning *et al.*, 1989). Furthermore, Denning and colleagues suggested that increasing the adhesion of either LFA-1 to ICAM-1 or CD2 to LFA-3 leading to a stronger interaction between T cell and monocyte might be the mechanism by which CD44 may enhance T cell activation (Denning *et al.*, 1990). This study demonstrated that the CD44 antibodies A3D8 and A1G3 enhances CD2-mediated T cell triggering by binding

to monocytes and augmenting monocyte-T cell adherence, by inducing monocyte IL-1 release, and by binding to T cells and stimulating T cell release of IL-2 (Denning *et al.*, 1990). The treatment of human T cells with antibodies against CD44 have been demonstrated to induce LFA-1-dependent homotypic aggregation (Pals *et al.*, 1989; Koopman *et al.*, 1990) that is sensitive to both 1-O-alkyl-2-O-methyl glycerol-3 phosphocholine and 1-(5-isoquinolinyl sulfonyl-2 methyl piperazide) treatment, both of which prevent protein kinase C (PKC) activation. Furthermore, cytochalasin B also inhibits this activation. These data suggest that PKC activation and cytoskeletal interaction are critical for the activation of the LFA-1 pathway via CD44 (Koopman *et al.*, 1990).

1.5.2.4 The role of CD44 in tumour metastasis

The most life threatening aspects of the oncogenic process are tumour invasion and metastasis. Metastasis is a complex process that consists of a cascade of sequential inter-dependent stages involving multiple host-tumour interactions (Fidler and Hart, 1982; Schirrmacher, 1985; Fidler, 1990). Metastatic cells must first be able to exit from the primary tumour site, invade the local host tissue and then enter the bloodstream or lymphatics. Once in the circulation, the malignant cell or group of cells must be able to survive within this harsh environment and arrest in a distant vascular bed. Upon adhesion to a secondary site, the cells must exit the vasculature and colonize in an ectopic organ site.

Studies have demonstrated an important role for hyaluronan in tumour migration. Knudson and Knudson have demonstrated that carcinomas are commonly associated with

local accumulation of hyaluronan (Knudson and Knudson, 1990). Tumour cells may directly release hyaluronan or may induce nearby fibroblast to secrete hyaluronan. The free hyaluronan may be incorporated into the ECM (Yoneda *et al.*, 1988; Knudson and Knudson, 1991) and may function to separate tissues, partially degrade the collagenous fibrillar framework, or affect adhesive forces between the cells and their substrate (Docherty *et al.*, 1989).

Continued tumour cell growth requires that tumour cells secrete angiogenic factors that promote the vascularization of the tumour mass. The degradation of the ECM and in particular hyaluronan is thought to be important for angiogenesis (Blood and Zetter, 1990). West and colleagues have suggested that the degradation products of hyaluronan promote angiogenesis (West *et al.*, 1985). The study by this group demonstrated that 4 to 25 disaccharide degradation products of hyaluronan could induce the formation of blood vessels on the chick chorio-allantoic membrane.

The evidence supporting the role for CD44 in tumour invasion and metastasis is mounting. Birch *et al* demonstrated that clones of the human melanoma cell line LT5.1 expressing high levels of CD44 gave 10 to 20 times the number of lung colonies in nu/nu mice than clones expressing low levels of CD44 (Birch *et al.*, 1991). An analysis of 107 cases of non-Hodgkin's lymphoma of various histologic and immunophenotypic subclasses revealed a correlation between CD44 expression and the degree of dissemination (Pals *et al.*, 1989). Furthermore, the expression of CD44 on non-Hodgkin's lymphoma is related to the clinical

stage, tumour spread, and poor response to treatment (Horst *et al.*, 1990; Jalkanen *et al.*, 1991).

The correlation between the expression of CD44 and tumour growth appears to be important. Many studies have demonstrated the upregulation of CD44 expression in human tumour cells (Picker *et al.*, 1989; Stamenkovic *et al.*, 1989; Dougherty *et al.*, 1991; Jackson *et al.*, 1992; Koopman *et al.*, 1993). Conversely, others have demonstrated that CD44 is either not expressed or is downregulated in some tumours (Shtivelman and Bishop, 1991; Cannistra *et al.*, 1993; Ariza *et al.*, 1995).

Isoforms of CD44 appear to play a role in metastasis. Gunthert and colleagues demonstrated that a variant form of CD44 designated pMeta-1 is expressed in the metastasizing rat pancreatic carcinoma cell line BSp73 and in the mammary adenocarcinoma 13762NF (Gunthert *et al.*, 1991). This isoform is not however expressed in the non-metastasizing BSp73AS cells, in other nonmetastatic derivatives of the same parental tumour or in most normal rat tissues (Gunthert *et al.*, 1991). The overexpression of this isoform in the nonmetastasizing BSp73AS cells confers full metastatic ability. Koopman and colleagues reported that this alternatively spliced isoform of CD44 contains sequences encoded by exon v6 (Koopman *et al.*, 1993). Furthermore, they demonstrated that many aggressive non-Hodgkin's lymphomas express the v6 containing CD44 variant. This particular isoform is expressed at low levels on normal resting lymphocytes, whereas it is transiently expressed on activated lymphocytes (Arch *et al.*, 1992; Koopman *et al.*, 1993). It is interesting to note that antibodies specific for the peptide encoded by the v6 containing variant CD44

sequences can inhibit *in vivo* activation of both B and T cells (Arch *et al.*, 1992). Seiter and colleagues demonstrated that an antibody (1.1ASML) specific for peptide sequences determined by v6 could inhibit the growth of lymph node and lung metastases (Seiter *et al.*, 1993). This retardation of metastatic behavior does not result from a 1.1ASML-dependent downregulation of this isoform or an activation of an immune response (Seiter *et al.*, 1993). They suggest that this mAb interferes with the interaction between the tumour cells and other cells and/or the ECM (Seiter *et al.*, 1993).

Sy and colleagues demonstrated that two isoforms of CD44 have distinct effects on tumour growth *in vivo* (Sy *et al.*, 1991). The two CD44 forms studied were CD44H and a higher molecular mass species CD44E that does not mediate adhesion to hyaluronan (Stamenkovic *et al.*, 1989; Stamenkovic *et al.*, 1991). The cDNAs encoding these isoforms were stably expressed in the human Burkitt lymphoma cell, Namalwa and injected into nu/nu mice. Only the transfectants expressing the CD44H form greatly enhanced both local tumour formation and metastatic capacity (Sy *et al.*, 1991). The *in vivo* tumour formation resulting from CD44H transfectants can be suppressed by treatment of the mice with a soluble human CD44H-immunoglobulin fusion protein (Sy *et al.*, 1991). The mechanism by which this inhibition occurred was not known however, this group suggested that binding of hyaluronan might be involved (Sy *et al.*, 1991). The evidence discussed in this section supports the notion that CD44 and its isoforms are involved in tumour formation and metastasis. However, the mechanism by which CD44 affects this function is not well defined. This issue is addressed further in Chapter III.

1.6 Regulation of CD44-mediated Adhesion to Hyaluronan

Although CD44 functions as a receptor for hyaluronan, there is not a one-to-one correlation between the expression of CD44 on the cell surface and the ability of cells to bind hyaluronan.

1.6.1 Evidence for regulation of hyaluronan binding

The evidence that the interaction of CD44 with hyaluronan was regulated first came from observations of the binding ability of murine hemopoietic cell lines expressing CD44. Many cell lines such as the CD44+ve T cell lines SAKRTLS 12, EL4 and the B cell lines 70Z/3 and RAW 253 were not able to bind hyaluronan (Lesley *et al.*, 1990). Lesley and coworkers further demonstrated that the inability to bind hyaluronan in these cell lines was not due to a masking of the hyaluronan receptor activity by endogenous hyaluronan (Lesley *et al.*, 1990). This conclusion was made following the observation that treatment of the nonbinding cell lines with hyaluronidase or chondroitinase ABC does not recover the hyaluronan binding capacity of the cell lines (Lesley *et al.*, 1990). These data suggest that the adhesive function of CD44 is not regulated simply by the expression of the molecule, and that other regulatory mechanisms must exist.

Some T cell lines could be induced to bind hyaluronan following treatment with phorbol ester (Lesley *et al.*, 1990). The cell lines used express CD44H and after stimulation, did not demonstrate an isoform shift. Furthermore, the induction required several hours at

37°C. The expression level of CD44 increased upon stimulation leading to the idea that upregulation of CD44 by phorbol ester is the mechanism that regulates the adhesive function. This notion is discounted by the observations of Hyman and colleagues by studying two variants of the SAKRTLS 12 cell line (Hyman *et al.*, 1991). One such variant was selected by fluorescence-activated cell sorting (FACS) for high CD44 expression whereas the other was selected for hyaluronan binding. Interestingly, the variant expressing high levels of CD44 was unable to bind hyaluronan. Furthermore, the hyaluronan-binding variant expressed lower levels of CD44 than the $CD44^{hi}$ variant (Hyman *et al.*, 1991). Thus the adhesive function of CD44 can be induced by phorbol esters and the upregulation of CD44 does not appear to be the mechanism by which the enhancement of binding occurs.

The hyaluronan binding capacity of CD44 can also be induced by treatment with certain mAbs directed against CD44. Lesley and Hyman demonstrated that mAb IRAWB 14 can "activate" the hyaluronan binding of several T cell lines including SAKRTLS 12 and EL4 (Lesley and Hyman, 1992). Unlike the induction seen with phorbol ester, the mAb treatment was rapid and occurred at 0°C (Lesley and Hyman, 1992). Moreover, Fab fragments of the inducing antibodies were unable to emulate the effect suggesting that crosslinking of the CD44 protein on the cell surface are required.

1.6.2 Other adhesion molecules that require activation

The failure of many cell lines expressing high levels of CD44 to adhere to hyaluronan (Hyman *et al.*, 1991; Lesley *et al.*, 1990) suggests that CD44 is one of many cell adhesion

molecules that require activation to most efficiently bind its ligand (Altieri and Edgington, 1988; Dustin and Springer, 1991; Graham and Brown, 1991; Neugebauer and Reichardt, 1991; Spertini *et al.*, 1991; Hynes, 1992). The requirement of some form of activation for optimal binding efficiency is not uncommon among adhesion molecules. Some of the adhesion proteins that function in this manner are L-selectin (Spertini *et al.*, 1991) and many of the integrins including LFA-1, Mac-1/CR3, and platelet integrin gpIIb-IIIa (Dustin and Springer, 1991; Hynes, 1991).

L-selectin is an adhesion molecule that belongs to a family of proteins that contain a lectin-like domain. It can recognize and bind to a carbohydrate ligand sialyl Lewis^x (sLe^x) (Berg *et al.*, 1992; Foxall *et al.*, 1992). Furthermore, it is expressed on the surface of neutrophils and lymphocytes and can be activated by certain stimuli (Spertini *et al.*, 1991). L-selectin expressed on neutrophils can be activated by G-CSF, GM-CSF, and TNF-α to bind to its ligand. The corresponding molecule found on lymphocytes can be stimulated by crosslinking the T cell receptor or CD2. This enhancement of the function of L-selectin occurs in a matter of minutes and does not involve the upregulation of the molecule.

Several integrins can also be activated to bind their ligands more efficiently. These molecules include the β2 integrins LFA-1, Mac-1/CR3, and the β3 integrin platelet integrin gpIIb-IIIa. LFA-1 (CD11a/CD18) is expressed on T lymphocytes and is critically important for the interaction between helper T cells and antigen presenting cells or between cytotoxic T cells and target cells (Dustin and Springer, 1989; van Kooyk *et al.*, 1989). The counter-receptors for this dimeric protein were determined to be intercellular cell adhesion molecule

(ICAM)-1, ICAM-2, ICAM-3 and ICAM-4 (Marlin and Springer, 1987; Staunton *et al.*, 1989; de Fougerolles and Springer, 1992). One important means by which the interaction between LFA-1 and ICAM-1 can be regulated, is by inducing the expression of ICAM-1 upon inflammation (Dustin *et al.*, 1988; Dustin and Springer, 1988). ICAM-1 is induced on a wide variety of cells by inflammatory cytokines such as interleukin-1, TNF-α, interferon-γ (IFN-γ), and lipopolysaccharide (reviewed in Springer, 1990). The upregulation of ICAM-1 expression is transcriptionally mediated and is first observed after 4 hours (Dustin *et al.*, 1988). However, cytotoxic T cells can interact with the appropriate target cells, deliver a lethal hit, and release the effected target in a far shorter time frame of 1 to 5 minutes (Poenie *et al.*, 1987). Thus other regulatory mechanisms appear to be involved in cytotoxic T cell engagement. The LFA-1-dependent adhesive function of T lymphocytes can be stimulated by the crosslinking of the T cell receptor. Activation of resting T lymphocytes with either mAbs against the T cell receptor or by phorbol ester treatment transforms cellular LFA-1 from a low to a high avidity state, with no alteration in the surface expression or distribution (Dransfield and Hogg, 1989; Dustin and Springer, 1989; van Kooyk *et al.*, 1989; Figdor *et al.*, 1990). Furthermore, a mAb directed against LFA-1 designated NK1-L16 could also activate LFA-1 dependent binding in some T cells (Keizer *et al.*, 1988). It has also been proposed that cells regulate the adhesive function of LFA-1 by way of interactions between the cytoplasmic domain of LFA-1 and cytoskeletal elements (Hibbs *et al.*, 1991).

Another regulated integrin is the platelet integrin gpIIb-IIIa (CD41/CD61). On resting circulating platelets, this adhesion molecule is unable to bind its soluble ligand, however it can adhere to immobilized fibrinogen. Platelet integrin gpIIb-IIIa can bind to soluble

fibrinogen following treatment of the platelet with phorbol ester. Moreover, this activation of the molecule appears to involve intracellular signaling (Shattil and Brass, 1987). The adhesive function of this molecule can also be activated by treatment with certain mAbs directed against this integrin (O'Toole *et al.*, 1990) or by binding of fibrinogen (Du *et al.*, 1991). The activation of this adhesion molecule by the mAb does not appear to involve crosslinking of the proteins on the platelet cell surface as O'Toole and coworkers demonstrated that solubilized integrin could be induced to bind soluble ligand upon stimulation with Fab fragments (O'Toole *et al.*, 1990). Furthermore, mAbs have been developed that recognize the activated molecule but not the molecule on resting cells (Shattil *et al.*, 1985). Thus these data suggest that causing a conformational change in the molecule induces the adhesive property of gpIIb-IIIa. As with LFA-1, the regulation of platelet integrin appears to also involve intracellular interaction of the cytoplasmic domain of this molecule (O'Toole *et al.*, 1991).

Mac-1 (CD11b/CD18) is a β2 integrin also known as complement receptor 3 (CR3). It is expressed on monocytes, macrophages, granulocytes, large granular lymphocytes, and immature and CD5+ B cells (De la Hera *et al.*, 1988). This adhesion protein has been demonstrated to bind the complement component, C3bi (Beller *et al.*, 1982). When expressed on monocytes and neutrophils, the adhesive function of Mac-1 is rapidly induced by phorbol esters (Wright and Silverstein, 1982). This activation process is thought to involve the microclustering of the molecules on the cell surface (Detmers *et al.*, 1987). Similar to the platelet integrin, the activation of Mac-1 appears to involve a conformational shift as mAbs can be generated specifically against the activated form (Altieri and Edgington, 1988).

1.7 Potential Mechanisms Regulating the Adhesive Function of CD44

The adhesive function of CD44 does not appear to be regulated simply by expression of the protein. Other potential regulatory mechanisms may exist to control the binding of CD44 to its ligand, hyaluronan. These mechanisms may include interactions between the cytoplasmic domain and cytoskeletal components, conformational changes, different binding affinities for alternate CD44 isoforms, masking or shedding of the CD44 on the cell surface, or interaction with other regulatory molecules. Two or more of these regulatory pathways may also function in concert with each other or with other unknown mechanisms.

1.7.1 Involvement of the cytoplasmic domain in regulation

The evidence that the cytoplasmic domain of CD44 may be involved in regulating the adhesion to hyaluronan first came from deletional mutagenesis studies (Lesley *et al.*, 1992; Thomas *et al.*, 1992). A CD44 mutant protein was generated lacking the entire cytoplasmic domain except for the first six amino acid residues. Wild-type CD44 expressed in a CD44-negative T lymphoma line designated AKR1, was able to bind hyaluronan. The truncated mutant expressed in the same cell line however was unable to bind soluble hyaluronan but did adhere to immobilized hyaluronan (Lesley *et al.*, 1992). Further studies by this group demonstrated that the mutant could be induced to bind soluble hyaluronan by treatment with mAb IRAWB directed against CD44 (Lesley *et al.*, 1992). The use of Fab fragments of this

mAb was unable to alter the adhesive function of CD44. These data suggest that aggregation of CD44 on the cell surface is important for the binding of hyaluronan, and that this clustering may involve the cytoplasmic domain of CD44.

Thomas and coworkers performed a similar study in which a cytoplasmic deletion mutant was transfected into melanoma cells (Thomas *et al.*, 1992). These transfectants were unable to bind immobilized hyaluronan whereas cells transfected with full-length CD44 bound avidly. The two studies suggest that the cytoplasmic domain of CD44 may be important for the regulation of CD44 adhesive function. Potentially, the cytoplasmic domain may regulate the cell surface distribution of CD44 or may alter the extracellular conformation into a state that can bind hyaluronan.

The cytoplasmic domain of CD44 has been demonstrated to bind cytoskeletal elements. Kalomiris and Bourguignon studied this interaction and showed that purified CD44 can bind to purified erythrocyte ankyrin (Kalomiris and Bourguignon, 1988). Furthermore, they demonstrated that the cytoplasmic domain of CD44 can be phosphorylated *in vitro* by PKC isolated from brain tissue, and that the addition of phosphate groups enhanced the ability of CD44 to bind ankyrin (Kalomiris and Bourguignon, 1988). The correlation between phosphorylation and the affinity of CD44 for cytoskeletal elements is however not absolute as Camp and colleagues observed that cytoskeletally-associated CD44 found in the detergent (Nonidet P-40) insoluble fraction of murine peritoneal macrophages was not phosphorylated, whereas non-cytoskeletally-associated detergent-soluble CD44 was phosphorylated (Camp *et al.*, 1991).

Neame and Isacke studied the role of the cytoplasmic domain and phosphorylation in the localization of CD44 to the basolateral membrane of polarized epithelial (MDCK) cells (Neame and Isacke, 1992). The localization of CD44 in these cells appeared to be regulated by the intracellular domain, as cytoplasmic deletion mutants expressed in these cells demonstrated a scattered and unclustered distribution. Furthermore, point mutations were generated in which two serines residues (Ser^{303} and Ser^{305}) implicated as targets of phosphorylation were replaced by either alanine or glycine. The CD44 distribution in cells transfected with the mutant constructs appeared normal suggesting that cytoplasmic phosphorylation is not important for localization.

The interaction of CD44 with cytoskeletal elements is also affected by the cellular activation state. In human peripheral blood leukocytes, Geppert and Lipsky observed that following treatment with phorbol myristate acetate (PMA), the percentage of CD44 associated with the cytoskeleton decreased (Geppert and Lipsky, 1991).

To date, the importance of cytoskeletal interaction and phosphorylation on the interaction between CD44 and hyaluronan remains largely undefined and will be discussed further in Chapter II. Intracellular molecules however have been observed in other systems to regulate adhesive function. Pullman and Bodmer (Pullman and Bodmer, 1993; Pullman and Bodmer, 1992) used a mammalian expression cloning system that enriched for collagen type I binding to isolate a regulator of integrin adhesive function. The cDNA clone isolated was designated cell adhesion regulator or CAR. This clone encoded a protein of 142 amino acids

that contained an N-terminal myristoylation motif suggesting a cytoplasmic sub-membrane location for the protein. Moreover, this molecule had a consensus tyrosine kinase phosphorylation site at the C-terminus. Site directed mutagenesis removing this tyrosine residue abolished the ability to enhance cell matrix binding (Pullman and Bodmer, 1993; Pullman and Bodmer, 1992).

Other cytoplasmic molecules that may be important in regulating the adhesive function of CD44 are the ERM family of proteins. These cytoplasmic linker proteins are involved in the redistribution of adhesion molecules and the organization of cell membrane structures. *In vitro* studies have demonstrated that the cytoplasmic domain of CD44 binds to ezrin, radixin and moesin. Furthermore, the Rho subfamily of small G proteins and phosphatidylinositol turnover appear to regulate the CD44-ERM (Hirao *et al.*, 1996). Although the ERM family of molecules interacts with the cytoplasmic domain of CD44, no studies to date have linked this interaction to an alteration of CD44 function.

1.7.2 Involvement of extracellular modification in regulation

CD44 is extensively post-translationally modified in the extracellular domain by chondroitin sulfate attachment, and both N- and O-linked oligosaccharides. A wide variety of glycosylation patterns on the CD44 molecule have been observed in different human cell lines (Brown *et al.*, 1991; Jalkanen *et al.*, 1988). A change in glycosylation pattern is influenced directly by the generation of alternative CD44 species as isoform specific additional peptide sequences often contain potential glycosylation signals (Figure 1).

Other adhesion molecules have been found to be regulated by glycosylation of the extracellular domain. Diamond and colleagues have suggested that the extent of glycosylation on ICAM-1 may regulate adhesion to LFA-1 or Mac-1 (Diamond *et al.*, 1991). Mutations in ICAM-1 that destroy consensus sequences for N-linked glycosylation enhanced binding to purified Mac-1 (Diamond *et al.*, 1991). Furthermore, Calvete and coworkers have demonstrated modification of the glycosylation pattern of boar spermadhesin served to modulate its ligand-binding capacity (Calvete *et al.*, 1993). This group observed that glycosylated boar spermadhesin was unable to bind seminal-plasma protease inhibitors as well as zona pellucida glycoproteins due to the presence of an oligosaccharide chain on a conserved asparagine residue. Non-glycosylated forms of spermadhesin bind avidly to the appropriate ligands (Calvete *et al.*, 1993). Two groups also showed an important role for glycosylation in the adhesive-function of human CD2 (Recny *et al.*, 1992; Parish *et al.*, 1993). The T-lymphocyte glycoprotein receptor, CD2, mediates cell-cell adhesion by recognizing and binding to the cell surface molecule, CD58 (LFA-3). Recny and colleagues (Recny *et al.*, 1992) demonstrated that high mannose oligosaccharides attached to Asn-65 on the CD2 molecule were required for CD2-CD58 interaction. The fact that other adhesion molecules can be regulated in this manner suggests that this form of post-translational modification may also regulate CD44.

An extensive array of glycosylated CD44 molecules has been studied to date. The basic non-glycosylated form does not appear to be able to bind hyaluronan (Lokeshwar and Bourguignon, 1991), however, the role of this form of modification on the function of CD44

is not well understood. While some studies have demonstrated that deglycosylation augments binding (Katoh *et al.*, 1995; Lesley *et al.*, 1995) others have found contrary results (Bartolazzi *et al.*, 1996; Sleeman *et al.*, 1996). Katoh *et al* demonstrated that tunicamycin treatment of Chinese hamster ovary cells, previously selected for non-binding, allowed the cells to recognize hyaluronan via CD44 (Katoh *et al.*, 1995). Lesley *et al* found similar results culturing a non-binding pre-B cell (RAW 253) or a fibroblast (L cells) in tunicamycin or p-nitrophenyl beta-D-xylopyranoside (Lesley *et al.*, 1995). Contrasting results are observed by Sleeman and colleagues in which tunicamycin treatment inhibited the hyaluronan binding function of a rat pancreatic carcinoma cell line BSp73 transfected with CD44v4-v7 cDNA (Sleeman *et al.*, 1996).

Enzymatic treatment of cells with tunicamycin not only deglycosylates CD44 but also other cell surface glycoproteins, some of which may work in conjunction with CD44 to bind hyaluronan. To determine whether glycosylation affects CD44 directly, Bartolazzi *et al* used human melanoma cells stably transfected with CD44 (Bartolazzi *et al.*, 1996). This group showed by site-specific mutagenesis that all five potential N-linked glycosylation sites within the N-terminal hyaluronan-binding domain of CD44 are critical for hyaluronan binding. Expression of a CD44 protein mutated in any one of these potential N-linked glycosylation sites abrogates CD44-mediated adhesion to hyaluronan-coated plastic, suggesting that all five sites are necessary to maintain the HA-recognition domain in the appropriate conformation (Bartolazzi *et al.*, 1996). Although the studies above conclusively demonstrate a role for glycosylation on hyaluronan binding by CD44, they, however, do not delineate the mechanisms by which these functional alterations occur.

Skelton *et al* attempted to better define the role of glycosylation on the hyaluronan-binding function of CD44 by using a system involving the isolation of soluble recombinant CD44-immunoglobulin fusion proteins from a mutant Chinese hamster ovary cell line ldl-D, which has reversible defects in both N- and O-linked oligosaccharide synthesis (Skelton *et al.*, 1998). CD44 glycoforms with defined oligosaccharide structures were generated by treating this cell line with a variety of recombinant glycosidases and metabolic glycosidase inhibitors. Since the ldl-D cells themselves express CD44, changes in cell avidity and soluble CD44 affinity induced by glycosylation differences can be compared. From the panel of distinct glycoproteins generated, only four were observed to effect CD44-mediated HA binding. Three specific oligosaccharide structures effected the affinity of soluble CD44 without altering cell avidity. Terminal α2,3-linked sialic acid on N-linked oligosaccharides inhibited binding, the first N-linked N-acetylglucosamine residue enhanced binding, whereas O-linked glycans on N-deglycosylated CD44 enhanced binding. N-acetylgalactosamine incorporation into non-N-linked glycans did not effect the affinity of soluble CD44 for hyaluronan but increased cellular hyaluronan binding (Skelton *et al.*, 1998) suggesting that this alters CD44 oligomer formation or the involvement of another glycoprotein. These results begin to explain the apparent discrepancies found in previous studies on the role of glycosylation, however, whether or not these glycoforms are physiologically relevant remains to be determined. Furthermore, it is important to note that glycosylation is not the only regulatory mechanism involved in CD44-hyaluronan binding. Regulation of ligand binding by the glycosylation of CD44 will be discussed further in Chapter II.

CD44 has also been demonstrated to be modified by the glycosaminoglycans chondroitin sulfate, heparan sulfate and keratin sulfate (Brown *et al.,* 1991). The addition of keratin sulfate on CD44H has been suggested to inhibit hyaluronan binding (Takahashi *et al.,* 1996). Heparin sulfate has been demonstrated to modify the SGSG motif found encoded by exon V3 (Greenfield et al., 1999). This modification confers upon exon V3 containing isoforms the ability to interact with hepatocyte growth factor (van der Voort *et al.*, 1999). Importantly, inhibition of sulfation using a potent inhibitor of adenosine triphosphate sulfurylase, abolished antibody induced hyaluronan binding by CD44 in fibroblasts (Esford *et al.,* 1998)

1.7.3 Involvement of CD44 isoforms in regulation

The role of higher molecular mass CD44 isoforms in hyaluronan binding is controversial to date with numerous conflicting reports. Stamenkovic and colleagues isolated CD44E, the major isoform expressed by the colon carcinoma cell line HT29 (Stamenkovic *et al.*, 1991). This group demonstrated that CD44E was unable to recognize and bind to hyaluronan and further suggested that the isoform specific sequences were responsible for the altered adhesive function of CD44 (Stamenkovic *et al.*, 1991). Conversely, the studies of He and colleagues which suggest that the murine homologue of CD44E, although it contains a 132 amino acid insertion is able to mediate attachment to hyaluronan upon transfection into a CD44-negative T lymphoma cell line (He *et al.*, 1992). CD44R1 an isoform differing from CD44E by just 3 amino acid substitutions, has been isolated from the human myelomonocytic leukemia cell line KG1a (Dougherty *et al.*, 1991). This isoform can

however, bind avidly and specifically to hyaluronan, whether expressed on the surface of transfected COS7 cells or as a soluble chimeric protein fused in-frame to the enzyme alkaline phosphatase (Dougherty *et al.*, 1994). These data suggest an important role for one or more of these amino acids in hyaluronan binding. One of the amino acid substitutions in CD44E (tyrosine at position 109) is present within the region of CD44 implicated in hyaluronan binding (Peach *et al.*, 1993). It is important to note that the serine residue found at this position in CD44R1 is also found at this position in all other human and animal CD44 cDNAs that have been reported to date. The serine to tyrosine substitution at this position may explain the inability of CD44E to bind hyaluronan however, the other amino acid substitution may be involved as well, perhaps to alter the conformation of CD44.

1.7.4 Involvement of CD44 masking or shedding in regulation

The reduction of CD44 on the cell surface by shedding of the extracellular domain is a potentially rapid mechanism by which the function of the molecule may be regulated. Treatment of human neutrophils with TNFα, PMA, calcium ionophore, and formyl-Met-Leu-Phe (fMLP) for 30 minutes downregulated the expression of CD44 presumably by proteolytic cleavage, as this reduction was blocked by protease inhibitors (Campanero *et al.*, 1991). Shedding of CD44 also appears to be involved in the downregulation of CD44 on granulocytes following stimulation with PMA or ionomycin for 12 hours (Bazil and Horejsi, 1992). Shedding seems to be the mechanism by which CD44 is downregulated as a lower molecular mass (compared to cell surface CD44) ^{125}I-labeled molecule reactive with mAbs directed against CD44 could be isolated from supernatants of ^{125}I-surface labeled stimulated

cells. Shedding of cell surface molecules, as a mechanism of regulating expression is not restricted to CD44. Other hemopoietic molecules are downregulated following stimuli such as CD23, CD6, L-selectin, TNF receptor, CD14, ICAM-1, and CD32 (reviewed in Lesley *et al.*, 1993).

1.7.5 Involvement of cell-specific molecules in regulation

There is increasing evidence that the functional activity of adhesion proteins can be regulated by interactions in the plane of the membrane with other cell surface molecules. We have demonstrated in the past that by using an expression cloning system, cell specific regulatory molecules can be isolated that activate the hyaluronan-binding capacity of CD44 upon transfection into the murine fibroblastoid cell line MOP8 (Chiu *et al.*, 1995). A transmembrane protein originally designated IL-2Rγ (now known as γc), that constitutes an integral component of the cell surface receptors that bind a number of cytokines, was observed to induce hyaluronan binding without increasing the overall expression of CD44.

1.8 Thesis objective

The adhesion protein CD44 has been suggested to play an important role in the development and pathogenesis of malignant disease. In particular, dramatic correlations have been noted between the expression of CD44, or the presence of certain alternatively spliced CD44 isoforms, and prognosis. Although present on most normal cell types, CD44 largely exists in a functionally inactive state. Thus, the major objective of this thesis is to better define the molecular mechanisms that regulate both the ligand binding activity and specificity of the CD44 molecule on normal and malignant cells. Such information is critical if the contribution that CD44 makes to the malignant process is to be fully understood.

1.8.1 Specific Aims

1. To define the mechanisms responsible for the differences observed in the hyaluronan binding activity of the myelomonocytic cell line KG1 and its phenotypically less mature, but closely related derivative KG1a.

2. To better characterize the unique adhesive interaction that occurs between exon v10 containing CD44 isoforms.

3. To define the role that soluble CD44 plays in the regulation of hyaluronan binding activity.

CHAPTER II

MOLECULAR MECHANISMS REGULATING THE HYALURONAN BINDING FUNCTION OF CD44

2.1 Introduction

Although the subject of much debate, at present, the precise molecular mechanisms that regulate the hyaluronan-binding function of CD44 remain unclear. There is, however, a general consensus that in common with many other adhesion proteins, the interaction between CD44 and hyaluronan is not regulated simply by expression. Thus, while most primary hemopoietic cells express high levels of CD44, in the absence of appropriate stimulation, only a small subset of these cells can recognize and bind either immobilized or soluble hyaluronan (Lesley *et al.*, 1993; Lesley *et al.*, 1994). Hemopoietic cell lines expressing CD44 are similarly heterogeneous in their hyaluronan binding activity (Lesley *et al.*, 1993).

Efforts designed to shed light on the mechanisms responsible for the functional heterogeneity of CD44 have yielded conflicting results (reviewed in Lesley and Hyman, 1998). In particular, apparently contradictory findings have been reported regarding the ability of various alternatively spliced CD44 isoforms to bind hyaluronan (He *et al.*, 1992; Liao *et al.*, 1993; Dougherty *et al.*, 1994; Bennett *et al.*, 1995; Galluzzo *et al.*, 1995; van der Voort *et al.*, 1995; Sleeman *et al.*, 1998) and the functional significance of interactions

between the cytoplasmic domain of CD44 and cytoskeletal proteins remains controversial (Lokeshwar *et al.*, 1994; Perschl *et al.*, 1995; Liu *et al.*, 1996). In part, such confusion undoubtedly reflects the wide range of experimental systems and cell lines employed in the study of CD44.

In order to provide a solid foundation upon which to base subsequent studies on the ligand binding function of CD44, an initial series of experiments were undertaken to define the mechanisms responsible for the differences observed in the hyaluronan binding activity of the myelomonocytic cell line KG1 and its phenotypically less mature, but closely related derivative KG1a. The results obtained emphasize that the cellular context in which CD44 is expressed plays a critical role in determining the functional activity of the molecule. It was further demonstrated, that CD44H and the alternatively spliced exon v10 containing CD44 isoforms CD44R1 and CD44R2 do not differ in their hyaluronan binding activity when expressed in K562 cells. Rather, the data obtained suggests that in cell lines where CD44 exists in a "functionally competent" state, it is the local concentration of the molecule in the plane of the membrane that appears to determine hyaluronan-binding activity. Increased local concentrations of CD44 generated by transcriptional mechanisms, or produced by an active process that involves interactions between the cytoplasmic domain of the molecule and the cytoskeleton, enhance hyaluronan-binding activity.

2.2 Materials and Methods

2.2.1 Cell lines

The erythroleukemic cell line K562, the histiocytic cell line U937, and the myelomonocytic cell line KG1 and its less mature derivative KG1a were all obtained from the American Type Culture Collection (ATCC) (Rockville, MD). K562 and U937 cells were cultured in Dulbecco's minimum essential medium (DMEM) (Stem Cell Technologies Inc., Vancouver, Canada) supplemented with 10% Cool Calf II (CCII) (Sigma, St. Louis, MO). KG1 and KG1a cells were grown in Iscove's modified Dulbecco's media (IMDM) supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). All cell lines were maintained at 37°C in an atmosphere containing 5% CO_2.

The murine lymphoma cell line TIL1 was derived from a tumour initiated in C3Hf/Sed//Kam mice by the subcutaneous inoculation of syngeneic Fsa-R fibrosarcoma cells that had been genetically engineered through retroviral-mediated gene transfer to express murine interleukin-7 (McBride *et al.*, 1992). Briefly, the tumour was disaggregated by mincing, and tumour pieces placed in a flask together with approximately 50 ml DMEM + 10% FBS. After 2 weeks in culture, nonadherent cells were transferred to a separate flask and this procedure repeated daily until all adherent fibroblastoid tumour cells were removed. The cell line obtained, designated TIL1, expresses CD4 and CD8, TcR, and LFA-1, but is negative for Mac-1, p150,95, and CD44. These cells do not contain retroviral vector-derived sequences, nor do they produce interleukin-7. They are, however, malignant and generate a

localized ascitic tumour following intraperitoneal injection into syngeneic C3H/HeJ mice. TIL1 cells were maintained in DMEM + 10% FBS or 10% CCII.

2.2.2 Monoclonal antibodies

The generation and characterization of the anti-CD44 mAbs 3G12 and 8D8, and the exon v10 specific mAb 2G1 has been described in detail previously (Dougherty *et al.*, 1994; Droll *et al.*, 1995). Previous studies have demonstrated that mAb 3G12 can inhibit cellular adhesion to immobilized hyaluronan while mAb 8D8 lacks this activity (Droll *et al.*, 1995). The R-phycoerythrin-conjugated anti-CD44 mAb G44-26 (PE-G44-26) was obtained from PharMingen, (San Diego, CA).

2.2.3 Cloning of CD44 cDNAs

A cDNA encoding the 85-95 kDa CD44H isoform expressed by U937 cells was generated by RT-PCR. Briefly, mRNA was isolated from U937 cells using a Stratagene mRNA Isolation Kit (Stratagene, La Jolla, CA). cDNA was synthesized using a Stratagene First Strand cDNA Synthesis Kit (Stratagene) and CD44H amplified by PCR (Hybaid OmniGene, Labnet, Woodbridge, NJ) (30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) using 10 µl of the first strand synthesis reaction and CD44 exon 1 (5'-GGTCTAGACCGTTCGCTCCGGACACCATGG-3')- and exon 20 (5'-GGTCTAGATT ACACCCCAATCTTCATGTCC-3')-specific primers. The PCR products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. The approximately

1.1 kb fragment corresponding in size to CD44H was excised, the DNA isolated using GeneClean (BIO 101, Vista, CA), "blunted" using T4 polymerase (Gibco BRL, Gaithersburg, MD) and ligated into the EcoRV site of the vector pZERO 2.1 (Invitrogen, San Diego, CA). Restriction enzyme analysis confirmed that the majority of clones corresponded to CD44H. pZERO.CD44H(U937) clone #7 was digested with XbaI and the and the full-length cDNA obtained, cloned into the NheI site of the EBV-based episomal expression vector, pCEP4 (Invitrogen). Further restriction enzyme analysis confirmed the presence of CD44H in the correct orientation for the clone pCEP4.CD44H(U937) #7.5.

cDNAs encoding CD44H and the 130 kDa exon v10 containing CD44 isoform CD44R1 were also generated from the cell line KG1a which can constitutively bind hyaluronan (see below). These cDNAs were isolated respectively, from the plasmids pCDM8.CD44 (clone 2.7) and pCDM8.CD44 (clone 2.3) (Dougherty *et al.*, 1991) and cloned into the EBV-based episomal vector pCEP4. Unless otherwise stated, CD44 cDNAs used in this thesis, are isolated from the cell line KG1a.

2.2.4 Construction of Retroviral Vectors encoding CD44H (JhCD44H) and CD44R1 (JhCD44R1)

The Moloney murine leukemia virus-based retroviral vector Jzen.1 (Laker *et al.*, 1987) was used to introduce and express human CD44H and CD44R1 in TIL1 cells. Briefly, full-length CD44H and CD44R1 cDNAs were inserted into the XbaI site of pTZ19R/Tk-neo (Hughes *et al.*, 1992). SmaI- HindIII cassettes containing CD44H or CD44R1 together

with Tk-neo were isolated and cloned into HpaI- HindIII cut Jzen.1. The plasmids obtained were transfected into the ecotropic packaging line GP+E-86 (Markowitz *et al.*, 1988) by calcium phosphate precipitation and transfected cells selected in G418 (0.5 mg/ml active weight) (Life Technologies Inc., Grand Island, NY). Supernatants conditioned by the packaging line for 24 h contained $>10^6$ colony-forming units/ml. TIL1 cells were infected with cell free JhCD44H or JhCD44R1 viral supernatants containing 5 μg/ml Polybrene (Sigma), as described previously (Hughes *et al.*, 1992). Infected cells were selected and maintained in medium containing G418 (0.3 mg/ml active weight). To ensure that any heterogeneity present within the starting TIL1 cell population was retained, at least 200 clones were pooled and expanded for further study. Transduced cells were regularly examined by FACS analysis to confirm continued high level expression of the introduced genes (see below).

2.2.5 Preparation of fluorescein-labeled hyaluronan

Fluorescein isothiocyanate conjugated hyaluronan (FITC-HA) was prepared using the protocol of de Belder and Ove Wik (de Belder and Ove Wik, 1975). Briefly, 100 mg human placental hyaluronan (Sigma) was dissolved in 20 ml formamide (Sigma-Aldrich) by overnight agitation in a 50 ml tube (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). After complete mixing, 25 ml methyl sulfoxide (Sigma-Aldrich), 50 mg sodium hydrogen carbonate (Fluka), 50 mg dibutyltin dilaurate (Sigma-Aldrich), and 150 mg fluorescein isothiocyanate (FITC) (Sigma) was added and the tube was stirred over a steam bath for 30 min. To remove unbound FITC, the contents were diluted with 25 ml deionized water and

1 l ethanol in a 2 l flask and then transferred to 500 ml centrifuge tubes. After centrifugation at 1000 rpm for 5 min, the product was collected and reprecipitated twice. After the last precipitation, the FITC-HA was dried and weighed.

2.2.5 K562 cell transfection

K562 cells were transfected with plasmid DNA purified using the BiggerPrep DNA Isolation Kit (5' 3', Boulder, CO, USA), and the BTX ECM 600 Electroporation System (BTX, San Diego, CA). Briefly, log-phase K562 cells were harvested and resuspended in phosphate buffered saline (PBS) at a final concentration of 1 x 10^7 cells/ml. 15 μg of plasmid DNA (pCEP4 or the CD44 constructs pCEP4.CD44H(U937), pCEP4.CD44H(KG1a), pCEP4.CD44R1 or pCEP4.CD44R2) (see Chapter III, Materials and Methods for the generation of this isoform) were added to a 400 μl aliquot of the cell suspension, transferred to a 2 mm gap cuvette and electroporated at 280 volts with a capacitance setting of 500 μF. The time constants obtained ranged from 3.0-3.6 ms. Immediately after electroporation, cells were diluted in 30 ml DMEM + 10% CCII, transferred to a 75 cm^2 tissue culture flask (Falcon; Becton Dickinson Labware) and incubated at 37°C in the presence of 5% CO_2. Hygromycin B (Sigma) was added 24 h later at a final concentration of 200 μg/ml. Transfected cells were selected for a minimum of 14 days and were maintained thereafter in DMEM + 10% CCII containing 200 μg/ml hygromycin.

2.2.6 Flow cytometric analysis of CD44

The expression of CD44 on retrovirally transfected-TIL1 cells, K562, U937, KG1 and KG1a cells as well as the K562 transfectants (pCEP4.CD44H(U937), pCEP4.CD44H(KG1a), pCEP4.CD44R1 or pCEP4.CD44R2), was determined by FACS analysis. In some experiments, cells were stained with a combination of PE-G44-26 and FITC-HA (see below). In other studies, $5x10^5$ cells were incubated with anti-CD44 mAbs 4A4, 3G12 or 2G1 tissue culture supernatant, or media alone, for 1 hour at 0°C. After 3 washes with ice-cold Hank's balanced salt solution (HBSS) containing 2% CCII, the cells were stained for 1 hour at 0°C with an FITC-conjugated goat anti-mouse antibody (PharMingen) at a final concentration of 10 μg/ml in HBSS+2% CCII. Following extensive washing, cells were resuspended in HBSS+2%CCII containing 1 μg/ml propidium iodide (PI) (Sigma) to facilitate the identification and exclusion of dead cells, and analyzed on a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA).

2.2.7 Soluble hyaluronan binding

$2x10^5$ of each of the cell lines K562, U937, KG1 and KG1a, or K562 transfected with various CD44 isoforms, were incubated at 0°C for 30 min with a combination of FITC-HA (approximately 50 μg/ml final) and mAb PE-G44-26 (10 μl/$2x10^5$ cells in a final volume of 1 ml). Following extensive washing, the cells were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems) for CD44 expression (FL2) and soluble hyaluronan binding (FL1).

2.2.8 Immobilized hyaluronan binding assay

10^6 K562, U937, KG1, or KG1a cells, or the K562 cells transfected with various CD44 isoforms, were incubated with DMEM + 10% CCII or with anti-CD44 mAb 8D8 or 3G12 tissue culture supernatants for 1 h at 4°C. $2x10^5$ cells in a final volume of 0.5 ml were added to each of 2 wells in a 24 well plate (Falcon) that had been coated overnight at 4°C with human placental hyaluronan (5mg/ml in PBS). After incubation for 10 min at room temperature, non-adherent cells were removed by gently washing each well 5 times with HBSS. The number of adherent cells per unit area was determined by digital analysis of captured well images (NIH Image, Research Services Branch, NIMH, Bethesda, MD) or by counting 5 random fields using an inverted phase microscope. Each point represents the mean ± S.D. of at least three independent determinations.

2.3 Results

2.3.1 Correlation between CD44 expression and hyaluronan-binding function

Although the myeloid cell lines U937, KG1 and KG1a express similar levels of CD44, they differ greatly in their ability to bind both soluble and immobilized hyaluronan. Thus U937 cells bind little if any soluble FITC-HA (Figure 4) and do not adhere to plastic surfaces coated with hyaluronan under the experimental conditions employed (Figure 5). In contrast, approximately 25% of KG1 cells can bind soluble FITC-HA (Figure 4) and

significant numbers of these cells also attach to hyaluronan-coated plastic (Figure 5). Such adhesion is mediated by CD44 since it can be blocked if cells are pre-treated with a mAb directed against the hyaluronan binding domain of CD44 (mAb 3G12). A control mAb (8D8) that recognizes an epitope on CD44 distinct from that involved in ligand-binding, had no effect on adhesion to hyaluronan. In comparison to KG1, a two to three fold higher percentage of KG1a cells bind soluble hyaluronan (Figure 4) and this is reflected in the increased proportion of these cells that bind to plastic surfaces coated with hyaluronan (Figure 5). Once again, mAb blocking studies confirmed that such attachment was mediated largely by CD44. K562 cells do not express CD44 and bind neither soluble or immobilized hyaluronan (Figure 5).

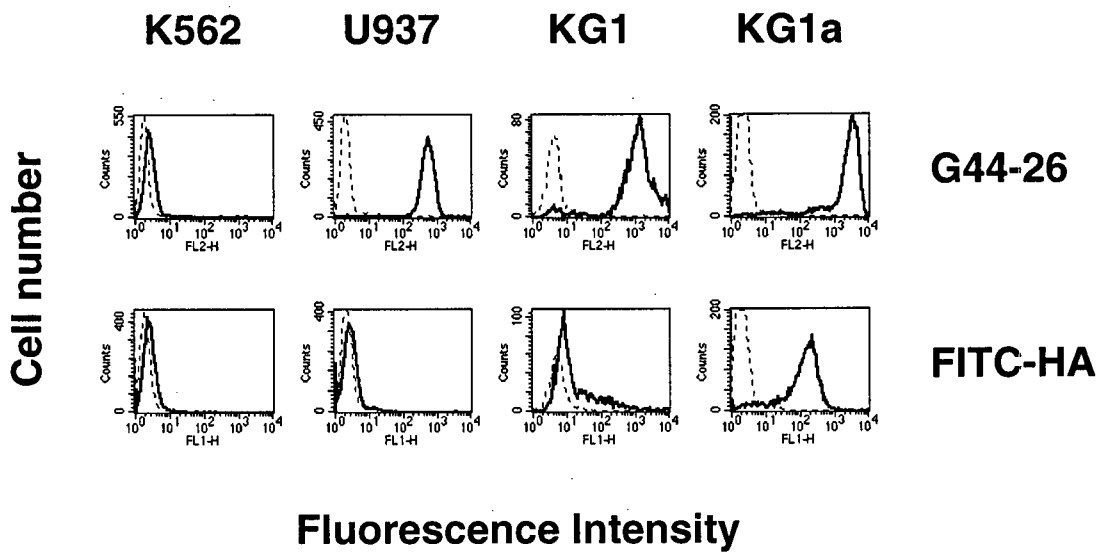


Figure 4: CD44 expression and soluble hyaluronan binding activity of various hemopoietic cell lines. K562, U937, KG1 and KG1a cells were incubated with a combination of FITC-HA and PE-G44-26. Following extensive washing, the cells were analyzed on a FACScan for CD44 expression (G44-26) and FITC-HA binding.

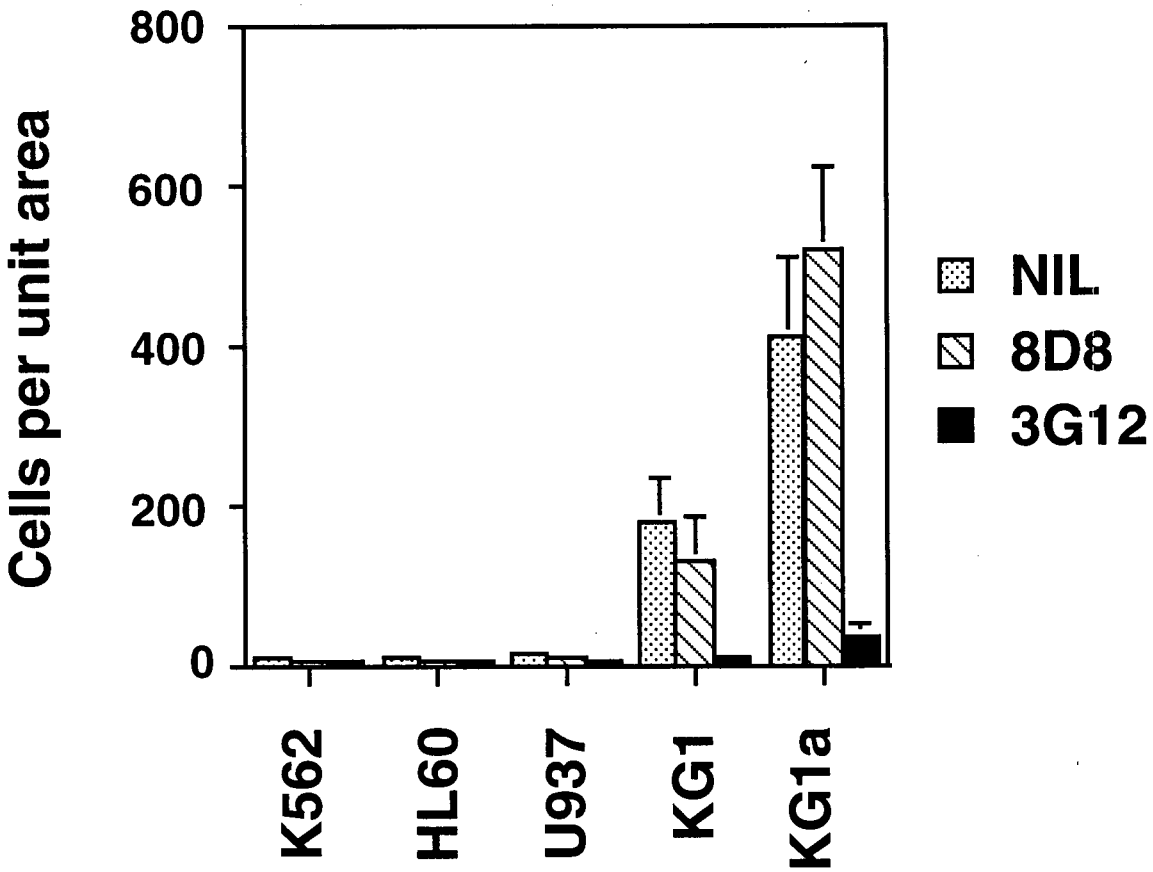


Figure 5: Adhesion of hemopoietic cell lines to immobilized hyaluronan. K562, U937, KG1, and KG1a cells were incubated with tissue culture medium (NIL) or with anti-CD44 mAb 8D8 or 3G12 tissue culture supernatants for 1 h at 4°C. Following extensive washing, the cells were assayed for their ability to adhere to hyaluronan-coated plastic. The number of adherent cells per unit area was determined by digital analysis of captured well images or by counting 5 random fields using an inverted phase microscope. Each point represents the mean ± S.D. of at least three independent determinations.

2.3.2 Impact of cellular context on the hyaluronan binding activity of CD44

Although U937 cells lack hyaluronan binding activity, the CD44 gene appears not to be functionally mutated in these cells. Briefly, a CD44H cDNA was amplified from U937 cells by RT-PCR and cloned into the EBV-based episomal expression vector pCEP4. pCEP4, pCEP4.CD44H(U937) or pCEP4.CD44H(KG1a) plasmid DNA was introduced into CD44-negative K562 cells by electroporation and transfected cells selected in hygromycin. FACS analysis confirmed moderate expression of CD44 in K562 cells transfected with pCEP4.CD44H(U937) or pCEP4.CD44H(KG1a) (Figure 6). Control K562 cells transfected with pCEP4 remained CD44-negative (Figure 6). Functional studies confirmed that although U937 cells lack hyaluronan binding activity (Figure 5), K562 cells transfected with a CD44H cDNA derived from U937 can adhere to hyaluronan coated plastic (Figure 7). These data emphasize that the cellular context in which CD44 is expressed is critical in determining the functional status of the molecule.

2.3.3 Hyaluronan binding activity of alternatively spliced CD44 isoforms

Although the total level of CD44 expressed by KG1 and KG1a cells as determined by reactivity with the CD44 mAbs 4A4 and 3G12 appeared very similar (Figure 8), the two closely related cell lines did differ with respect to the proportion of their CD44 molecules that contain the alternatively spliced exon v10. Thus KG1 cells are essentially unreactive with the v10-specific mAb 2G1, while KG1a cells exhibit significant levels of reactivity (Figure 8). The relative reactivity of mAbs 4A4 and 2G1 with CD44-negative murine TIL

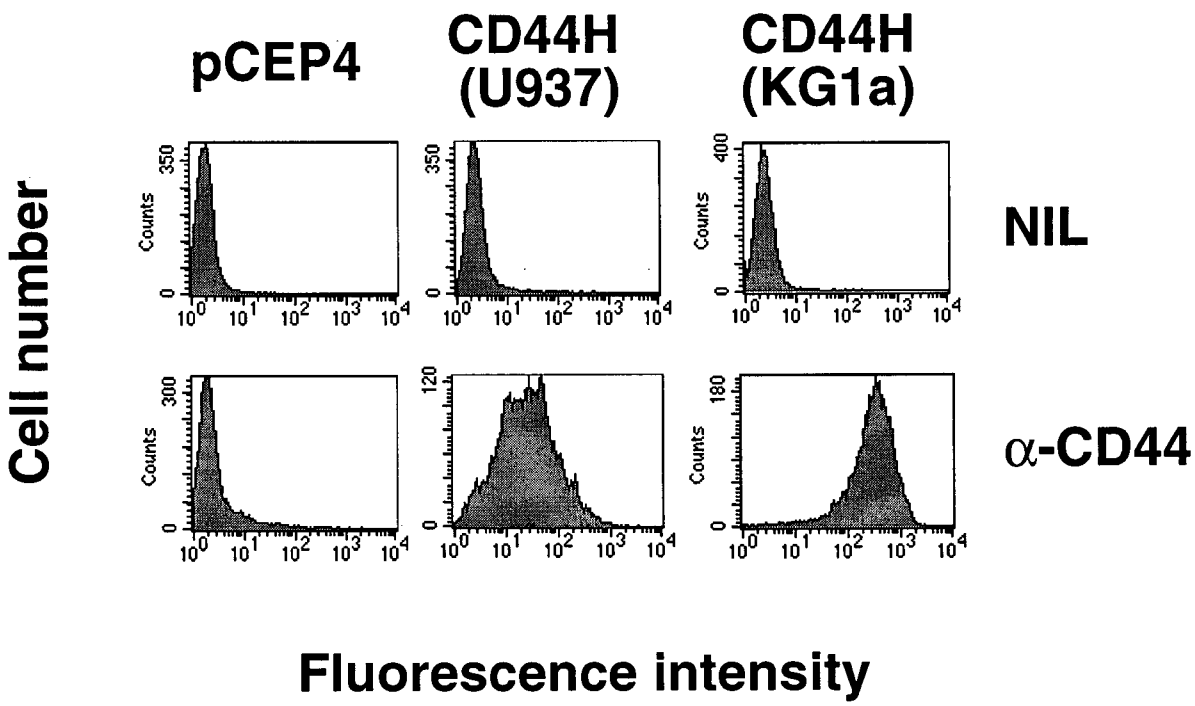


Figure 6: Expression of CD44 on K562 cells transfected with CD44H cDNAs isolated from U937 and KG1a cells. The expression of CD44 in K562 cells transfected with pCEP4 or pCEP4 containing a CD44H isolated from U937 [CD44H(U937)], or KG1a [CD44H(KG1a)], was determined by FACS analysis. Cells were incubated with tissue culture medium alone (NIL) or mAb 4A4 tissue culture supernatant (mAb 4A4), washed then incubated with an FITC-conjugated goat anti-mouse secondary antibody. Following additional washing, stained cells were analyzed on a FACScan.

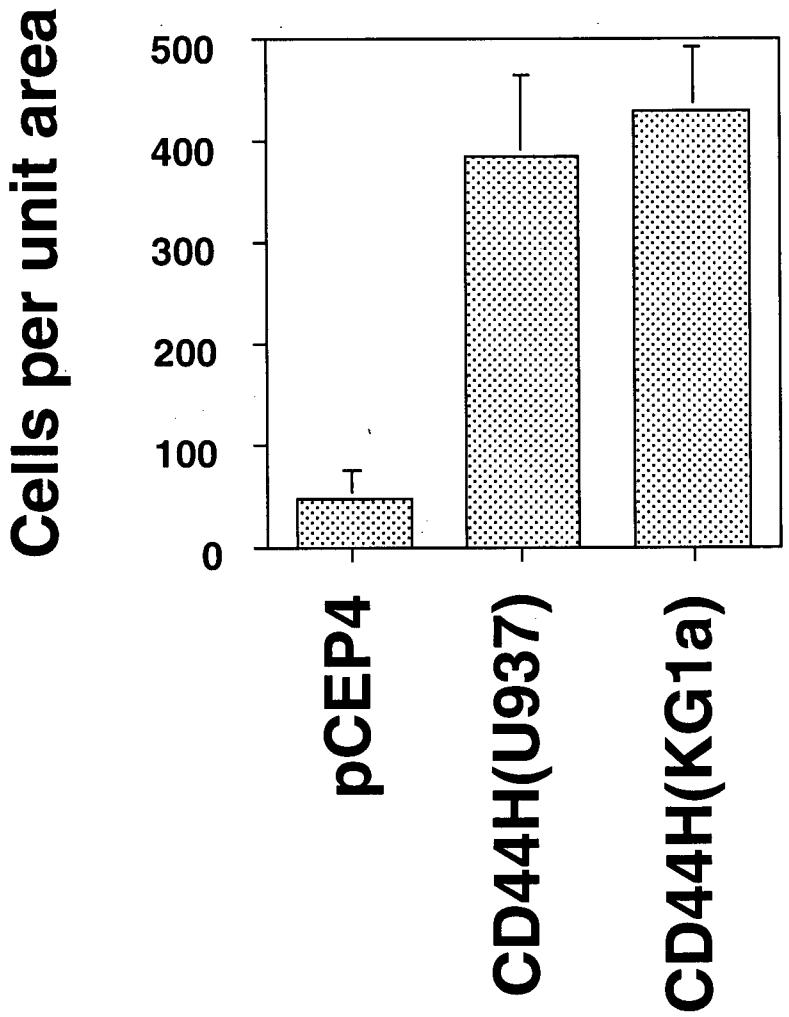


Figure 7: Hyaluronan-binding activity of a CD44H cDNA isolated from U937 cells. K562 cells transfected with the pCEP4 vector alone or with pCEP4 containing CD44H isolated from U937 [CD44H(U937)] or KG1a [CD44H(KG1a)] cells, were assayed for their ability to adhere to hyaluronan-coated plastic. The number of adherent cells per unit area was determined by digital analysis of captured well images or by counting 5 random fields using an inverted phase microscope. Each point represents the mean ± S.D. of at least three independent determinations.

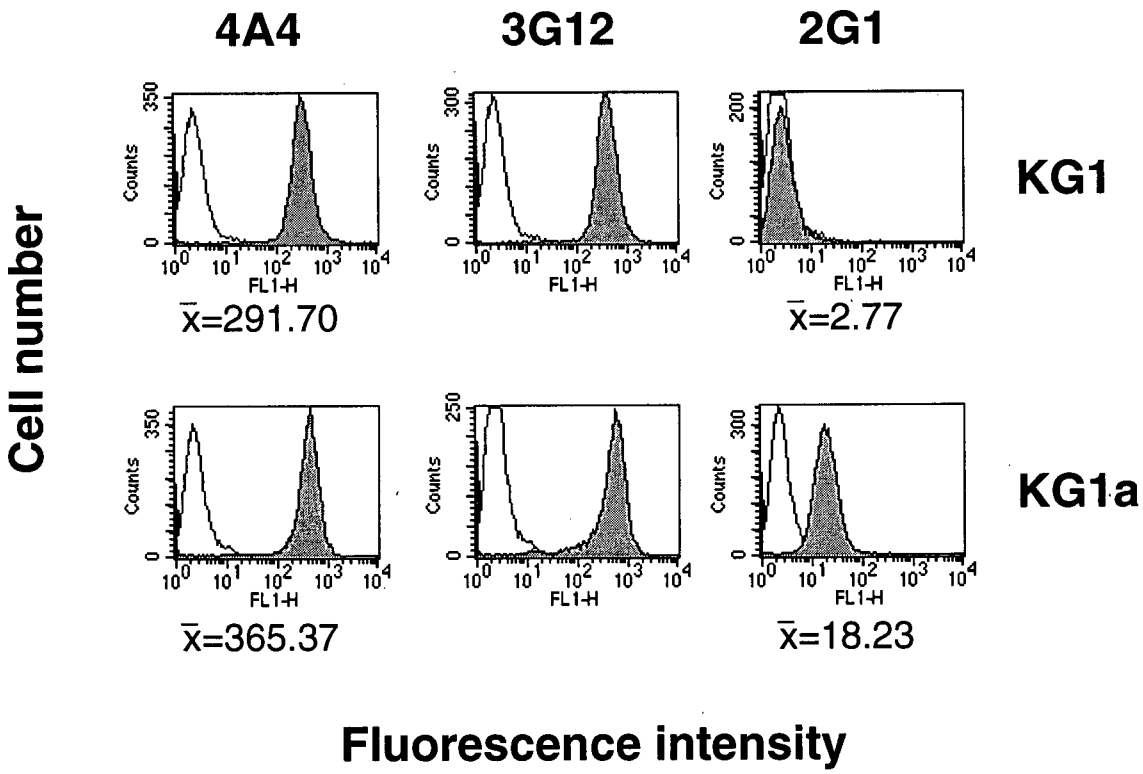


Figure 8: Expression of exon v10 containing CD44 isoforms on the cell lines KG1 and KG1a. $5x10^5$ KG1 or KG1a cells were incubated with tissue culture medium alone (NIL) or with mAb 4A4, 3G12 or 2G1 tissue culture supernatants for 1 hour at 4°C. After 3 washes with HBSS, the cells were incubated for a further 1 hour at 4°C with an FITC-conjugated goat anti-mouse secondary antibody. Following additional washing, stained cells were analyzed on a FACScan.

cells transduced with retroviral vectors encoding CD44H or CD44R1 (Figure 9) enables one to derive a constant that can be used to correct for differences in antibody affinity, allowing the proportion of CD44 molecules present on KG1 and KG1a that contain exon v10 to be estimated. Thus all of the CD44 molecules expressed by TIL cells transduced with JhCD44R1 contain exon v10 and the difference observed in the mean fluorescence obtained with mAb 4A4 and 2G1 (Figure 9) can be attributed largely to differences in the affinity of these two antibodies for CD44. By dividing the mean fluorescence obtained with mAb 4A4 by that obtained with mAb 2G1 it is possible to derive a constant which if applied to the mean fluorescence obtained with mAb 2G1 corrects for the lower affinity of this antibody. Based on the staining of CD44R1 transduced TIL cells the value of this constant is 3.14 (Figure 9). The mean fluorescence intensities obtained with mAbs 4A4 and 2G1 on KG1 cells are 365.37 and 13.23 respectively (Figure 8). Multiplying the value obtained with mAb 2G1 by the constant calculated as described above, gives an "affinity corrected" mean fluorescence value of 57.24. When divided by the value obtained for mAb 4A4 (representing the total amount of CD44 present on the cell surface) it would appear that approximately 16% of the CD44 molecules expressed by KG1a contain exon v10. Using the same approach the equivalent value for KG1 cells is only 3%.

Although the subject of much debate, it has been suggested that alternatively spliced CD44 isoforms may differ in their ability to bind hyaluronan. To determine whether variation in the expression of v10 containing isoforms may contribute to differences in the hyaluronan binding activity of KG1 and KG1a cells, cDNAs encoding the major CD44 isoforms expressed by KG1a cells (CD44H, CD44R1 and CD44R2) were cloned into the

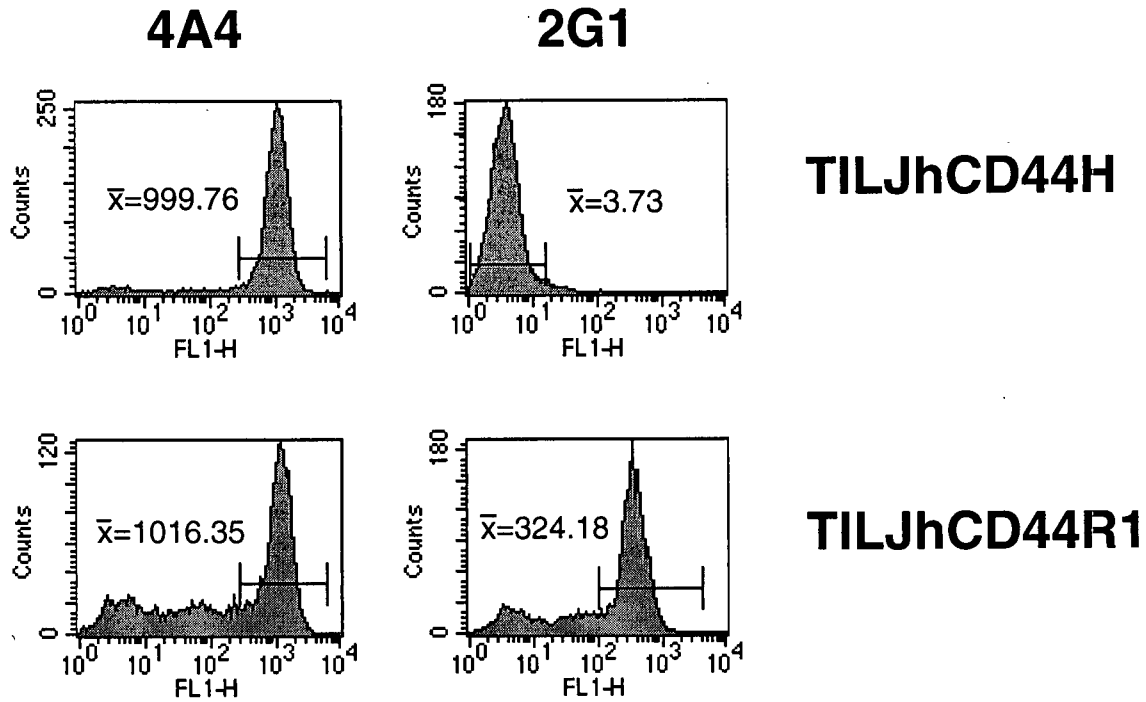


Figure 9: Reactivity of mAb 4A4 and 2G1 with TILJhCD44H, TILJhCD44R1. Transduced TIL cells were incubated with mAb 4A4 or 2G1 tissue culture supernatants for 30 min at 0°C. After 3 washes with HBSS+2% FCS, the cells were incubated for a further 30 min at 0°C with an FITC-conjugated goat anti-mouse secondary antibody. Following additional washing, stained cells were resuspended in HBSS+2% FCS containing 1 μg/ml propidium iodide and analyzed on a FACScan.

EBV-based episomal expression vector pCEP4. Plasmid DNA was introduced into CD44-negative K562 cells by electroporation and transfected cells selected in hygromycin.

FACS analysis of transfected cells following double labeling with a PE-conjugated anti-CD44 mAb and FITC-HA revealed an interesting relationship between CD44 expression and FITC-HA binding (Figure 10). Specifically, there is no simple linear correlation between the level of CD44 present on a cell and hyaluronan binding. Rather, it is necessary that cells express a minimum or threshold level of CD44 before any hyaluronan binding can be detected. Importantly, this threshold level appears the same regardless of whether K562 cells were transfected with CD44H, CD44R1 or CD44R2. Above the binding threshold, there is an obvious linear relationship between CD44 expression and the amount of hyaluronan bound.

To calculate the linear regression values for CD44H, CD44R1 and CD44R2, data points corresponding to cells binding hyaluronan were extracted from gated CellQuest data files using FCS Assistant 1.3.1a beta and imported into CricketGraph via Microsoft Excel. As shown in Figure 11, the slope of the lines obtained appears nearly identical for CD44H ($y = 0.355x + 142.248$; $r = 0.759$), CD44R1 ($y = 0.430x + 138.472$; $r = 0.684$) and CD44R2 ($y = 0.351x + 138.433$; $r = 0.737$). Taken together, these data strongly suggest that CD44H and the exon v10 containing CD44 isoforms CD44R1 and CD44R2 do not differ greatly in their hyaluronan binding activity, at least when expressed in the context of K562 cells.

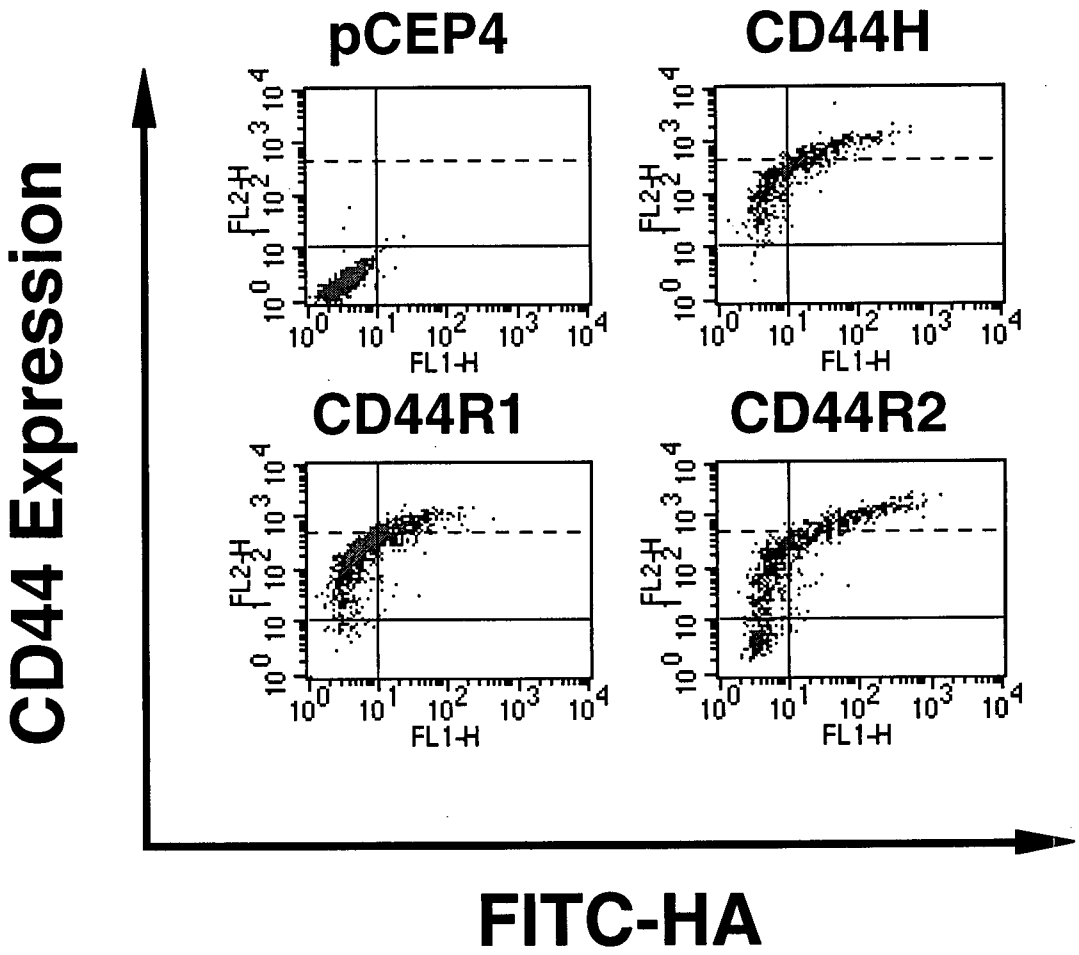


Figure 10: Binding of soluble hyaluronan to K562 cells transfected with various alternatively spliced CD44 isoforms. K562 cells transfected with the pCEP4 vector alone (pCEP4) or with pCEP4 containing CD44H, CD44R1 or CD44R2 cDNAs were simultaneously tested for CD44 expression and soluble hyaluronan binding. $2x10^5$ of each transfectant were incubated at 0°C for 30 min with a combination of FITC-HA (approximately 50 μg/ml final) and PE-G44-26 (10 μl/ml). Following extensive washing, the cells were analyzed on FACScan for CD44 expression (FL2) and FITC-HA binding (FL1).

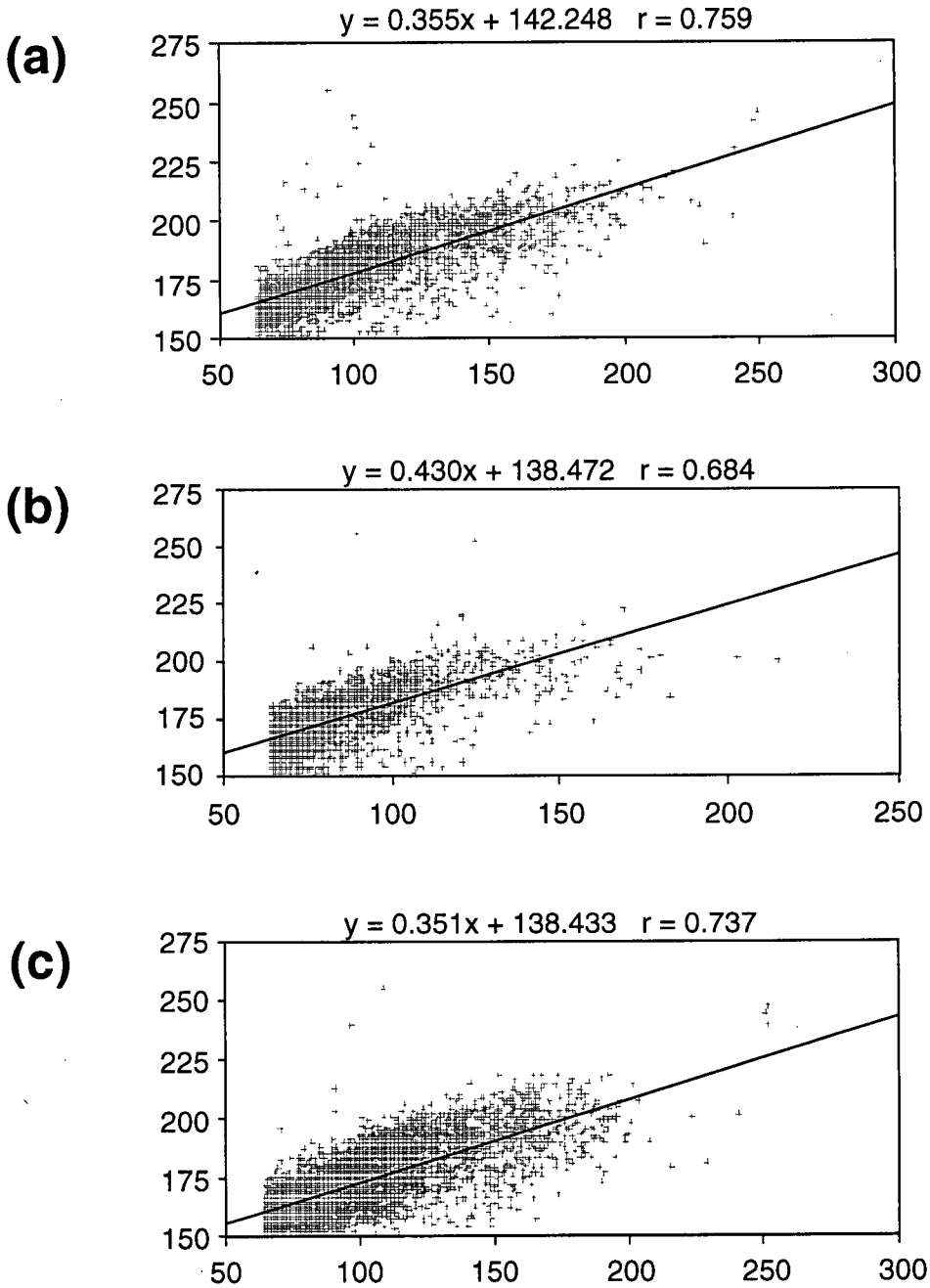


Figure 11: Linear regression analysis of the correlation between CD44 isoform expression and soluble hyaluronan binding. To determine the relationship between CD44 expression and hyaluronan-binding activity, data points corresponding to the subset of transfected K562 cells expressing CD44H (a), CD44R1 (b) and CD44R2 (c) that bind hyaluronan were extracted from appropriately gated CellQuest data files using FCS Assistant 1.3.1a beta and imported into CricketGraph via Microsoft Excel. The linear regression equations and r values obtained for each cell line are shown. The x- and y-axis represents FL1 and FL2 respectively.

2.3.4 Involvement of cytoskeletal associations in the regulation of the hyaluronan binding activity of CD44

Although FACS analysis suggests that KG1 and KG1a cells express similar overall levels of CD44, indirect immunoperoxidase staining of acetone fixed cytospin preparations revealed obvious differences in the distribution of CD44 on the surface of these two cell lines. In KG1 cells, CD44 is distributed fairly evenly with some limited aggregation evident as punctated staining (Figure 12). In contrast, in KG1a cells, CD44 is usually localized to large clusters or patches that are often associated with distinct membrane projections or uropods (Figure 12). Such clusters may be found closely opposed at sites of cell-cell contact. As might be expected, FITC-HA co-localized with CD44 and was found preferentially associated with uropods in KG1a cells (Figure 13).

The clustering of CD44 on KG1a cells appears to involve direct or indirect associations with the cytoskeleton. Overnight treatment with cytochalasin D effectively disrupted the large CD44 clusters that are found associated with uropods on KG1a cells although smaller aggregates of protein remained distributed over the cell surface (Figure 14). Emphasizing the important role of cytoskeleton-dependent CD44 clustering in the regulation of hyaluronan binding, treatment with cytochalasin D also dramatically inhibited the ability of KG1a cells to bind FITC-HA (Figure 15).

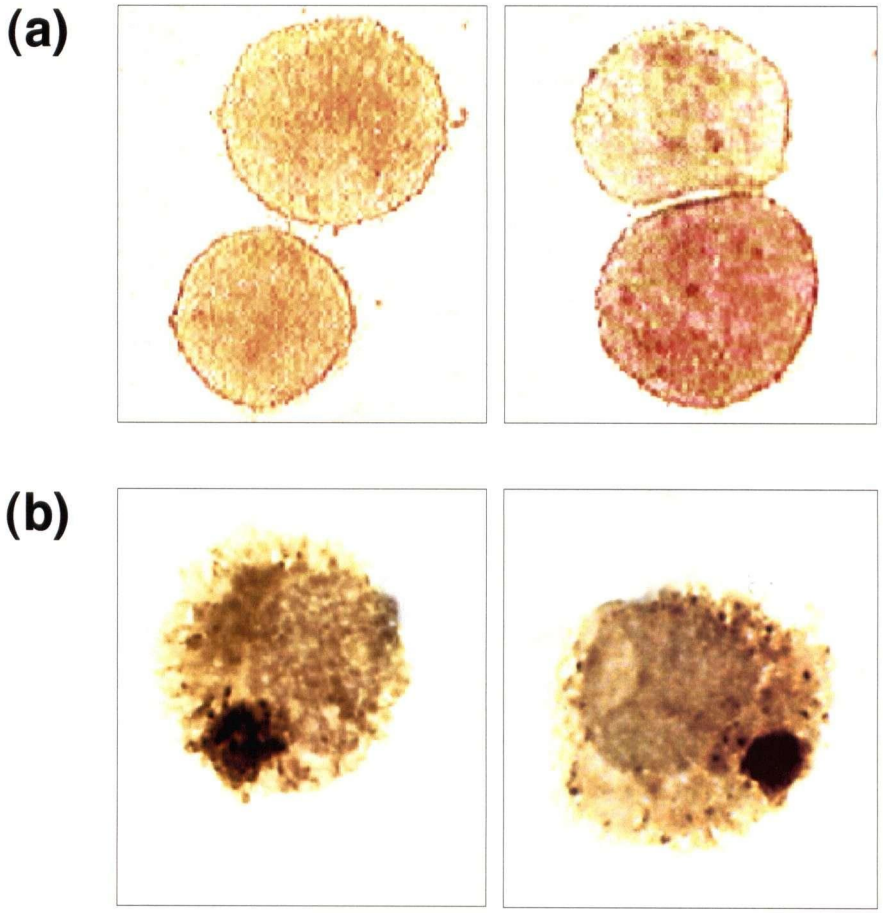


Figure 12: Distribution of CD44 on KG1 and KG1a cells. Cytospin preparations of KG1 cells (a) or KG1a cells (b) were fixed in acetone and stained for CD44 expression using an indirect immunoperoxidase technique.

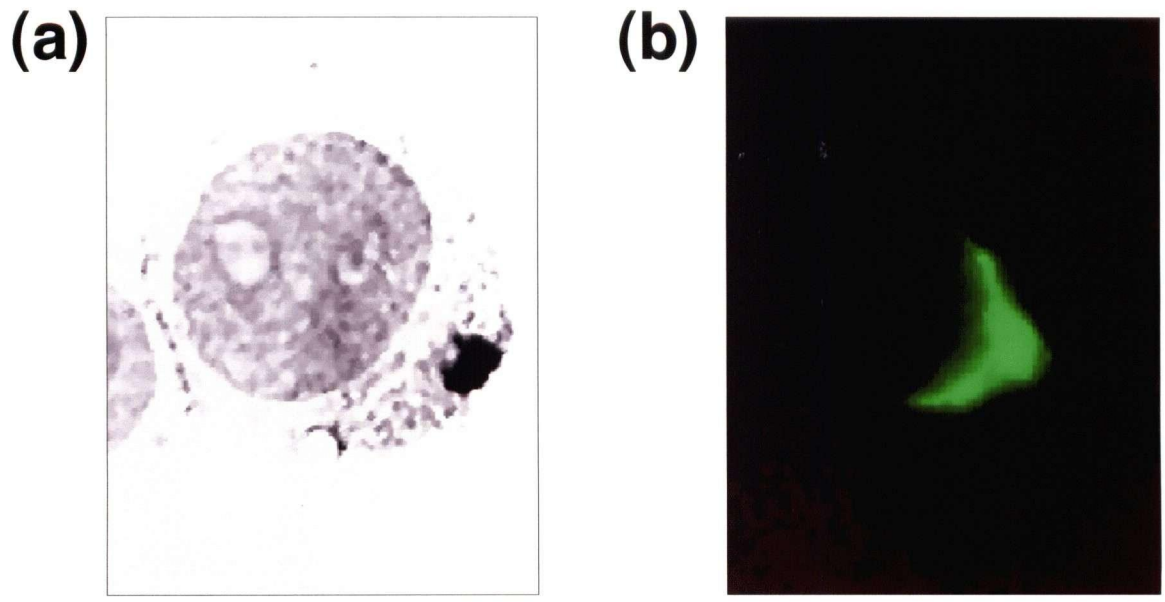


Figure 13: Co-localization of CD44 and soluble hyaluronan to uropods on the surface of KG1a cells. KG1a cells were fixed in acetone and stained for CD44 expression using an indirect immunoperoxidase technique (a), or pre-incubated with FITC-HA prior to the preparation of cytospins (b).

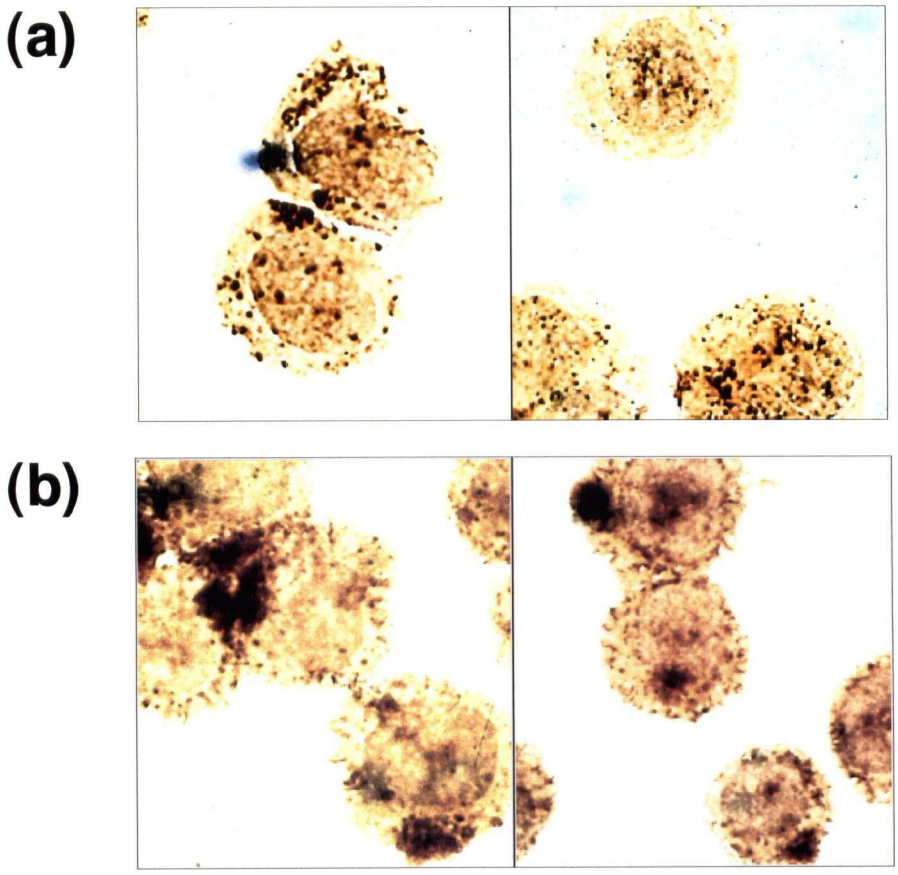


Figure 14: Distribution of CD44 on KG1a cells treated with cytochalasin D. Cytospin preparations of KG1a cells treated with (a), or without (b) cytochalasin D (5mM), were fixed in acetone and stained for CD44 expression using an indirect immunoperoxidase technique.

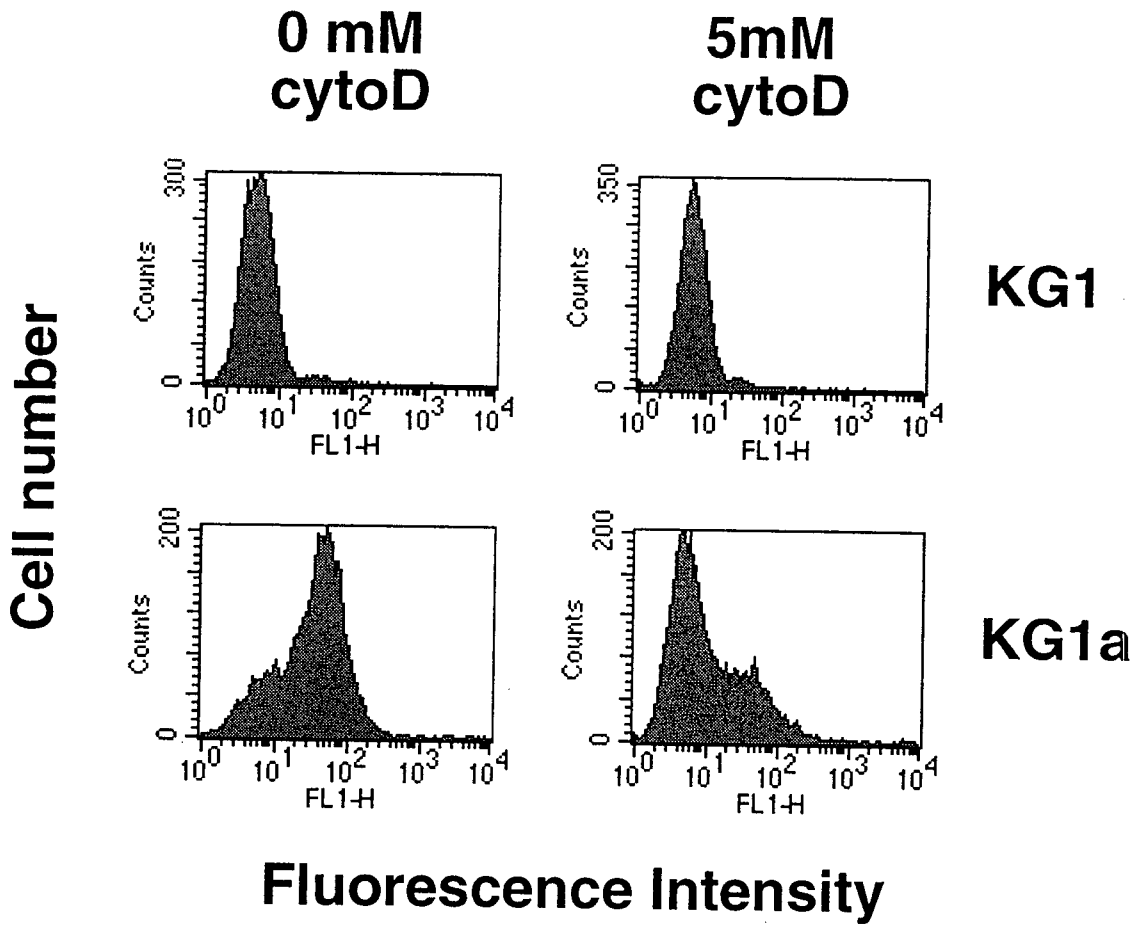


Figure 15: Binding of soluble hyaluronan by KG1a cells treated with cytochalasin D. KG1 and KG1a cells pre-treated with 0 mM or 5 mM cytochalasin D (cytoD) were incubated with FITC-HA (approximately 50 μg/ml final) for 30 min at 0°C. Following extensive washing, the cells were analyzed on a FACScan.

2.4 Discussion

Although a topic of much interest and speculation, at present, the molecular mechanisms that regulate the hyaluronan-binding activity of the adhesion protein CD44 remain poorly defined. In this chapter, we demonstrate that changes in avidity achieved through increased transcription and/or aggregation of CD44 in the plane of the membrane play a critical role in regulating the hyaluronan binding activity of the molecule.

Our studies demonstrate that a minimum threshold level or concentration of CD44 is required before hyaluronan binding can occur. Importantly, cell transfection studies indicate that the precise level of this threshold is the same for each of three major alternatively spliced CD44 isoforms expressed by normal and malignant hemopoietic cells (CD44H, CD44R1 and CD44R2) (Dougherty *et al.*, 1991). These data suggest that even modest changes in overall CD44 expression that increase the total amount of the protein present on the cell surface above the required threshold are likely to have dramatic effects on hyaluronan-binding activity.

A number of stimuli have been shown to increase CD44 expression (Haegel and Ceredig, 1991; Ahrens, 1993; Murakami *et al.*, 1994; Zhang *et al.*, 1997; Lamb *et al.*, 1997; Legras *et al.*, 1997; Liu and Sy, 1997). For example, treatment with phorbol ester can enhance the expression of CD44 on both myeloid and lymphoid cell lines and induce hyaluronan binding (Murakami *et al.*, 1994; Legras *et al.*, 1997; Liu and Sy, 1997). Transient increases in hyaluronan binding activity and CD44 expression have also been noted following

activation of primary T cells *in vitro* (Lesley *et al.*, 1994). Treatment with IL-5 can enhance the ability of resting B cells to bind hyaluronan (Hathcock *et al.*, 1993) while IL-3 can induce increased expression of CD44 on fibroblastoid and other cell types (McBride *et al.*, 1994).

A similar threshold effect has been observed in studies in which soluble CD44-Ig fusion proteins were bound to inert beads (English *et al.*, 1998). Interestingly, in these studies the cell line in which the soluble fusion proteins were produced had a dramatic effect on the minimum density of CD44 required for FITC-HA binding. Thus the threshold concentration for CD44-Ig produced by AKR1 cells, which constitutively bind hyaluronan, is far lower than that for CD44-Ig produced by RAW253 cells, which lack hyaluronan binding activity. CD44-Ig produced by XJ(3) cells which can be induced to bind hyaluronan following crosslinking with mAbs had a threshold that lay between AKR1 and RAW253. Using site directed mutagenesis to inactivate individual N-linked glycosylation sites, the variation in hyaluronan binding was attributed to differences in the ability of different cell lines to glycosylate CD44. These findings are in general agreement with previous studies which demonstrated an inverse correlation between N-linked glycosylation of CD44 and hyaluronan binding activity (Lesley *et al.*, 1995). Treatment of cells with tunicamycin, an inhibitor of N-glycan addition, has also been shown to activate the hyaluronan binding function of CD44 (Katoh *et al.*, 1995; Lesley *et al.*, 1995). Neuraminidase can also activate CD44 suggesting that sialic acid may be an important regulator of the ligand binding function of the molecule (Katoh *et al.*, 1995; English *et al.*, 1998).

Based on such findings, it seems likely that the inability of U397 to bind hyaluronan as observed in this chapter can be attributed to cell-specific post-translational modification of CD44. Indeed when expressed in a different cellular context (i.e. K562 cells) a CD44H cDNA cloned from U937 cells was fully functional. Such studies are important because they emphasize that for any cell type, hyaluronan binding activity will be dictated not only by the total amount of CD44 expressed, but also by the proportion of these molecules that are in a "functionally competent" state.

Oligomerization or clustering has also been suggested to play an important role in regulating the functional activity of various adhesion proteins (Dougherty *et al.*, 1988; Dustin and Springer, 1991; Miller *et al.*, 1995). Such clustering might be expected to enhance the avidity of interactions between adhesion proteins and their ligands, particularly if these are of low affinity. Clustering may also be involved in ensuring the transduction of appropriate intracellular signals following receptor ligation.

A number of mAbs have been shown to rapidly activate the hyaluronan-binding activity of CD44 and to induce the hyaluronan-dependent adhesion of lymphocytes to endothelial cells *in vitro* (Lesley *et al.*, 1993; Toyama-Sorimachi *et al.*, 1993; Cao *et al.*, 1996). Multivalent antibody binding was generally required and monovalent Fab fragments exhibited dramatically reduced or absent activating activity (Lesley *et al.*, 1993; Cao *et al.*, 1996). Fab fragments could, however, activate the hyaluronan binding activity of CD44 if crosslinked with anti-immunoglobulin (Lesley *et al.*, 1993). Although there are other possibilities, such data has been interpreted as suggesting that the capping or clustering of

CD44, which is induced by multivalent antibodies, plays an important role in regulating the functional activity of the molecule.

The molecular events that control the capping of CD44 under physiological conditions remain to be determined. The cytoplasmic domain of CD44 has been shown to interact with various cytoskeletal components including ankyrin (Kalomiris and Bourguignon, 1988) and the ERM family members ezrin, radixin and moesin (Tsukita *et al.*, 1994; Hirao *et al.*, 1996), which could potentially link CD44 to the actin-based cytoskeleton. The sequence present within the cytoplasmic domain of CD44 responsible for ankyrin (Asn^{304}-Leu^{318}) (Lokeshwar *et al.*, 1994) and ERM (Lys^{298}-Lys^{300}) (Yonemura *et al.*, 1998) binding have been identified. Deletion analysis have demonstrated that these interactions may (Lokeshwar *et al.*, 1994) or may not (Perschl *et al.*, 1995) play a pivotal role in hyaluronan binding dependent on the cell lines used.

Further supporting the involvement of cytoskeletal interactions in regulating the cell surface distribution and functional activity of CD44, deletion mutants lacking all but 6 amino acids of the cytoplasmic domain were unable to bind FITC-HA when expressed in AKR1 T-lymphoma cells (Lesley *et al.*, 1992). Dimerization of CD44 by substituting the membrane spanning domain of the molecule with the membrane spanning domain of the CD3 zeta chain which contains a cysteine residue capable of forming disulfide bridges, reconstituted hyaluronan-binding function even in the absence of a cytoplasmic domain (Perschl *et al.*, 1995).

Domain swapping studies have also suggested an important role for the cytoplasmic tail in the localization of cell surface CD44. Transfection of CD44 and L-selectin into pre-B cells have demonstrated a contrasting surface distribution in which L-selectin is concentrated on microvilli and CD44 dispersed on the cell body. Chimeric proteins were generated in which intracellular and transmembrane domains of either CD44 or L-selectin were fused with the extracellular domain of the other molecule. These studies demonstrate that CD44 transmembrane and/or cytoplasmic domains targeted the L-selectin extracellular expression to the cell body, whereas L-selectin transmembrane and intracellular segments conferred CD44 extracellular clustering on microvilli (von Andrian *et al.*, 1995).

Although phosphorylation of CD44 on serine residues by brain-derived protein kinase C *in vitro* has been shown to enhance its ability to bind ankyrin (Kalomiris and Bourguignon, 1989), there is no obvious correlation between the phosphorylation status of the molecule and either its association with the cytoskeleton or its functional activity. Indeed, CD44 molecules in which the target serine residues were mutated to neutral amino acids to generate a molecule that cannot be phosphorylated, or acidic amino acids in order to mimic a fully phosphorylated state, did not differ from the wild-type molecule in their hyaluronan binding activity (Uff *et al.*, 1995). Although, these data suggest that phosphorylation of CD44 does not play a role in ligand binding, it may be involved in post-ligand binding events necessary for cell migration (Peck and Isacke, 1996; Peck and Isacke, 1998).

Interactions between the cytoplasmic domain of CD44 and the cytoskeleton are not the only way in which CD44 molecules could potentially be localized within particular regions of the cell membrane. Although detergent insolubility is frequently taken as strong evidence that a protein is associated with the cytoskeleton, recent studies have shown that sequences within the transmembrane domain of CD44 determine its solubility in Triton X-100 (Neame *et al.*, 1995; Perschl *et al.*, 1995). Importantly, treatment of L cells with cytochalasin D did not alter the proportion of CD44 molecules that are detergent insoluble (Perschl *et al.*, 1995). In some cells, CD44 may be palmitoylated (Bourguignon *et al.*, 1991). This particular form of the molecule has been reported to show increased binding to ankyrin and the incorporation of palmitic acid into CD44 is greatly stimulated during lymphoma cap formation (Bourguignon *et al.*, 1991).

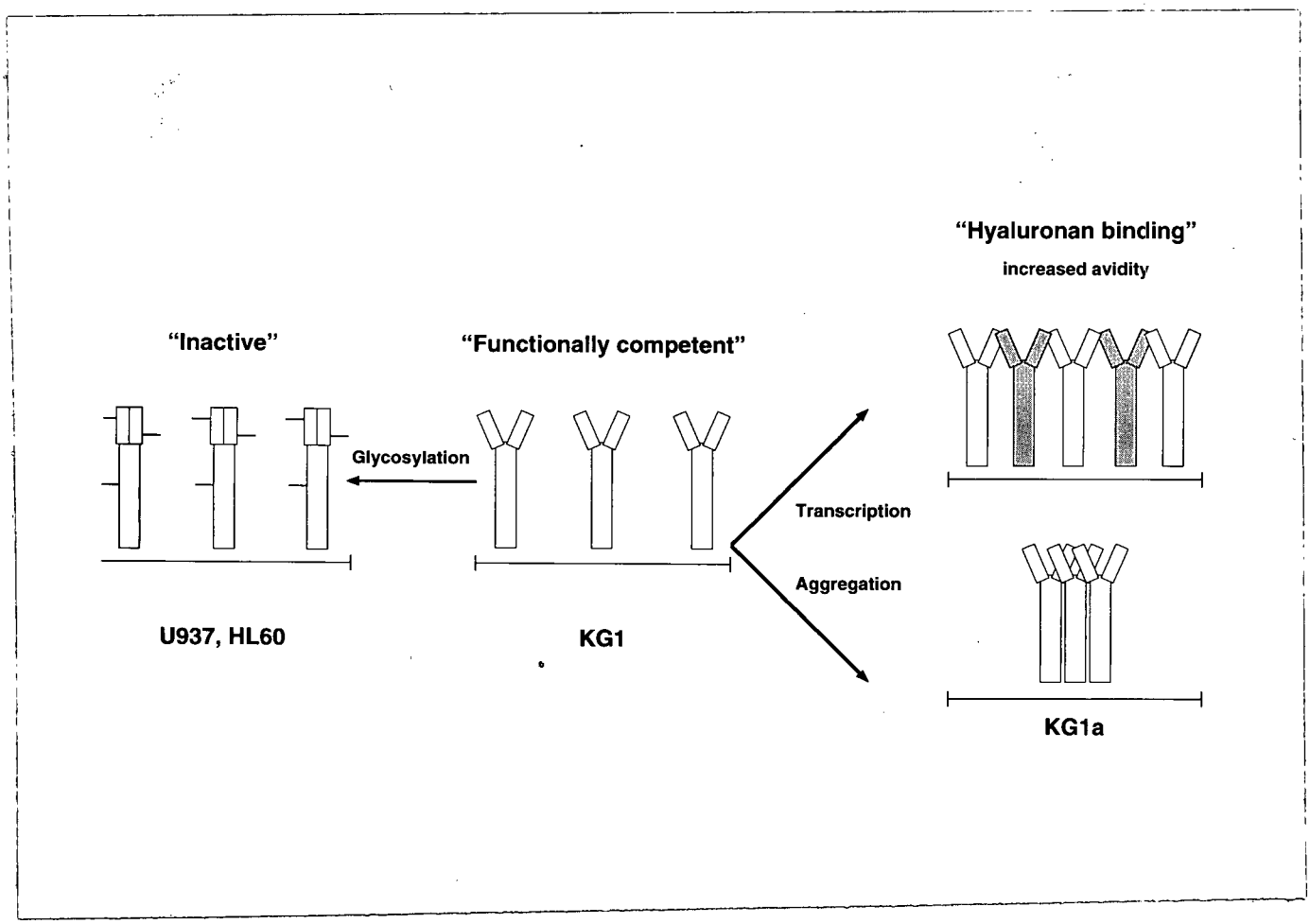


Figure 16: Model of hyaluronan binding by CD44. Functionally competent CD44 can be inactivated by mechanisms such as N-linked glycosylation, or the activity of the molecule enhanced by processes that increase receptor concentration. This can be achieved by altering transcriptional rate such that the overall expression of CD44 is increased above the critical threshold concentration or density required for hyaluronan binding. Alternatively, on some cell types, CD44 can aggregate into distinct clusters or caps generating a locally high concentration of the molecule without changing overall expression. Examples of cell lines which fall into each of these categories are shown.

CHAPTER III

ALTERNATIVELY SPLICED CD44 ISOFORMS CONTAINING EXON V10 PROMOTE CELLULAR ADHESION THROUGH THE RECOGNITION OF CHONDROITIN SULFATE MODIFIED CD44

3.1 Introduction

The gene encoding the broadly distributed cell surface glycoprotein CD44 includes a series of 10 exons (v1-v10) that can be alternatively spliced to generate various higher molecular mass CD44 isoforms that contain peptide sequences of differing lengths inserted into a single site within the extracellular domain of the molecule (Cooper and Dougherty, 1995). Several recent studies have demonstrated a correlation between the expression of certain CD44 isoforms and the metastatic propensity of various hemopoietic and non-hemopoietic tumour cell types (East and Hart, 1993; Gunthert, 1993; Herrlich *et al.*, 1993; Tanabe and Saya, 1994; Sleeman *et al.*, 1995). Although an area of much speculation (Cooper and Dougherty, 1995), at present, the precise molecular mechanism by which particular CD44 isoforms influence the metastatic process remains to be determined.

One obvious possibility is that the presence of the additional peptide sequences encoded by the alternatively spliced exons may somehow alter the functional activity and/or ligand-binding specificity of the CD44 molecule. In this regard, we have recently demonstrated that while CD44H, the major CD44 isoform expressed by resting hemopoietic

cells, and CD44R1, an isoform containing the alternatively spliced exons v8-v10 (Dougherty *et al.*, 1991) can both bind the glycosaminoglycan hyaluronan, only CD44R1 was able to promote homotypic cellular aggregation when expressed in the CD44-negative murine lymphoma cell line TIL1 (Droll *et al.*, 1995). Further studies demonstrated that this adhesive interaction involved the recognition by CD44R1 of a determinant expressed on both CD44H and CD44R1 molecules (Droll *et al.*, 1995). Importantly, CD44H lacked this unique ligand binding activity. Although dependent upon the presence of the domain encoded by exon v8-v10, the molecular nature of the interaction between CD44R1 and CD44H/CD44R1, was not further explored in these studies.

In this chapter, CD44R2, a CD44 isoform that contains only the 69 amino acid insert encoded by the alternatively spliced exon v10, is also demonstrated to be able to recognize and bind both CD44H and CD44R1. Furthermore, this interaction involves the recognition by CD44R2 of chondroitin sulfate side chains presented in association with CD44 molecules on the surface of opposing cells. It is proposed that such interactions may help explain the correlation between the expression of exon v10 containing CD44 isoforms and the metastatic propensity of certain tumour cells.

3.2 Materials and Methods

3.2.1 Cell lines

The SV40-transformed simian fibroblastoid cell line COS7 (Gluzman, 1981) was obtained from the ATCC. The CD4/CD8 double-positive T cell line TIL1 was isolated from an IL-7 transduced murine fibrosarcoma and has been described in detail previously (Chapter II, Materials and Methods). TIL1 cells expressing an "empty" retroviral vector (TILJneo), CD44H (TILJhCD44H) and CD44R1 (TILJhCD44R1) were generated by retroviral-mediated gene transfer as previously in Chapter II, Materials and Methods. All cell lines were maintained in DMEM + 10% FBS with or without G418 (Gibco BRL, Burlington, ON, Canada) at a final concentration of 0.5 mg/ml (active weight).

3.2.2 Monoclonal antibodies

The mAbs 8D8 and 2G1 have been described in Chapter II (Materials and Methods). The generation and characterization of the anti-CD44 mAb 3C12 has also been described previously (Dougherty *et al.*, 1994).

3.2.3 Construction of pCDM8.CD44R2

Peripheral blood mononuclear cells (PBMC) were prepared from fresh heparinized blood donated by healthy adult volunteers by centrifugation through Ficoll-Hypaque (Pharmacia Inc., Quebec, Canada). T cells were isolated by passage of PBMC over a nylon-wool column as previously described (Julius *et al.*, 1973) and cultured at $1x10^6$ cells/ml in RPMI 1640 (Stem cell Technologies Inc.) containing 10% fetal clone I (Hyclone) (RPMI + 10% FCI) with or without phytohemagglutinin (PHA) (Gibco BRL) at a final concentration of 4 μg/ml. After 48 h total RNA was isolated from these cells using the guanidine-isothiocyanate/CsCl-method (Chirgwin *et al.*, 1979).

1 μg of total cellular RNA was reverse transcribed using the Pharmacia First-Strand-cDNA-Synthesis Kit (Pharmacia) and random hexanucleotide primers. Exon v10 containing CD44 isoforms were amplified by PCR using the following primer pair: CGCTCCGGACACCATGGAC (5' primer; exon 1) and CCGCTCGAGGCGATTGACAT TAGAGTTGG (3' primer, exon v10). The PCR reactions (95°C for 30 s, 55°C for 30 s and 72°C for 90 s, 30 cycles) were carried out in an Biocycler Oven (BIOS Corp., New Haven, CT, USA). Controls included RNA from both unstimulated T cells and the CD44R1 positive cell line KG1a (Dougherty *et al.*, 1991).

Amplified PCR products were gel purified, blunted with T4 polymerase, and the fragments subcloned into the Sma 1 site of pBluescript KS+. Restriction enzyme analysis, Southern blotting using exon-specific probes, and DNA sequencing confirmed that the

majority of the clones obtained corresponded to CD44R1 (v8-v10) and CD44R2 (v10). One of the CD44R2 clones was digested with the restriction enzymes Nco 1 and Tth111 I, and a 805 bp fragment containing exon v10 and part of the constant region of CD44 was isolated and ligated into the corresponding sites of a CD44R1 cDNA, producing a full length CD44R2 cDNA.

For expression studies, the CD44R2 cDNA was cloned into the episomal expression vector pCDM8 (Invitrogen). pCDM8.CD44H (clone 2.7) and pCDM8.CD44R1 (clone 2.3) encoding respectively the 90 kDa CD44H and 130 kDa CD44R1 isoforms of CD44 have been described previously (Chapter II, Materials and Methods).

3.2.4 COS7 cell transfection

COS7 cells were transfected with plasmid DNA by electroporation using the Bio-Rad Gene-Pulsar System (Bio-Rad, Richmond, CA, USA). Briefly, cells were trypsinized and resuspended in ice-cold PBS at a final concentration of $1x10^7$ cells/ml. 20 µg of plasmid DNA were added to a 400 µl aliquot of the cell suspension, transferred to a 0.4 cm cuvette and electroporated at 280 volts with a capacitance setting of 250 µF. The time constants obtained ranged from 6.2-7.5 ms. After electroporation, cells were incubated for 5 min on ice, diluted in 30 ml DMEM + 10% FCS, plated in a 15 cm Integrid tissue culture dish (Falcon) and incubated for 3 days to allow replication and expression of the introduced cDNAs.

3.2.5 Western blot analysis

Transfected COS7 cells were harvested by brief incubation in PBS containing 2.5 mM EDTA, washed extensively with PBS and resuspended at $2x10^7$ cells/ml in PBS containing 1% (v/v) NP40, 5 mM EDTA and 10 mM phenylmethyl-sulphonyl fluoride. After incubation on ice for 15 min, the lysates were microfuged for 5 min to pellet nuclei and other cellular debris, and aliquots stored at –70°C until required. Samples were rapidly thawed, added to an equal volume of non-reducing sample buffer (125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8) and incubated for 5 min at 100°C. Total cellular proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Filters were blocked with PBS containing 5% (w/v) milk protein overnight at 4°C. After extensive washing in HBSS, they were incubated with mAb 3C12 or 2G1 tissue culture supernatants for 4 h at room temperature, washed in HBSS for 30 min, and then incubated for a further 1 h with a 1:100 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako Corporation, Carpinteria, California, USA) in DMEM+10% FCS. After extensive washing in HBSS, the reaction was developed in PBS containing 0.06% (w/v) 3-3'-diaminobenzidine (Sigma) and 0.012% (v/v) hydrogen peroxide (Sigma).

3.2.6 FACS analysis

Approximately 10^6 transfected COS7 cells were incubated at 0°C for 30 min with a combination of fluorescein isothiocyanate-conjugated hyaluronan (approximately 50 μg/ml final) and affinity-purified mAb 8D8 (10 μg/ml final). Following extensive washing, the cells

were incubated for 30 min on ice in a 1:100 dilution of biotinylated goat-anti-mouse IgG (BIO/CAN Scientific, Mississauga, Ontario, Canada), washed again and incubated for another 30 min at 0°C with streptavidin-conjugated phycoerythrin (PE) (BIO/CAN Scientific). After additional washing, labeled cells were analyzed on a FACSort .

3.2.7 Cellular aggregation assay

$5x10^4$ COS7 cells harvested 48 h after transfection with pCDM8 or pCDM8.CD44R2, were added to the wells of a 24 well plate (Falcon). After incubation at 37°C overnight, supernatants were removed and $5x10^5$ TILJneo, TILJhCD44H or TILJhCD44R1 cells were added and allowed to adhere for 15 min. After washing three times in PBS, the cells were fixed in methanol and incubated for 2 h at room temperature with mAb 3C12 tissue culture supernatant. After a further 1 h incubation with horseradish peroxidase-conjugated rabbit-anti-mouse IgG (Dako) diluted 1:100 in DMEM+10% FCS and extensive washing with HBSS, the reaction was developed by incubating the wells for 5-10 min at room temperature in PBS containing 0.06% (w/v) 3-3'-diaminobenzidine (Sigma) and 0.012% (v/v) hydrogen peroxide (Sigma). The percentage of CD44-positive COS7 cells binding 3 or more transduced TIL1 cells was determined by counting on an inverted phase microscope. In some experiments, the transduced TIL1 cells were treated with chondroitinase ABC (Sigma, 1u/ml final) or leech-derived hyaluronidase (Sigma, 10 u/ml final) for 1 h at 37°C, or the transfected COS7 cells incubated with chondroitin-4-sulfate, chondroitin-6-sulfate or hyaluronan (all from Sigma) at a final concentration at 1 mg/ml on ice for 1 h, prior to being added to the adhesion assay, and the effect on binding to CD44R2 transfected COS7 cells

determined. In order to exclude the possibility that the inhibitory effect of chondroitinase ABC on cellular adhesion may be due to proteolytic activity, transduced TIL1 cells were incubated with a 10 fold excess of the enzyme (10 u/ml) for 1 h at 37°C and CD44 expression determined by FACS analysis using mAbs directed against both a common region of the CD44 molecule and exon v10. No significant changes in the expression of CD44 resulted from this treatment (data not shown).

3.3 Results

3.3.1 Generation of a full length CD44R2 cDNA

Using exon 1 and v10 specific primers two distinct products corresponding to CD44R1 and CD44R2 were amplified by RT-PCR from total cellular RNA isolated from PHA-stimulated peripheral blood T cells. These were subcloned into pBlueScript(KS)+ and used to generate a full length CD44R2 cDNA as described in the Materials and Methods (Section 3.2.3). The alternative splicing event and variant exon usage in producing CD44R2 is shown in Figure 17. Western blot analysis using mAb 3C12 confirmed that COS7 cells transfected with pCDM8.CD44H, pCDM8.CD44R1 or pCDM8.CD44R2 expressed CD44 proteins of the appropriate size (Figure 18). As expected, only CD44R1 and CD44R2 were detected by the v10 specific mAb 2G1 (Figure 18). No CD44 species were detected in COS7 cells transfected with the pCDM8 vector alone using either of these two mAbs.

3.3.2 Hyaluronan binding capacity of CD44R2

As shown in Figure 19, FACS analysis indicated that approximately 15-20% of COS7 cells transfected with the pCDM8.CD44R2 vector expressed CD44 as determined by reactivity with mAb 8D8. FITC-HA bound almost exclusively to the subpopulation of these cells that expressed the highest levels of CD44, with little if any binding detected on cells expressing CD44 below a certain critical level. Similar results were obtained for COS7 cells transfected with pCDM8.CD44R1 and pCDM8.CD44H confirming our recent observation that these CD44 isoforms do not differ in their ability to recognize and bind hyaluronan (Chapter II).

3.3.3 Adhesive interactions between CD44R2 and other CD44 isoforms

To determine whether CD44R2 can recognize and bind other CD44 isoforms, control CD44-negative TIL1 cells or TIL1 cells expressing CD44H or CD44R1 were tested for their ability to adhere to COS7 cells transfected with either pCDM8.CD44R2 or the pCDM8 vector alone. As shown in Table 1, TIL1 cells expressing CD44R1 and to a lesser extent those expressing CD44H, bound avidly to the CD44R2 transfected COS7 cells. TILJneo cells bound very poorly, while none of the three TIL1 cell lines examined (TILJneo, TILJhCD44H or TILJhCD44R2) adhered to control COS7 cells transfected with the pCDM8 vector alone.

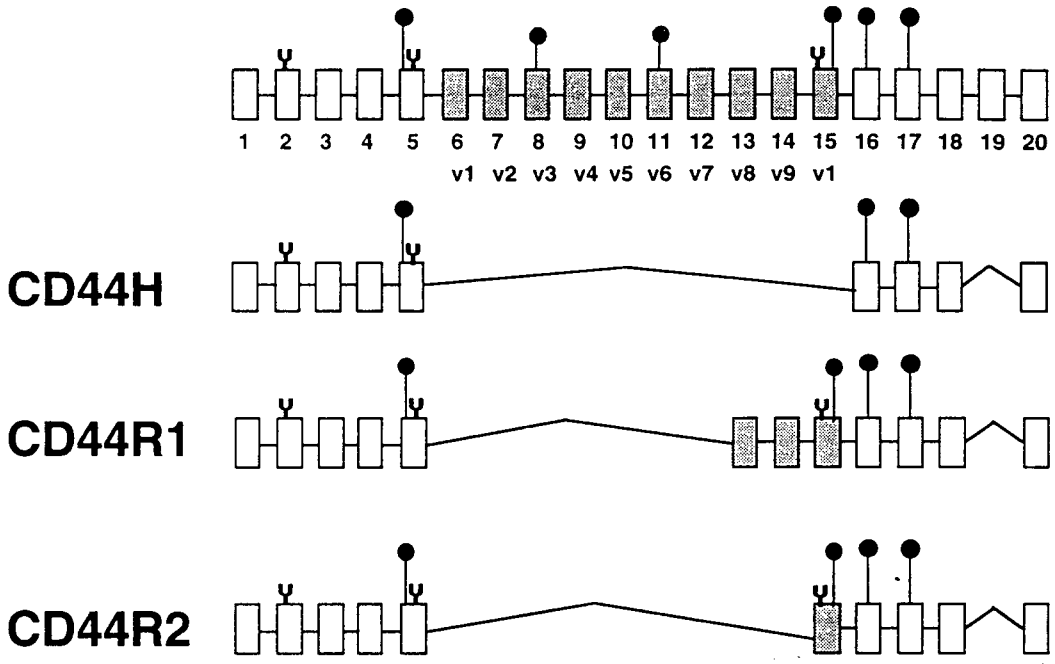


Figure 17: Alternative splicing of the CD44 gene leading to the generation of CD44R2. To date, the human genomic CD44 structure is known to consist of 20 exons. Exon 1 encodes the leader peptide whereas exon 2 and 3 represent the putative hyaluronan binding domain (**Y**). Exons 6 through 15 correspond with the variant exons 1 to 10 (v1-v10). These exons can be alternatively spliced to generate higher molecular mass CD44 isoforms. The isoforms CD44R1 (containing exons v8, v9, and v10) and CD44R2 (containing exon v10) are also shown. Exons 18 encodes the transmembrane and exon 20 the cytoplasmic domains. Sites of potential chondroitin sulfate addition are marked with a ( ).

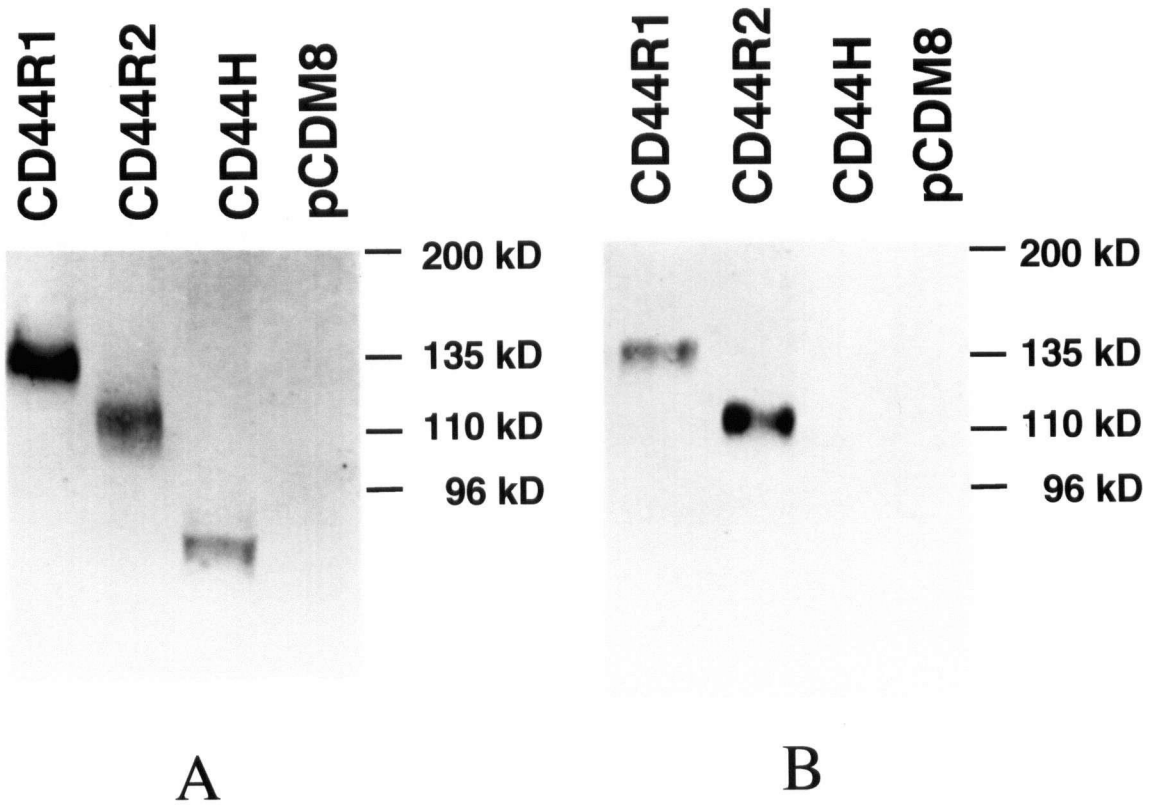


Figure 18: Western blot analysis of CD44 expression in transfected COS7 cells. COS7 cells were transfected with plasmid DNA by electroporation. 72 h later, cells were harvested with PBS containing 2.5 mM EDTA and detergent lysates prepared. Approximately $2x10^5$ cell equivalents were run in each lane of a 5% SDS-PAGE gel, transferred to nitrocellulose and probed with either the CD44 mAb 3C12 (Panel A) or the exon v10 specific mAb 2G1 (Panel B) followed by a horseradish peroxidase-conjugated rabbit anti-mouse IgG. The reaction was developed in PBS containing 0.06% (w/v) 3-3'-diaminobenzidine and 0.012% hydrogen peroxide.

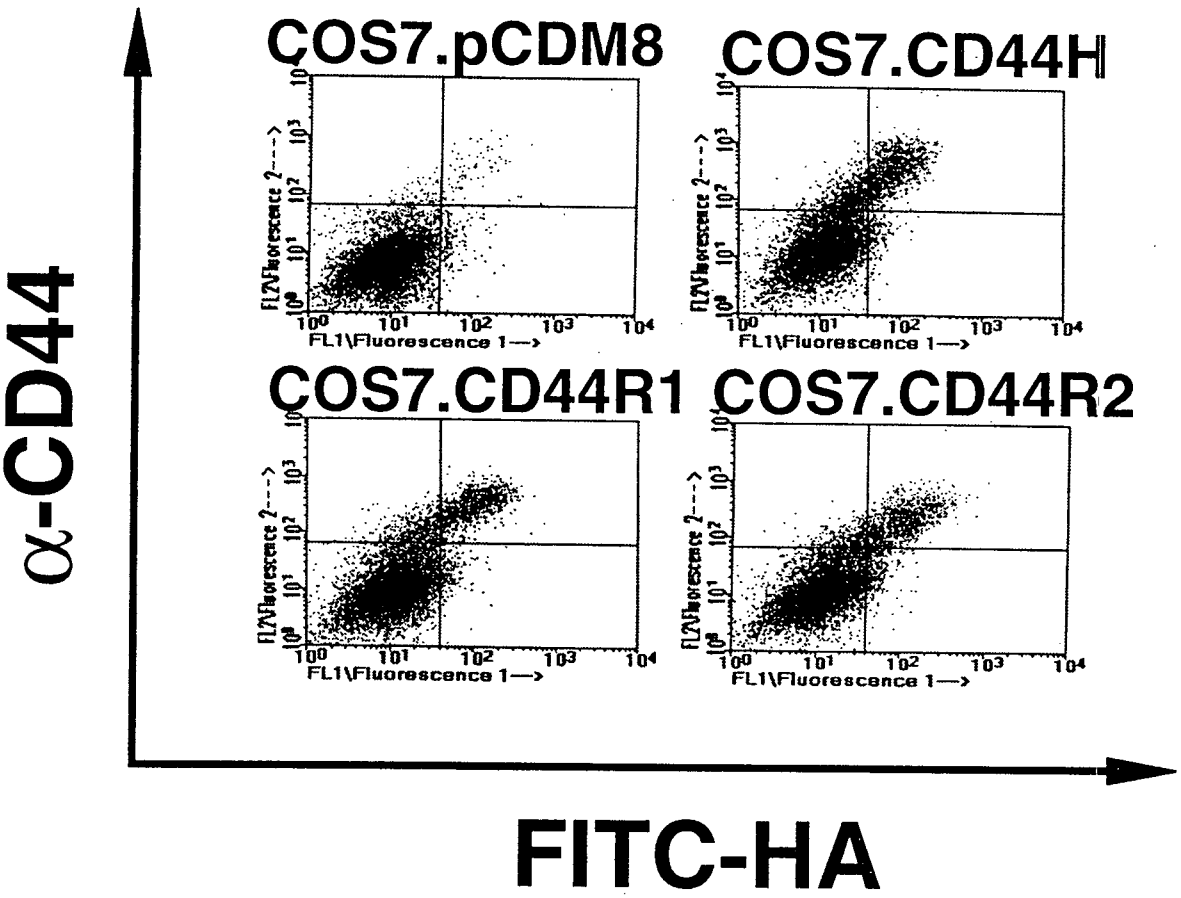


Figure 19: Binding of soluble hyaluronan to transfected COS7 cells. COS7 cells were transfected with pCDM8, pCDM8.CD44H, pCDM8.CD44R1 or pCDM8.CD44R2 plasmid DNA by electroporation. 72 h later, cultures were harvested with PBS containing 2.5 mM EDTA and approximately 10^6 cells incubated at 0°C for 30 min with a combination of FITC-HA (approximately 50 μg/ml final) and affinity-purified mAb 8D8 (10 μg/ml final). Following extensive washing, the cells were incubated for 30 min on ice in a 1:100 dilution of biotinylated goat-anti-mouse IgG, washed again and incubated for another 30 min at 0°C with streptavidin-conjugated PE. After additional washing, labeled cells were analyzed on a FACSORT for CD44 expression (FL2) and FITC-HA binding (FL1).

Table 1. Binding of Transduced TIL1 Cells to CD44R1 Transfected COS7 Cells

	% COS7 cells binding 3 or more TIL cells	
	pCDM8	pCDM8.CD44R2
TILJneo	<1	<1
TILJhCD44H	<1	11.5 ± 2.4
TILJhCD44R1	<1	57.6 ± 2.0

COS7 cells were transfected with pCDM8 or pCDM8.CD44R2 and tested for their ability to bind TILJneo, TILJhCD44H or TILJhCD44R1 cells. Unbound TIL1 cells were removed by washing 3 times with PBS and the adherent COS7 cell monolayers fixed in methanol for 5 min at room temperature, air dried and CD44 expressing cells identified by immunohistochemical staining with mAb 3C12. The percentage of CD44-positive COS7 cells binding 3 or more transduced TIL1 cells was determined by counting on an inverted phase microscope. Values represent the mean ± SEM of 3 experiments.

3.3.4 Adhesive interactions between CD44R2 and other CD44 isoforms involve the recognition of chondroitin sulfate

To further characterize the molecular nature of the adhesive interaction between CD44R2, and CD44H and CD44R1, TIL1 cells transfected with CD44R1 were treated with chondroitinase ABC or leech hyaluronidase at final concentrations of 1 u/ml and 10 u/ml respectively and then tested for their ability to bind to COS7 cells transfected with CD44R2. As shown in Figure 20, pretreatment of TILJhCD44R1 cells with chondroitinase ABC almost completely inhibited their attachment to CD44R2-positive COS7 cells. In contrast, hyaluronidase treatment had virtually no inhibitory effect. Similar studies were also carried out using TILJhCD44H cells. Although these cells bind far less well to COS.CD44R2 than equivalent TILJhCD44R1 cells, once again treatment with chondroitinase almost completely abrogated attachment while hyaluronidase treatment had little effect. Taken together, these results suggest that CD44R2 can recognize and bind a chondroitin sulfate moiety presented on the surface of the transfected TIL cells. Moreover, the inability of TILJneo cells to bind to CD44R2 transfected COS7 cells strongly suggests that it is the CD44 molecules expressed by the transduced TIL1 cells that present chondroitin sulfate to CD44R2.

Interestingly, pre-incubation of COS7.CD44R2 cells with high concentrations of chondroitin-4-sulfate or chondroitin-6-sulfate (1 mg/ml) had little effect on their ability to bind TILJhCD44H or TILJhCD44R1 cells (Figure 20) suggesting that chondroitin sulfate must be presented in an appropriate context in order to be recognized by CD44R2. In striking contrast, pre-incubation of COS7.CD44R2 cells with hyaluronan (1 mg/ml) greatly

increased their ability to bind TILJhCD44H presumably as a result of hyaluronan acting as a bridge between the CD44 species expressed on the surface of the two interacting cell types. Adhesion of TILJhCD44R1 cells to CD44R2 expressing COS7 cells is already extremely efficient and is increased only modestly by the addition of hyaluronan ($p>0.5$).

3.4 Discussion

Numerous recent studies have demonstrated a correlation between the expression of certain alternatively spliced CD44 isoforms and the metastatic propensity of various hemopoietic and non-hemopoietic tumour cell types (East and Hart, 1993; Gunthert, 1993; Herrlich *et al.*, 1993; Tanabe and Saya, 1994; Sleeman *et al.*, 1995). Although much of this work has focused on exon v6 containing isoforms, which have been shown to play a pivotal role in the metastatic spread of the rat pancreatic tumour cell line BSp73ASML (Gunthert *et al.*, 1991), it is important to emphasize that other CD44 species, and in particular those containing exons v8-v10 (CD44R1), may also be upregulated in malignant cells and play a role in the metastatic process. For example, we have previously demonstrated a correlation between the expression of CD44R1 and poor prognosis in colorectal carcinoma (Finn *et al.*, 1994). Takeuchi *et al* have similarly reported that CD44R1 expression is significantly higher in colorectal carcinomas associated with liver metastasis (Takeuchi *et al.*, 1995). CD44 isoforms containing exons v8-v10 are transiently induced during both T cell (Mackay *et al.*, 1994) and B cell (Salles *et al.*, 1993) activation, and are stably expressed on a significant proportion of hemopoietic malignancies (Dougherty *et al.*, 1991; Ghaffari *et al.*, 1995; Ghaffari *et al.*, 1996). In comparison to primary nodal large cell lymphomas, extranodal and

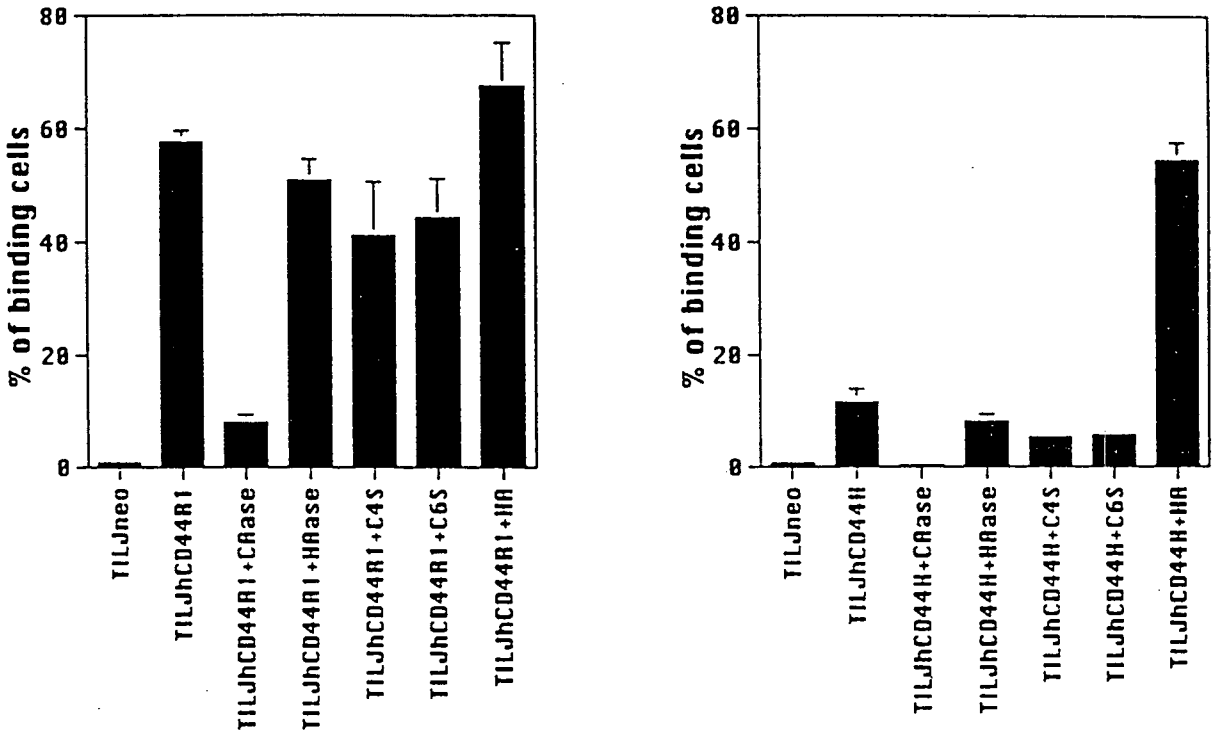


Figure 20: Involvement of chondroitin sulfate and hyaluronan in adhesion between CD44R2 and CD44H/CD44R1. $5x10^4$ COS7 cells harvested 48 h after transfection with pCDM8 or pCDM8.CD44R2, were added to the wells of a 24 well plate. After incubation at 37°C overnight, supernatants were removed and $5x10^5$ TILJneo, TILJhCD44H or TILJhCD44R1 cells were added and allowed to adhere for 15 min. After washing three times in PBS, the cells were fixed in methanol and stained for CD44 expression using an indirect immunohistochemical technique. The percentage of CD44-positive COS7 cells binding 3 or more transduced TIL1 cells was determined by counting on an inverted phase microscope. Transduced TIL1 cells were also treated with chondroitinase ABC (CAase) or leech hyaluronidase (HAase) for 1 h at 37°C, or the COS7 cells incubated with chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S) or hyaluronan (HA) at a final concentration at 1 mg/ml on ice for 1 h, prior to being added to the adhesion assay and the effect on cell binding determined. The data presented demonstrates binding of transduced TIL cells to COS cells transfected with pCDM8.CD44R2 only. No binding was found to COS transfected with the pCDM8 vector alone.

disseminated lymphomas had increased levels of v10 containing CD44 isoforms (Salles *et al.*, 1993).

The precise molecular mechanism(s) by which particular CD44 isoforms influence the metastatic process remains to be determined. Although CD44H has been shown to function as a receptor for the glycosaminoglycan hyaluronan (Aruffo *et al.*, 1990; Miyake *et al.*, 1990) there is increasing evidence that not all adhesion-dependent processes mediated by CD44 can be attributed to the recognition of this particular ligand (Culty *et al.*, 1990; Sugimoto *et al.*, 1994). CD44 has been shown to bind a number of ligands in addition to hyaluronan including fibronectin, collagen types I and IV, laminin, and a 60 kDa cell surface glycoprotein expressed by mucosal vascular endothelial cells termed mucosal vascular addressin. Moreover, we have previously demonstrated that the CD44R1 molecules expressed on the surface of interacting cells can also directly bind to one another (Droll *et al.*, 1995). CD44H molecules lacked this ability although they could function as a ligand for CD44R1 (Droll *et al.*, 1995). These data suggest that the additional peptide sequences present within CD44R1, encoded by the alternatively spliced exons v8-v10, confer the ability to recognize and bind a common determinant shared by both CD44H and CD44R1.

In this chapter, we demonstrate that CD44 isoforms containing exon v10 promote cell-cell adhesion and these interactions involve the recognition of chondroitin sulfate presented by CD44 itself. CD44R2, an alternatively spliced CD44 isoform that differs from CD44H only by the presence of sequences encoded by exon v10, can also recognize and bind

both CD44H and CD44R1. The finding that TIL1 cells expressing CD44R1 bound very much more efficiently to CD44R2 than TIL1 cells expressing CD44H, can be explained if the two v10 containing isoforms (CD44R2 and CD44R1) interact with one another in a reciprocal fashion while the interaction between CD44R2 and CD44H is nonreciprocal in nature (Figure 21). Hyaluronan is not involved in this process as all three CD44 isoforms (CD44H, CD44R1 and CD44R2) can bind FITC-HA and the adhesive interaction between CD44R2 and CD44H/CD44R1 is not inhibited by hyaluronidase. Adhesion between CD44R2 transfected COS7 cells and CD44H or CD44R1 expressing TIL1 cells can, however, be abrogated by treatment with chondroitinase.

In the presence of exogenous hyaluronan, adhesion between CD44 expressing cells is greatly enhanced. Such adhesion is not dependent upon the presence of chondroitin sulfate as it is resistant to the effects of chondroitinase. Perhaps the simplest explanation for this finding is that hyaluronan can function as a linker, bridging CD44 molecules present on the surface of interacting cells. However, the possibility that the interaction of CD44 with hyaluronan may alter the functional activity of the molecule in other more subtle ways can not be excluded. In particular, hyaluronan may induce the redistribution or capping of CD44 in the plane of the membrane generating locally high concentrations of molecule thereby enhancing the avidity of adhesive interactions between the various isoforms. Similarly, binding to hyaluronan could conceivably give rise to conformational or other changes in CD44 that expose novel functionally important epitopes.

Recently, CD44 has been shown to recognize and bind serglycin, a high molecular weight cell surface proteoglycan that consists of a small protein core that is extensively modified by the addition of chondroitin sulfate side chains (Toyama-Sorimachi and Miyasaka, 1994a; Toyama-Sorimachi and Miyasaka, 1994b; Toyama-Sorimachi *et al.*, 1995). The adhesion of CD44 to serglycin required the presence of the chondroitin sulfate moieties and could be readily inhibited by chondroitinase treatment (Toyama-Sorimachi *et al.*, 1995). In agreement with the results obtained in the present study, free chondroitin-4-sulfate or chondroitin-6-sulfate, even when present at high concentration, had little effect on the binding (Toyama-Sorimachi *et al.*, 1995) suggesting that the molecular context in which chondroitin sulfate is presented may be important in its recognition by CD44. Toyama-Sorimachi *et al* have proposed that the "clustering" of chondroitin sulfate on serglycin may increase the avidity of its interaction with CD44 (Toyama-Sorimachi *et al.*, 1995). Interestingly, the invariant chain of class II which has also been suggested to function as a ligand for CD44 (Naujokas *et al.*, 1993) is also modified by the addition of chondroitin sulfate (Sant *et al.*, 1985a; Sant *et al.*, 1985b). Although only a single side chain is present (Miller *et al.*, 1988), class II molecules have been reported to associate in the plane of the membrane (Brown *et al.*, 1993) and could in this way present a higher concentration of chondroitin sulfate moieties to potential receptors.

Since only cells expressing CD44R1 or CD44H bind to CD44R2 it must be assumed that the principle molecule presenting chondroitin sulfate side chains on the surface of transduced TIL1 cells is CD44 itself. CD44H contains 4 serine-glycine motifs (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989) which in other proteins have been shown to serve as the

sight of attachment of chondroitin sulfate (Miller *et al.*, 1988; Mann *et al.*, 1990). An additional serine glycine motif is found at the junction between exon v10 and the constant exon 16 in CD44R1 and CD44R2 (Dougherty *et al.*, 1991). The identity of the site(s) actually used for chondroitin sulfate attachment remains to be determined. However, based on the molecular weight of the CD44 species detected in transduced TIL1 cells by Western blot analysis only a small subset of the CD44 molecules expressed in TIL1 cells appear to be modified by the addition of chondroitin sulfate side chains (Droll *et al.*, 1995). Similar results have been reported for the invariant chain (Sant *et al.*, 1985).

The binding of hyaluronan by CD44 appears to be largely ionic in nature and mediated by the interaction between negatively charged carboxyl groups on the glycosaminoglycan and clusters of positively charged basic amino acids in the protein (Laurent and Fraser, 1992). The extracellular domain of CD44 contains two copies of the amino acid motif [$B(X_7)B$] which in other proteins, such as RHAMM, has been implicated in the recognition and binding of hyaluronan (Yang *et al.*, 1994). Whether these same regions are involved in attachment to chondroitin sulfate remains to be determined, although the observation that CD44H transduced TIL1 cells are unable to adhere to one another, although they can bind soluble hyaluronan (Droll *et al.*, 1995), points toward the existence of functionally distinct chondroitin sulfate and hyaluronan binding domains. Alternatively, it is conceivable that the differential ability of CD44R1 and CD44R2 to bind chondroitin sulfate may simply reflect the fact that exon v10 encodes an additional [$B(X_7)B$] motif (Dougherty *et al.*, 1991) the presence of which increases the avidity of the protein for this particular ligand. Such considerations may be less important in the binding of hyaluronan since the

minimum structure recognized by CD44 is presented in a highly multivalent fashion (Laurent and Fraser, 1992). It is also possible, that positioning and/or context of the exon v10 [B(X_7)B] motif in CD44R1 and CD44R2 may allow chondroitin sulfate to be bound more efficiently than similar motifs present elsewhere in the CD44 molecule (Figure 21).

There are several ways in which cellular adhesion events mediated by alternatively spliced CD44 isoforms could potentially influence the metastatic process. Specifically, the expression of chondroitin sulfate modified exon v10 containing CD44 isoforms could allow tumour cells to both homotypically aggregate and bind other cell types, promoting the formation of microemboli that survive better than single cells in the circulation and which trap more readily in capillary beds. Alternatively, the expression of certain CD44 isoforms on tumour cells could, perhaps, directly enhance their adhesion to vascular endothelial cells in target organs since these constitutively express CD44H and can be induced to express other CD44 isoforms upon appropriate stimulation. Finally, it is possible that crosslinking of CD44 isoforms as a result of the interaction with other CD44 molecules could transduce signals that may directly affect tumour cell growth and/or the expression or functional activity of other cell surface adhesion proteins. In this regard, it is interesting to note that the crosslinking of CD44 with mAbs or hyaluronan has been shown to enhance the proliferation of T cells in response to stimulation with antigen or mitogen, and can protect thymocytes from certain pro-apoptotic stimuli (Ayroldi *et al.*, 1995).

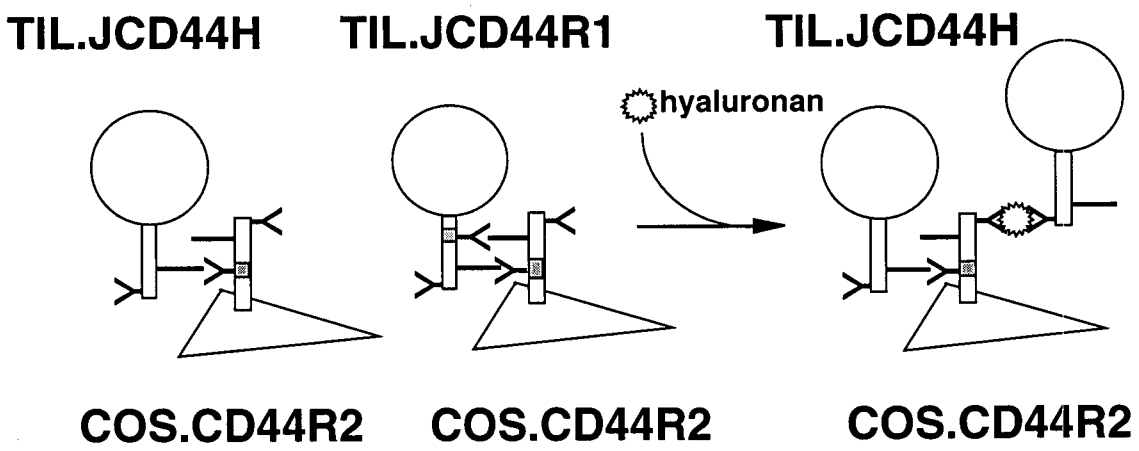


Figure 21: Model for the involvement of CD44 isoforms in cellular aggregation. TIL1 cells expressing CD44R1 bound very much more efficiently to CD44R2 than TIL1 cells expressing CD44H. These interactions appear to be mediated by chondroitin sulfate presented by CD44. As v10 contains an additional [B(X_7)B] motif, the contact between v10 containing isoforms are reciprocal while CD44R2 and CD44H adhesion is nonreciprocal in nature. Hyaluronan may act as a bridging molecule to increase this heterotypic association.

CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL ALTERNATIVELY SPLICED SOLUBLE CD44 ISOFORM THAT CAN POTENTIATE THE HYALURONAN BINDING ACTIVITY OF CELL SURFACE CD44

4.1 Introduction

Soluble proteins reactive with CD44 monoclonal antibodies can be readily detected in the serum of normal individuals. Interestingly, elevated levels are often seen in patients with various malignant diseases including lymphoma, chronic lymphocytic leukemia and cancers of the breast, colon and stomach (Guo *et al.*, 1994; Harn *et al.*, 1996; Martin *et al.*, 1997; De Rossi *et al.*, 1997; Fichtner *et al.*, 1997). Similar increases are also observed in various autoimmune and inflammatory conditions including rheumatoid arthritis (Katoh *et al.*, 1994; Kittl *et al.*, 1997; Oertli *et al.*, 1998). In the case of malignant disease, it has been proposed that the concentration of CD44 in the serum may be a sensitive indicator of tumour burden (Guo *et al.*, 1994; Kan *et al.*, 1996; Zeimet *et al.*, 1997). In patients with lymphoma, decreases in soluble CD44 paralleled responsiveness to chemotherapy, with control levels being attained in individuals exhibiting a complete response (Ristamaki *et al.*, 1994). Similarly, in animal models, the level of soluble CD44 in the circulation varied according to the rate of tumour growth and the magnitude of the anti-tumour immune response induced (Katoh *et al.*, 1994).

At present, neither the mechanism by which soluble CD44 is generated nor the precise role that the molecule plays in tumour growth and/or metastasis are clear. In normal individuals and lymphoma patients, the primary CD44 species detected in serum has a molecular mass of 70-80 kDa (Ristamaki *et al.*, 1994). However, in patients with gastric or colon cancer, species of 130-190 kDa may predominate (Guo *et al.*, 1994). Several studies have demonstrated that CD44 can be shed from the surface of primary lymphocytes and lymphoma cells maintained in culture (Bazil and Horejsi, 1992; Ristamaki *et al.*, 1997). Treatment with TNF-α or IFN-γ increased release from primary lymphocytes but not lymphoma cells (Ristamaki *et al.*, 1997). An endogenous protease may be involved in this process since treatment of cells with various protease inhibitors including aprotinin and phenylmethylsulfonyl fluoride (PMSF) prior to culture substantially decreased the amount of soluble material that could be detected (Campanero *et al.*, 1991; Katoh *et al.*, 1994). Introduction of a cDNA encoding CD44H into the CD44-negative Burkitt lymphoma cell line Namalwa confirmed that the standard 90 kDa CD44 isoform expressed by most resting hemopoietic cells (CD44H) could give rise to soluble CD44 (Ristamaki *et al.*, 1997). Presumably the presence of higher molecular mass soluble CD44 proteins in the circulation of patients with various epithelial malignancies reflects the fact that such tumour cells may differentially express various alternatively spliced CD44 isoforms that can also serve as substrates for endogenous or exogenous proteases. Although the precise site at which CD44 is cleaved by proteolytic enzymes remains to be identified, the size of the soluble protein and the fact that the molecule retains hyaluronan binding activity and can antagonize the function of the corresponding cell surface receptor (Dougherty *et al.*, 1994; Ristamaki *et al.*, 1997; Yu *et al.*, 1997) suggests that it is located close to the membrane.

Proteolytic cleavage of the corresponding cell surface receptor is clearly not the only mechanism by which soluble CD44 is generated. Specifically, there is evidence that aberrant splicing events resulting in intron retention can occur in certain tumour cell lines giving rise to mRNA transcripts that encode soluble CD44 proteins that terminate prior to the membrane spanning domain (Matsumura *et al.*, 1995; Yoshida *et al.*, 1995; Yoshida *et al.*, 1996). When expressed in tumour cells such proteins can bind to hyaluronan and other potential CD44 ligands blocking cellular attachment and preventing tumour growth and metastasis *in vivo* (Matsumura *et al.*, 1995; Yoshida *et al.*, 1995; Yoshida *et al.*, 1996).

In this chapter, a novel naturally occurring alternatively spliced CD44 transcript, designated CD44RC, that encodes a soluble form of the CD44 molecule is characterized. In contrast to previously described soluble CD44 proteins, CD44RC was found to markedly enhance the hyaluronan binding function of cell surface CD44. Evidence was obtained suggesting that CD44RC mediates this effect by binding to and crosslinking chondroitin sulfate modified cell surface CD44, inducing aggregation or clustering of the molecule thereby generating a multivalent complex with increased avidity for hyaluronan.

4.2 Materials and Methods

4.2.1 Cell lines and monoclonal antibodies

The source and maintenance of the erythroleukemic cell line K562, the myelomonocytic cell line KG1 and its less mature derivative KG1a have been described previously (Chapter II, Materials and Methods). The generation and characterization of the anti-CD44 mAb 3G12 has also been described previously (Chapter II, Materials and Methods).

4.2.2 Cloning of CD44RC

mRNA was isolated from the myelomonocytic cell line KG1a using a Stratagene mRNA Isolation Kit (Stratagene). cDNA was synthesized using a Stratagene First Strand cDNA Synthesis Kit (Stratagene) and CD44 amplified by PCR (Hybaid OmniGene) (30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) using 10 µl of the first strand synthesis reaction and CD44 exon 1 (5'-GGTCTAGACCGTTCGCTCCGGACACCATG G-3') and exon 20 (5'-GGTCTAGATTACACCCCAATCTTCATGTCC-3') specific primers. A full length CD44H cDNA template isolated from the plasmid pCDM8.CD44H (Dougherty *et al.*, 1991) by digestion with XhoI was used as a control. The PCR products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. The approximately 500 bp fragment was excised, the DNA isolated using GeneClean (BIO 101), "blunted" using T4 polymerase (Gibco BRL) and ligated into the EcoRV site of the vector

pZERO 2.1 (Invitrogen). Restriction enzyme analysis confirmed that the majority of clones obtained had an identical insert. pZERO.CD44RC clone #2 was digested with XbaI and the fragment obtained, cloned into the XbaI site of pBlueScript (KS)+. One of the clones obtained (pBS.CD44RC clone # 2.2) was completely sequenced in both directions at the University of British Columbia DNA Sequencing Facility (UBC, Vancouver, Canada) using T3 and T7 primers.

4.2.3 Cellular expression of CD44RC

The cellular expression of CD44RC was determined by RT-PCR analysis. mRNA was isolated and cDNA generated from peripheral blood leukocytes (PBL) obtained from 2 healthy adult volunteers and from the cell lines KG1, KG1a, HL60 and U937 as described above. A common 5' primer corresponding to CD44 exon 1 (5'-GGTCTAGACCGTTCGC TCCGGACACCATGG-3') was used together with two different 3' primers corresponding respectively to CD44 exon 20 (5'-GGTCTAGATTACACCCCAATCTTCATGTCC-3') or to the junction between exon 2 and the middle of exon 18 found uniquely in CD44RC (5'-GCAATGCAAACTGCAGGTCTC-3'). The conditions employed were exactly as described previously (Chapter 2, Materials and Methods). Ten µl of each PCR reaction were electrophoresed on a 1% agarose gel and the products visualized by ethidium bromide staining.

4.2.4 Production of CD44RC conditioned media

In order to define the functional activity of CD44RC, the cDNA was subcloned into the eukaryotic episomal expression vector pCEP4 (Invitrogen). Briefly, pBS.CD44RC clone # 2.2 was digested with XbaI. A fragment of approximately 500 bp containing the full-length CD44RC cDNA was isolated and ligated into the NheI site of pCEP4. The correct orientation of the CD44RC cDNA was confirmed by restriction enzyme analysis and an appropriate clone (pCEP4.CD44RC #2.2.1) was picked, further amplified and plasmid DNA purified using the BiggerPrep DNA Isolation Kit (5' 3', Boulder, CO, USA).

K562 cells were transfected with plasmid DNA by electroporation using 15 μg of pCEP4 or pCEP4.CD44RC plasmid DNA as per the protocol described in Chapter II, Materials and Methods (section 2.2.4). The time constants obtained ranged from 3.0-3.3 ms. Transfected cells were selected for a minimum of 14 days and were maintained thereafter in DMEM+10% CCII containing 200 μg/ml hygromycin. Expression of CD44RC mRNA was determined by RT-PCR analysis using the conditions and primers as described above.

In order to generate soluble CD44RC for use in further studies, 5 x 10^6 K562 cells transfected with pCEP4 or pCEP4.CD44RC plasmid DNA were resuspended in 10ml DMEM without serum or hygromycin and cultured at 37°C in a 25 cm^2 flask (Falcon). 24 h later, the entire cell suspension was harvested and centrifuged at 1000 rpm for 10 min. The supernatant was collected, centrifuged for a further 5 min at 10000 rpm to remove any

cellular debris, concentrated 10 fold using a Centricon-10 Concentrator (Amicon; Millipore, Bedford, MD), aliquoted and stored at –20°C until needed.

4.2.5 Effect of CD44RC on cellular adhesion to hyaluronan

KG1 and KG1a cells from log phase cultures were harvested and resuspended at a final concentration of $1x10^6$ cells/ml in medium conditioned by K562 cells transfected with pCEP4 or pCEP4.CD44RC of 1 h at 37°C. In some experiments, the cells were incubated for 1 h at 4°C with the anti-CD44 mAb 3G12 at a final concentration of 10 μg/ml and washed once with DMEM before being added to the conditioned supernatants. After incubation in the conditioned supernatants, $2x10^5$ cells in a final volume of 0.5 ml were added to each of 2 wells in a 24 well plate (Falcon) that had been coated overnight at 4°C with human placental hyaluronan (Sigma) (5mg/ml in PBS). After incubation for 10 min at room temperature, non-adherent cells were removed by gently washing each well 5 times with HBSS. The number of adherent cells per unit area was determined by digital analysis of captured well images (NIH Image) or by counting 5 random fields using an inverted phase microscope.

4.2.6 Mechanism of CD44RC-mediated enhancement of cellular adhesion to hyaluronan

In order to define the mechanism by which CD44RC enhances CD44-mediated adhesion to hyaluronan, KG1 cells were pretreated for 1 h at 37°C with 0.5 units of chondroitinase ABC (Sigma), or 1 unit of hyaluronan lyase (Sigma) prior to incubation in

CD44RC containing supernatants. The treated cells were then washed 3 times with DMEM, incubated with CD44RC containing supernatant and tested for attachment to hyaluronan as described above.

Cytospin preparations of KG1 cells, treated with K562.pCEP4.CD44RC or control K562.pCEP4 conditioned supernatants were also prepared, fixed in acetone and stained with mAbs directed against CD44 using an indirect immunoperoxidase technique as previously described (Dougherty *et al.*, 1994).

4.3 Results

4.3.1 Cloning and nucleotide sequencing of a novel soluble CD44

Using primers specific for CD44 exons 1 and 20, full length CD44 cDNAs were amplified by RT-PCR from mRNA isolated from the myelomonocytic cell line KG1a. Two major products were obtained, a 1.1 kb fragment corresponding in size to CD44H and an unknown band of approximately 500 bp (Figure 22). The smaller cDNA was isolated and subcloned into pZERO2.1 and then pBlueScript (KS)+. Sequence analysis revealed a cDNA of 484 nucleotides with an ATG initiation codon at position 19 followed by an open reading frame of 420 residues (Figure 23). The first 233 nucleotides exhibit 100% sequence identity with CD44 exons 1 and 2, while the final 187 nucleotides corresponded to the last 27 residues of CD44 exon 18 and all of exon 20. Thus, this transcript appears to be generated as

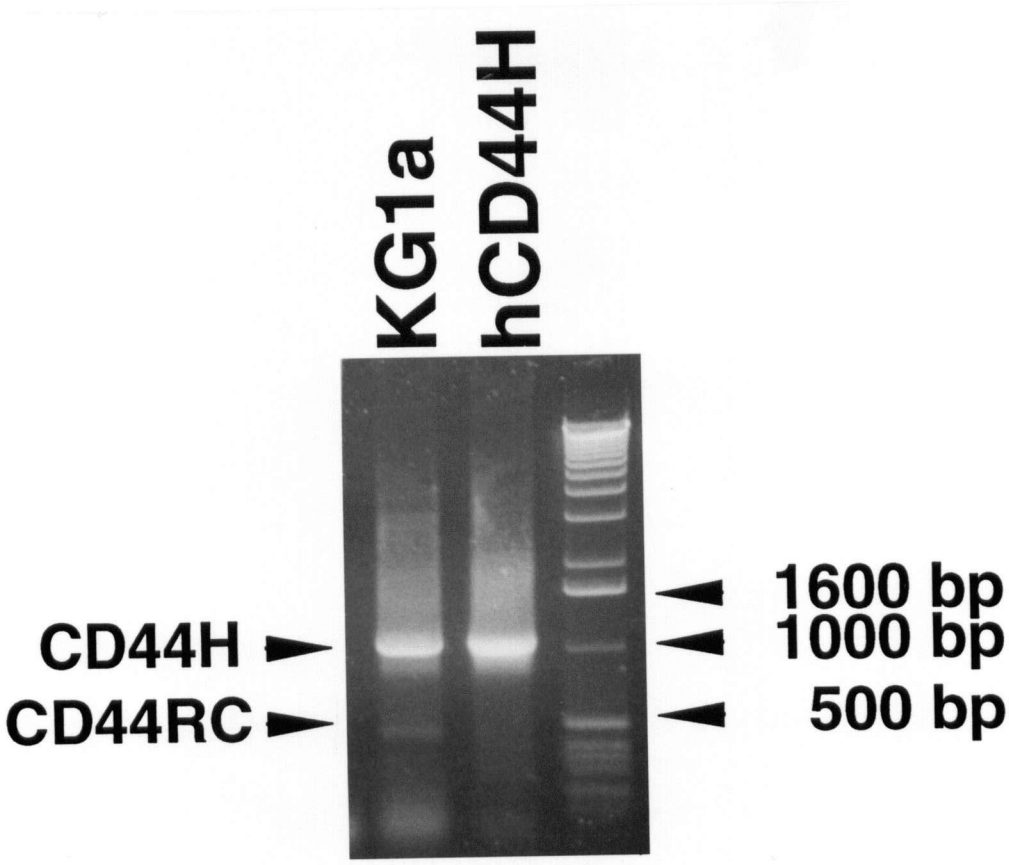


Figure 22: Expression of CD44 isoforms in KG1a cells. CD44 cDNAs were amplified by RT-PCR from mRNA isolated from the myelomonocytic cell line KG1a using primers specific for CD44 exons 1 and 20. As a control, a CD44H cDNA isolated from the plasmid pCDM8.CD44H by digestion with XhoI was used as a template and similarly amplified using the same primer pair.

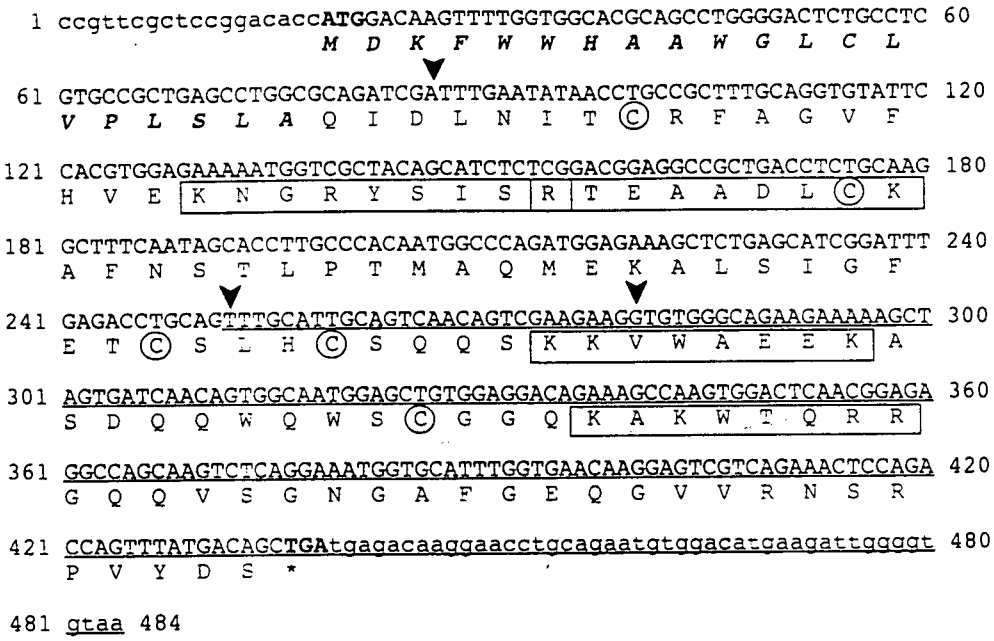


Figure 23: Nucleotide and predicted amino acid sequences of CD44RC. pBS.CD44RC clone # 2.2 was fully sequenced and the predicted amino acid sequence determined. The start and stop codons are shown in bold and the putative signal peptide is bold and italicized. Exon boundaries are marked with an (▼). Cysteine residues important for secondary structure are circled, and the putative hyaluronan binding domains boxed. The novel reading frame produced by the alternative splicing of CD44 exon 2 into exon 18 is underlined.

a result of alternative splicing occurring between the normal exon 2 splice donor and an alternative splice acceptor site (GCAG) found at position 48 within exon 18 (Figure 24). Utilization of this site generates a frame shift producing a novel CD44 molecule with a unique COOH terminus. We have designated this CD44 isoform CD44RC.

Upon cleavage of the 20 amino acid signal peptide, the mature CD44RC protein would consist of 119 amino acids with a predicted molecular weight of 13.1 kDa. The protein is predominantly hydrophilic (Figure 25) suggesting that it would be soluble in aqueous solution. The tandem hyaluronan binding domain KNGRYSISRTEAADLCK encoded by exon 2 which is present in all CD44 isoforms described to date is also present in CD44RC (Figure 23). In addition, however, the novel COOH-terminus of CD44RC contains two further basic amino acid motifs starting at position 92 (KKVWAEEK) and 113 (KAKWTQRR) that could potentially function as hyaluronan-binding domains (Figure 23 and Table 2).

4.3.2 Cellular expression of CD44RC

The expression of CD44RC in primary PBL and in the myeloid cell lines KG1, KG1a, HL60 and U937 was determined by RT-PCR analysis using a 5' primer specific for CD44 exon 1 and a 3' primer specific for exon 20. In addition to full length CD44H, a band of approximately 500 bp corresponding in size to CD44RC can be seen in all of the cell lines and both of the primary PBL samples tested (Figure 26). The intensity of this band relative

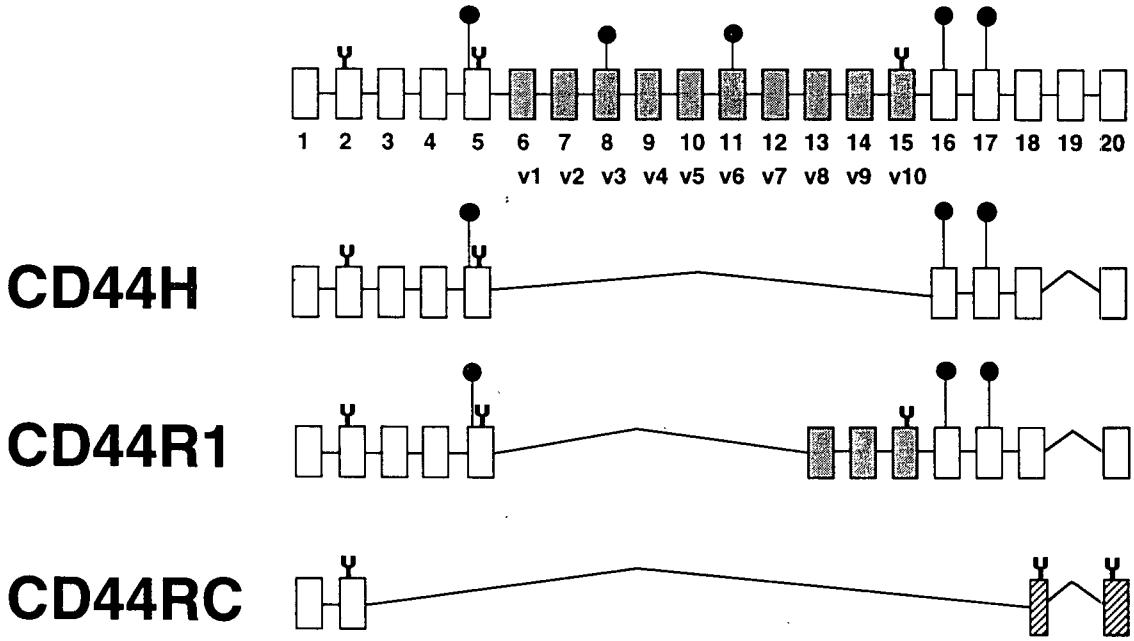


Figure 24: Splicing of the CD44 gene leading to the generation of CD44RC. To date, the human genomic CD44 structure is known to consist of 20 exons. Variant exons 1 to 10 (v1-v10) can be alternatively spliced to generate higher molecular mass CD44 isoforms. Interestingly, the cDNA for CD44RC is generated by a novel alternative splicing scheme. The splice donator motif at the end of exon 2 recognizes a secondary splice acceptor site of GCAG within exon 18 thereby creating a frame shift for the remainder of the molecule.

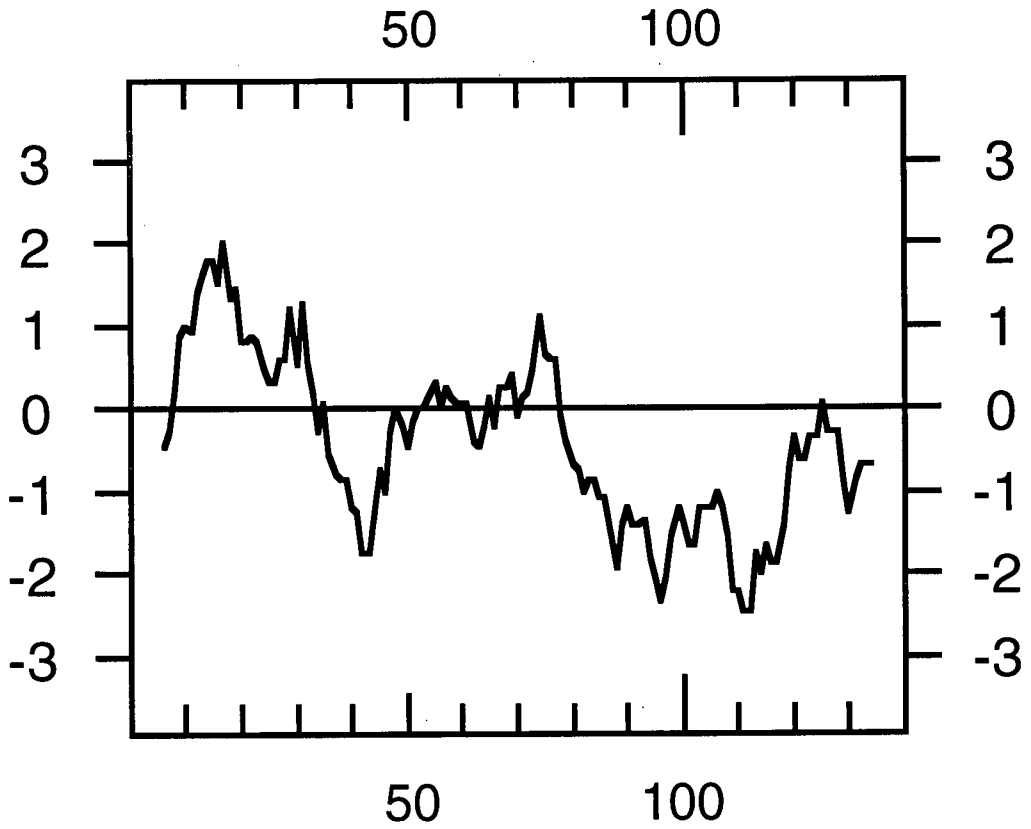


Figure 25: Hydrophobicity graph of the CD44RC protein. A Kyte Doolittle hydrophobicity analysis of the predicted amino acid sequence of CD44RC was performed using DNA Strider 1.2. Hydrophobicity is represented as positive numbers and hydrophilicity as negative. Following the 20 amino acid hydrophobic signal peptide, the mature CD44RC protein is predominantly hydrophilic.

Source	Hyaluronan binding sequence	motif	Hyaluronan-binding
RHAMM	423**K**LRSQLVK**R**K432	B(X_7) B	+++
RHAMM mutant#1	**K**LRSQLVK**RI**	B(X_7) B	+++
RHAMM mutant#2	**K**LRSQLV**K**SK	B(X_6) B	++
RHAMM mutant#3	NL**R**SQLVKR**K**	B(X_6) B	+
CD44	38**K**NGRYSIS**R**46	B(X_7) B	+++
CD44	46**R**TEAADLC**K**54	B(X_7) B	+++
CD44RC	92**K**KVWAEE**K**99	B(X_6) B	?
CD44RC	113**K**AKWTQR**R**120	B(X_6) B	?

Table 2: Comparison of binding ability for various hyaluronan binding sequences. The hyaluronan binding motif from RHAMM, and site specific mutants of RHAMM are aligned with the tandem hyaluronan binding sequences from CD44 and the putative ligand binding domain of CD44RC. The general motif (e.g. [B(X_7)B]) and relative hyaluronan binding capacity as determined by Yang *et al* (Yang *et al.*, 1994) are shown.

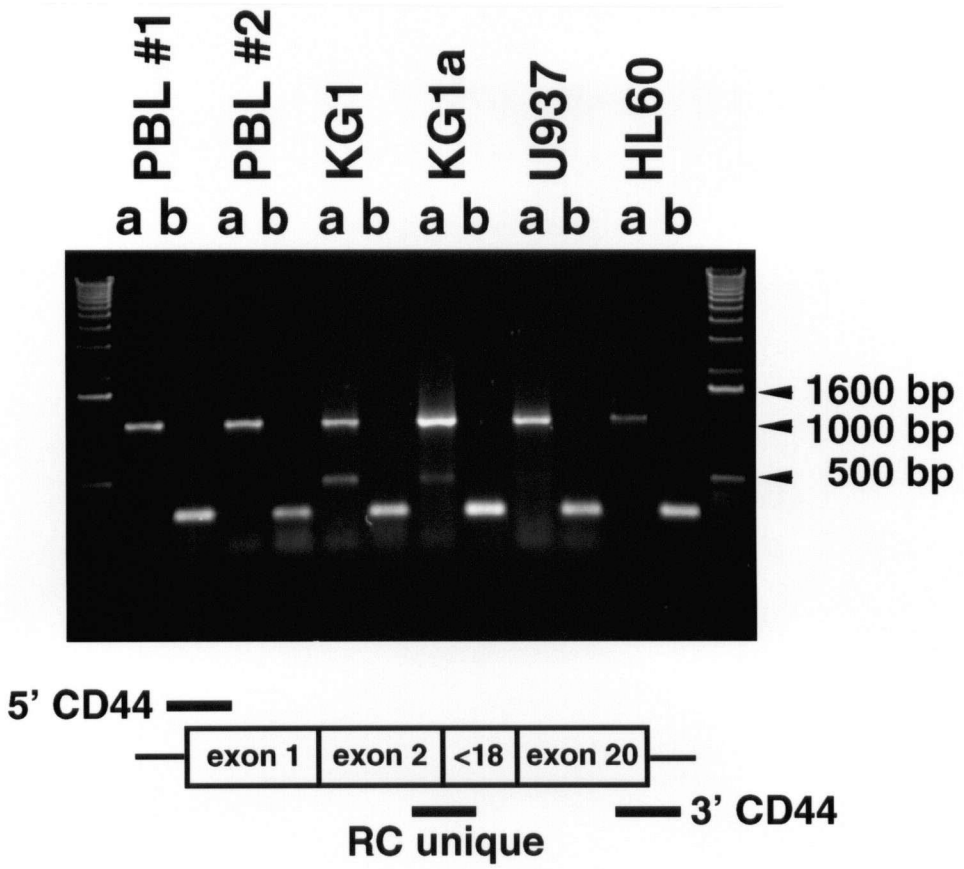


Figure 26: Expression of CD44RC in normal PBL and various hemopoietic cell lines. RT-PCR analysis was used to determine the expression of CD44RC in normal PBL and in the hemopoietic cell lines KG1, KG1a, U937 and HL60. Two primer pairs were used, (a) full length CD44 cDNAs were amplified using primers specific for exons 1 (5' CD44) and 20 (3' CD44) and (b) CD44RC transcripts were specifically amplified using primers specific for exon 1 (5' CD44) and the unique junction between exon 2 and exon 18 (RC unique) found only in CD44RC .

to CD44H varied considerably between the different cell types with the lowest levels being seen in primary PBL. Additional RT-PCR analysis using a 3' primer corresponding to the unique junction created by the splicing of exon 2 into the middle of exon 18 (see Material and Methods Section 4.2.3) confirmed the presence of CD44RC in all of the cell types tested (Figure 26).

4.3.3 Functional activity of CD44RC

The episomal expression vector pCEP4 was used to express CD44RC in the hemopoietic cell line K562. In the absence of available antibodies directed against determinants encoded by CD44 exons 1 or 2, expression of CD44RC in the transfected cells was once again determined by RT-PCR analysis using the primer sets described above. As shown in Figure 27, CD44RC mRNA is readily detected in the transfected cells. Although generally considered CD44 negative, a weak band corresponding in size to CD44H can be seen in K562 cells transfected with the control pCEP4 vector using a 5' primer specific for CD44 exon 1 and a 3' primer specific for exon 20. Using the 3' primer specific for the unique junction between exon 2 and exon 18 present in CD44RC, high levels of CD44RC mRNA could be detected in K562 cells transfected with pCEP4.CD44RC. Very low but detectable levels of CD44RC were also observed in control K562 cells transfected with pCEP4.

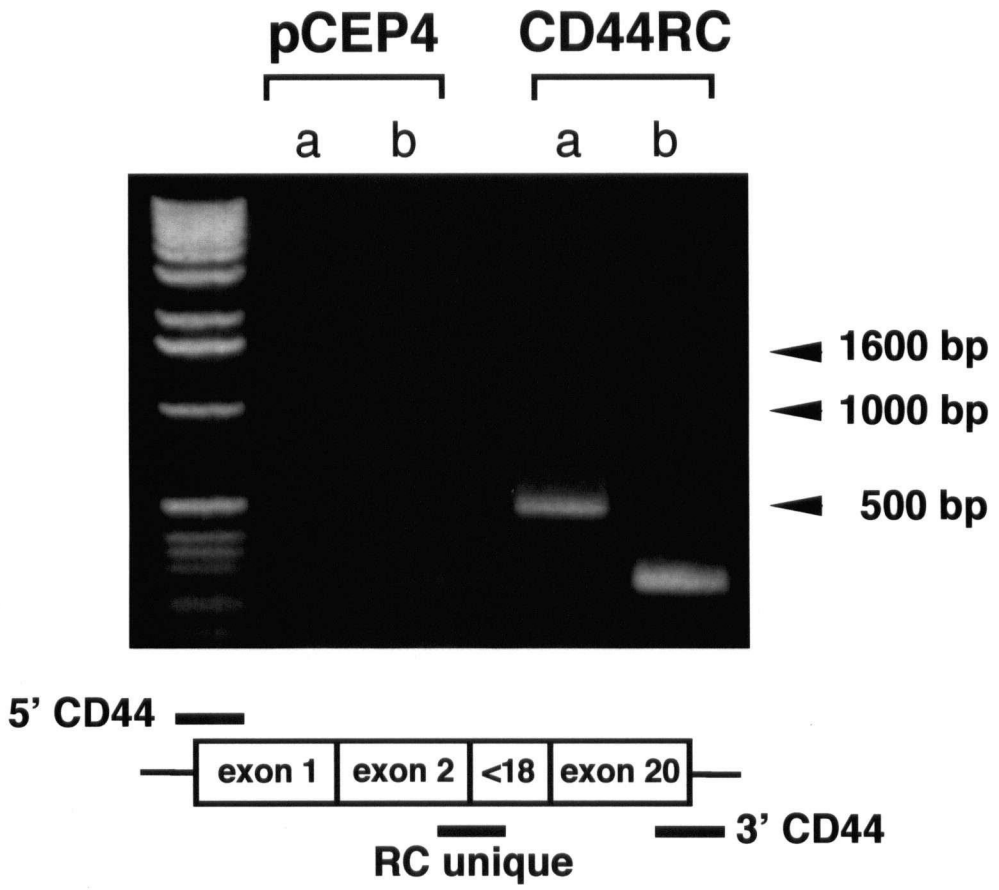


Figure 27: Expression of CD44RC in K562.CD44RC. The expression of CD44RC in vector alone control K562 cells (pCEP4) and K562 cells transfected with pCEP4.CD44RC (CD44RC) was determined by RT-PCR. Two primer pairs were used, (a) full length CD44 cDNAs were amplified using primers specific for exons 1 (5' CD44) and 20 (3' CD44) and (b) CD44RC transcripts were specifically amplified using primers specific for exon 1 (5' CD44) and the unique junction between exon 2 and exon 18 (RC unique) found only in CD44RC .

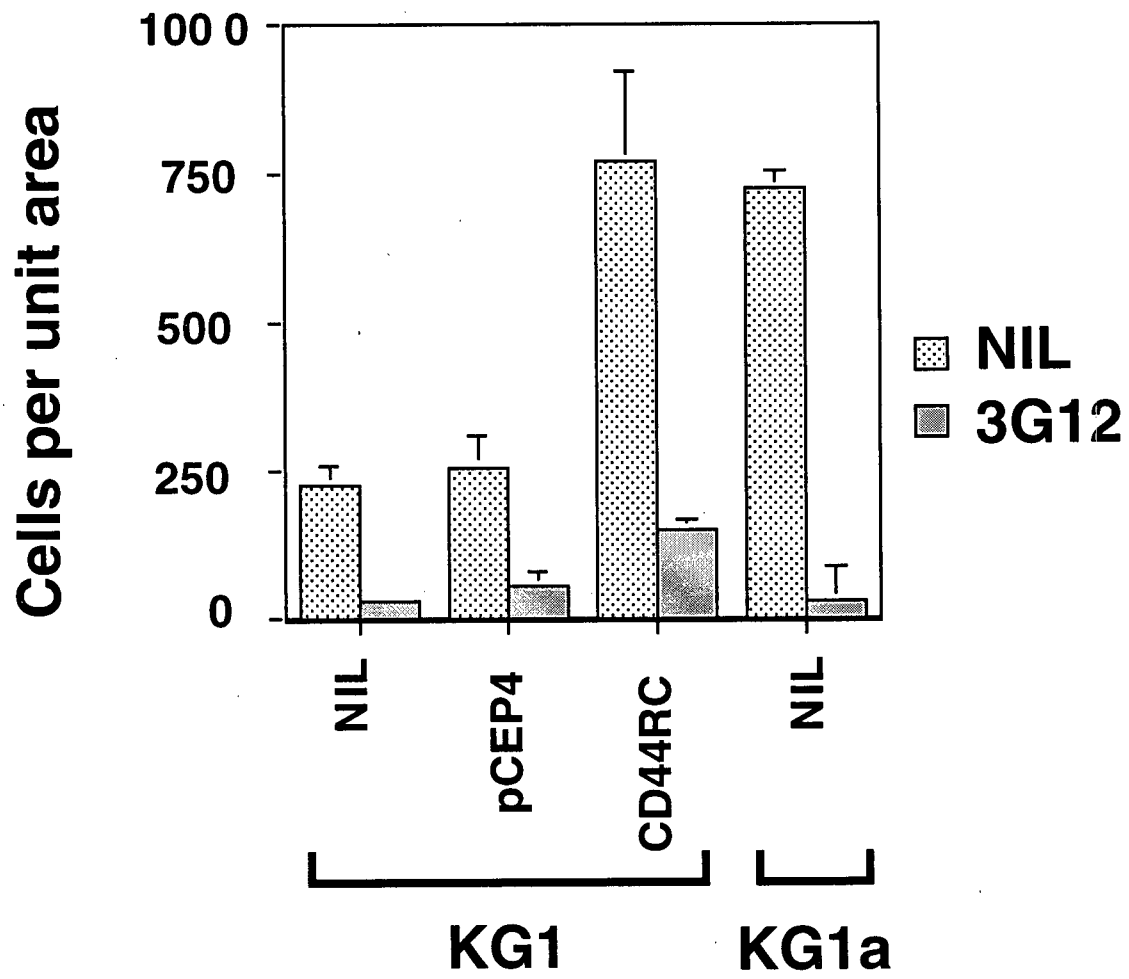


Figure 28: Effect of CD44RC on cellular adhesion to hyaluronan. KG1 and KG1a cells were incubated with tissue culture medium or with anti-CD44 mAb 3G12 tissue culture supernatant for 1 h at 4°C and washed prior to treatment with control tissue culture medium (NIL) or media conditioned by K562.CD44RC or K562.pCEP4 as described in Materials and Methods. Following extensive washing, cells were assayed for their ability to adhere to hyaluronan-coated plastic. Each point represents the mean ± S.D. of at least three independent determinations.

To determine the effect of CD44RC on hyaluronan binding, KG1 cells were incubated with medium conditioned by K562.pCEP4 or K562.pCEP4.CD44RC and tested for their ability to bind to hyaluronan-coated plastic. As shown in Figure 28, pre-treatment of KG1 cells with CD44RC increased by approximately 3 fold the proportion of these cells that bind to hyaluronan under the conditions employed. Similar treatment of KG1a cells, which exhibit a higher basal level of attachment to hyaluronan than KG1 cells, had no effect on binding.

4.3.4 Induction of hyaluronan binding by CD44RC involves the recognition of chondroitin sulfate presented by endogenous CD44

Previously, we have demonstrated in Chapter III, that CD44 can recognize and bind chondroitin sulfate moieties when presented in association with CD44 or other cell surface proteins. In order to further characterize the molecular mechanism by which CD44RC enhances the hyaluronan binding ability of KG1, cells were treated with chondroitinase ABC or hyaluronan lyase prior to incubation with CD44RC and then assayed for their ability to bind to hyaluronan-coated plastic. As shown in Figure 29, treatment with chondroitinase ABC almost completely inhibited the induction of hyaluronan binding observed following the addition of CD44RC. In contrast, treatment with hyaluronan lyase had virtually no inhibitory effect. These results suggest that CD44RC potentiates adhesion to hyaluronan by recognizing and binding to a chondroitin sulfate modified protein present on the surface of the KG1 cells. Since attachment of KG1 cells to hyaluronan can be almost completely blocked by mAbs directed against CD44, it is likely that it is chondroitin

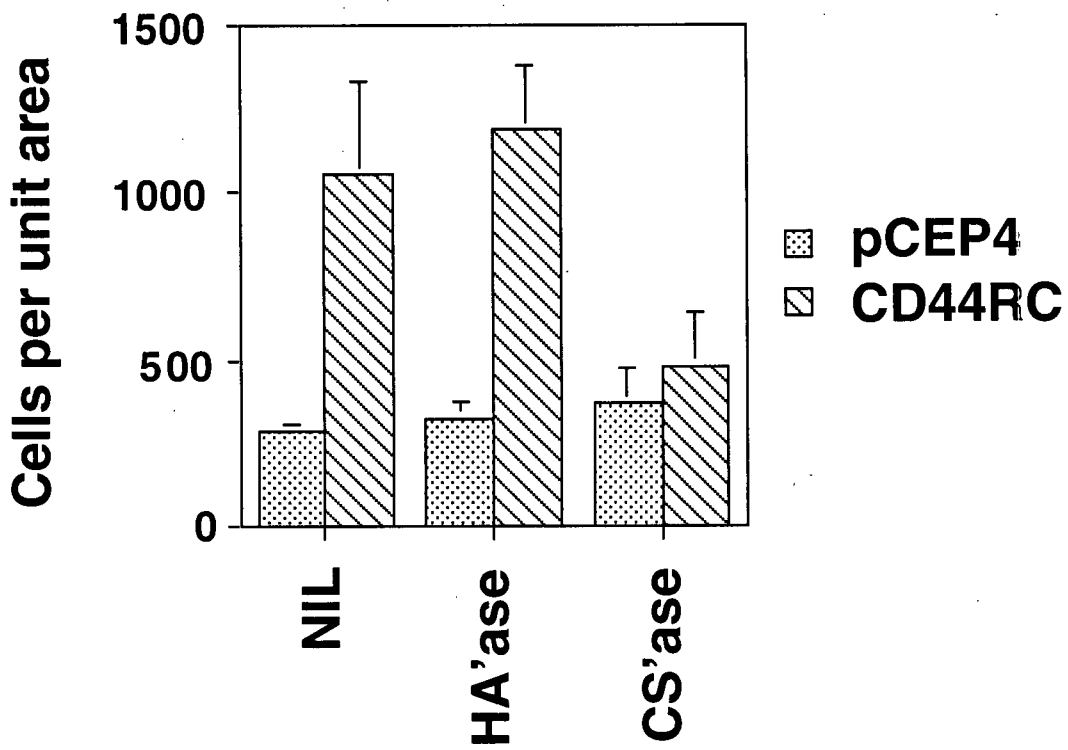


Figure 29: Effect of chondroitinase treatment on cellular adhesion to hyaluronan induced by CD44RC. KG1 cells were pretreated for 1 h at 37°C with control tissue culture medium (NIL), chondroitinase ABC (CS'ase), or hyaluronan lyase (HA'ase) prior to incubation with K562.pCEP4 or K562.CD44RC conditioned tissue culture supernatants as described in Materials and Methods. Treated cells were washed extensively and then assayed for their ability to adhere to hyaluronan-coated plastic. Each point represents the mean ± S.D. of at least three independent experiments.

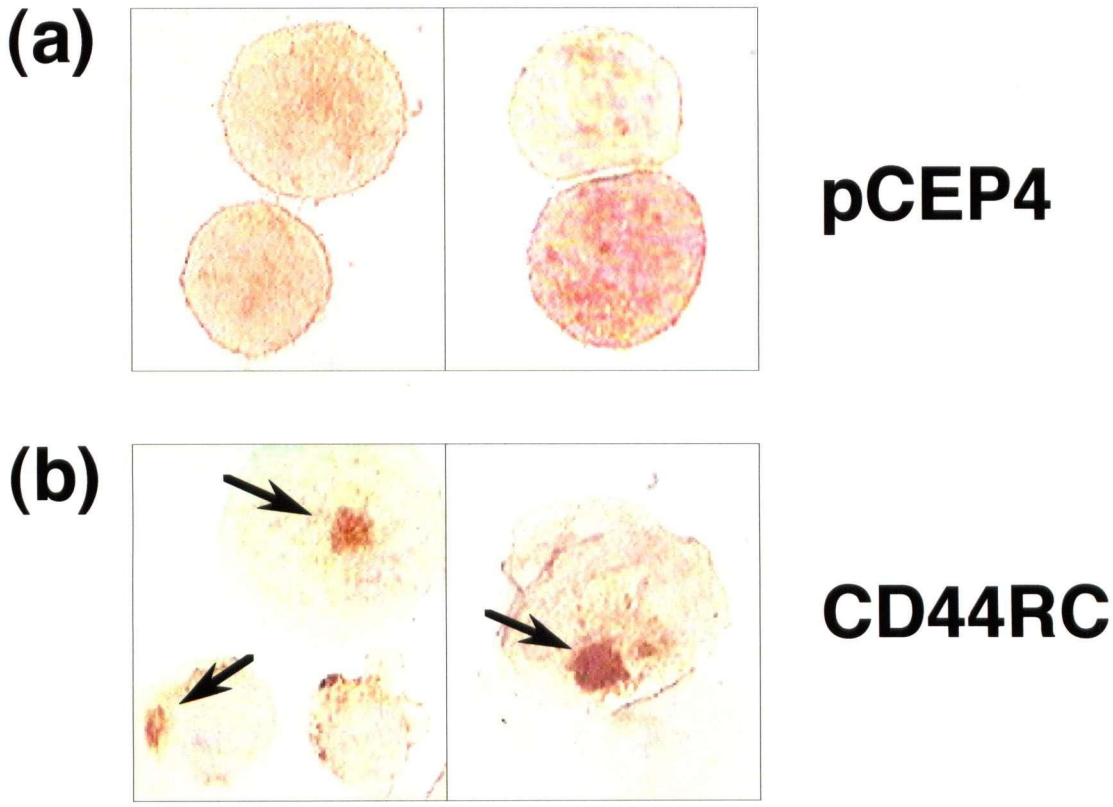


Figure 30: Effect of CD44RC on the cell surface distribution of CD44. Cytospin preparations of KG1 cells treated with tissue culture supernatants conditioned by (a) K562.pCEP4 or (b) K562.CD44RC, were fixed in acetone and stained for CD44 expression using an indirect immunoperoxidase technique. Clusters of CD44 induced by CD44RC are indicated with an arrow.

sulfate modified CD44 itself that serves as a ligand for CD44RC. Evidence in support of this suggestion is provided by studies in which the cell surface distribution of CD44 was determined by indirect immunohistochemical staining. As shown in Figure 30, CD44 is distributed fairly evenly on the surface of KG1 cells treated with K562.pCEP4 conditioned supernatants. In contrast, on cells similarly treated with K562.pCEP4.CD44RC conditioned supernatant, CD44 is aggregated to distinct regions of the cell membrane. KG1a cells, which bind avidly to hyaluronan-coated plastic even in the absence of added CD44RC, spontaneously show a similar localized distribution of CD44 (Dougherty *et al.*, 1995).

4.4 Discussion

Studies using genetically engineered proteins have suggested an important role for soluble CD44 in various cellular processes including tumour progression and metastasis (Sy *et al.*, 1992; Thomas *et al.*, 1992; Bartolazzi *et al.*, 1994; Yu *et al.*, 1997). Sy and colleagues, for example, have shown that the enhanced growth of the CD44-negative human lymphoma line Namalwa in SCID mice induced by the transfection of CD44H can be suppressed by treatment with soluble CD44 (Sy *et al.*, 1992). In another recent study, Yu *et al* have similarly demonstrated that expression of a cDNA encoding a soluble CD44 protein within the highly metastatic murine mammary carcinoma TA3/St blocked the CD44-mediated binding and internalization of hyaluronan, inhibited tumour metastasis and induced apoptosis *in vivo* (Yu *et al.*, 1997). Such studies are in agreement with a model in which soluble CD44 antagonizes the function of the corresponding cell surface receptor by binding to and preventing recognition of the ligand hyaluronan.

CD44RC, the novel naturally occurring soluble CD44 molecule identified in the present study, had a very different effect on hyaluronan binding. Rather than blocking adhesion, pretreatment of KG1 cells with CD44RC increased the proportion of cells that bound to immobilized hyaluronan. CD44RC appears to mediate this effect by binding to and crosslinking chondroitin sulfate side chains attached to cell surface CD44, inducing the clustering or aggregation of the protein in the plane of the membrane, thereby enhancing the local concentration CD44 and thus its avidity for hyaluronan (Figure 31). It is likely that this effect will be critically dependent upon the concentration of both CD44RC and cell surface CD44.

CD44RC includes exon 1 and 2 of the CD44 gene followed by the 3' end of exon 18 and all of exon 20. Analysis of the sequence of exon 18 reveals a surprisingly good internal consensus splice acceptor site that appears to be utilized to generate the CD44RC cDNA. Importantly, although exon 18 encodes the transmembrane domain of cell surface CD44, utilization of the alternative exon 18 splice acceptor generates a frame shift producing a predominantly hydrophilic soluble protein. Although CD44RC has not previously been described, there are a number of other examples where alternative splicing events have been shown to result in alterations in reading frame producing biochemically and functionally distinct protein isoforms (Ge *et al.*, 1991; Sham *et al.*, 1992; Zhang *et al.*, 1992; Grumont and Gerondakis, 1994; Quelle *et al.*, 1995).

Previous studies have demonstrated that adhesion of CD44 and other proteins to hyaluronan is mediated by an amino acid motif in which two basic amino acid residues are separated by a stretch of 7 non-acidic residues [B(X_7) B] (Yang *et al.*, 1994). The presence of additional basic amino acids within this motif further increases its avidity for hyaluronan (Yang *et al.*, 1994). A tandem arrangement of the [B(X_7) B] motif is encoded by CD44 exon 2 and is present in all isoforms that have been described to date including CD44RC. Similar motifs are also encoded by exon 5 and the alternatively spliced exon v10. There is evidence that the motif present in exon v10 can contribute to the unique ability of CD44 isoforms containing this exon to promote cell-cell adhesion via the recognition of chondroitin sulfate side chains presented on CD44 and other cell surface proteins (Droll *et al.*, 1995; Chiu *et al.*, 1998).

Interestingly, the unique region present at the COOH-terminus of CD44RC generated by the frame shift produced by the use of the alternative splice acceptor site present in exon 18, contains two [B(X_6)B] motifs. Although perhaps of lower affinity than the [B(X_7)B] motif, there is convincing evidence that peptides containing [B(X_6)B] can also bind hyaluronan (Yang *et al.*, 1994). Thus, it is possible that CD44RC contains two spatially distinct regions that can interact with hyaluronan. While the precise mechanism by which CD44RC potentiates adhesion to hyaluronan remains to be determined, the data obtained in the present study suggests a model in which CD44RC appears to mediate this effect by binding to and crosslinking chondroitin sulfate side chains attached to cell surface CD44 inducing the clustering or aggregation of the protein in the plane of the membrane thereby enhancing the local concentration CD44 and thus its affinity for hyaluronan. The

ability of CD44RC to "activate" the hyaluronan binding activity of cell surface CD44 would thus vary depending upon the concentration of the soluble protein in the local microenvironment and the proportion of cell surface CD44 molecules that are modified by the addition of chondroitin sulfate side chains. Both of these variables could in turn be altered by cellular activation state and/or differentiation stage and could conceivably be affected by malignant transformation.

Since both CD44H and CD44RC contain sequences encoded by exon 1 and exon 20, RT-PCR analysis using 5' and 3' primers specific for these exons constitutes a semi-quantitative means of determining the relative expression level of both isoforms within a particular cell line. Using such an approach, it appears that while primary PBL express fairly low levels of CD44RC substantially higher levels are seen in various transformed hemopoietic cell lines. As might be expected given the ability of glycosylation and other changes to modulate the functional activity of CD44 and the requirement for CD44 to be modified by the addition of chondroitin side chains before the molecule can be crosslinked by CD44RC, there is no simple correlation between the expression of CD44RC and the ability of a particular cell line to bind either immobilized and/or soluble hyaluronan.

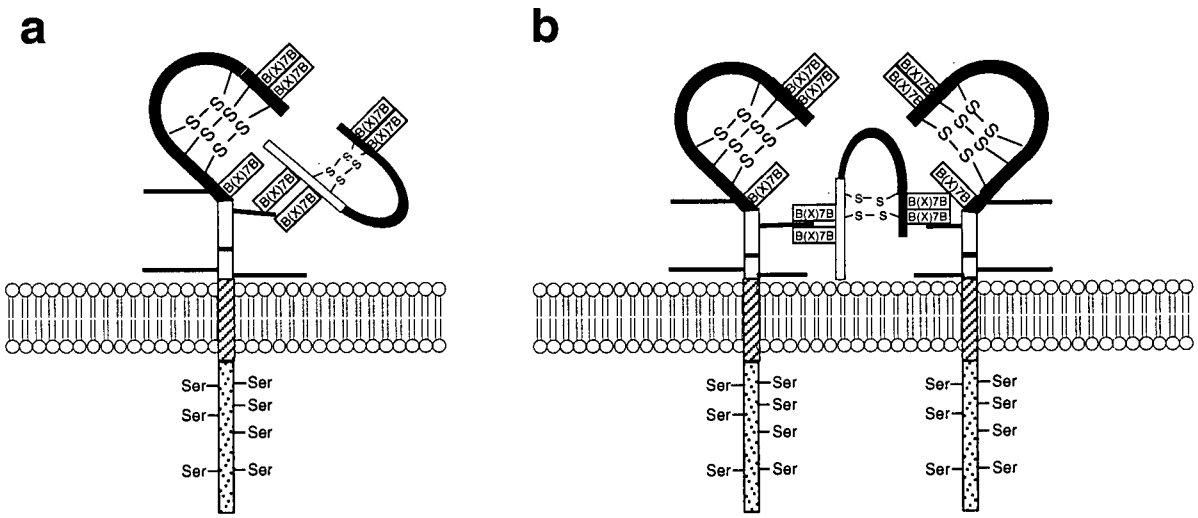


Figure 31: Model of functional regulation by CD44RC. The novel soluble CD44 molecule increased the proportion of cells that bound to immobilized hyaluronan. CD44RC appears to mediate this effect by binding to and crosslinking chondroitin sulfate side chains presented by cell surface CD44 effectively increasing the local concentration of this receptor.

CHAPTER V

DISCUSSION

It has recently become clear that certain modalities used in the treatment of cancer, including ionizing radiation and certain chemotherapeutic agents, mediate their cytotoxic effects at least in part through the induction of apoptosis (D'Amico and McKenna, 1994; Arceci, 1996). A corollary of such studies is that changes in the expression of genes involved in the regulation of apoptosis are likely to contribute not only to the development and/or pathogenesis of malignant disease, but also the response of malignant cells to therapy.

Although cytokines are the best studied regulators of hemopoietic cell apoptosis (Park, 1996; Sachs, 1996), it is clear that signals transduced via the interaction of cell surface adhesion proteins with their natural ligands may also play an important role in the control of cell survival and apoptosis (Meredith *et al.*, 1993; Re *et al.*, 1994; Bates *et al.*, 1994; Bates *et al.*, 1995). Loss of adhesion to specific extracellular matrix components can induce a variety of cell types to undergo apoptosis (Meredith *et al.*, 1993; Re *et al.*, 1994; Bates *et al.*, 1995). Heterotypic and homotypic cell-cell interactions may also inhibit the induction of apoptosis (Bates *et al.*, 1994), perhaps explaining the increased resistance of multicellular aggregates and tumour spheroids to chemotherapeutic agents and ionizing radiation (Kerbel *et al.*, 1994). Specifically with respect to hemopoiesis, a number of studies have shown that both normal and malignant hemopoietic cells show increased survival if plated on stromal layers derived from bone marrow or other tissues (Bendall *et al.*, 1994; Manabe *et al.*, 1994).

Although a role for cytokines bound to extracellular matrix proteins or presented on the surface of stromal cells cannot be excluded, studies in which hemopoietic cells and stromal elements are separated by a permeable filter suggest that direct contact is required if apoptosis is to be inhibited (Manabe *et al.*, 1994).

Monoclonal antibody blocking studies have implicated integrins as important determinants in contact-dependent inhibition of apoptosis (Bates *et al.*, 1995). Signals transduced via other cell surface proteins may also be involved (Hanaoka *et al.*, 1995; Bazil *et al.*, 1996; Fujita *et al.*, 1996). Of particular relevance to this thesis, mAbs directed against the broadly distributed polymorphic cell surface glycoprotein CD44 have been shown to block lymphopoiesis and myelopoiesis in murine long term cultures (Miyake *et al.*, 1990a; Miyake *et al.*, 1990b). Importantly, numerous recent studies have also demonstrated a dramatic correlation between the overall expression of CD44, or the presence of particular alternatively spliced CD44 isoforms, and the metastatic propensity of certain hemopoietic and non-hemopoietic malignancies (Herrlich *et al.*, 1993; Tanabe and Saya, 1994; Gunthert *et al.*, 1995). At present, the molecular mechanisms by which CD44 contributes to tumourigenesis remain unclear. Namalwa (Burkitt lymphoma) cells transfected with human CD44 were shown to grow faster than the parental cell line when injected s.c. into nu/nu mice, and colonized various organs more efficiently when injected i.v., although their tissue-specific pattern of metastasis was not altered, and their growth rate *in vitro* was unchanged (Sy *et al.*, 1991; Sy *et al.*, 1992). These data suggest that rather than simply promoting the dissemination of tumour cells, CD44 functions primarily to enhance tumour cell survival and/or proliferation at various sites *in vivo*.

As emphasized throughout this Thesis, although CD44 is present on many cell types the functional activity and ligand binding specificity of the molecule is highly regulated. Many cell types express significant levels of CD44 on their surface, but completely lack the capacity to bind hyaluronan (reviewed in Lesley *et al.*, 1993). Thus in order to better understand the involvement of CD44 in the malignant process it is necessary not only to characterize changes in the expression and/or alternative splicing of CD44, but also to determine whether the molecules that are expressed, are present in a functionally active state.

The work reported in this Thesis emphasizes that the density of CD44 on the cell surface plays a critical role in regulating the ligand binding function of the molecule. A simple model would predict that individual CD44 molecules have a reasonably low affinity for hyaluronan but that binding is enhanced by cellular processes that increase the local concentration of the molecule in the plane of the membrane thereby raising the avidity of the interaction between CD44 and its ligand. As discussed in Chapter 2, two main mechanisms appear to be involved. Firstly, alterations in transcriptional rate can increase the overall expression of CD44 above the critical threshold concentration or density required for hyaluronan binding (Figure 16). Alternatively, on some cell types, CD44 can aggregate into distinct clusters or caps generating a locally high concentration without increases in overall expression. In KG1a cells such localization appears to be dependent upon interaction between the cytoplasmic domain of CD44 and cytoskeletal proteins. As discussed in Chapter 4, there are clearly other ways in which CD44 can be induced to aggregate and these alternative mechanisms may be important in some cell lines. Thus, the conflicting data that

has been obtained regarding the involvement of phosphorylation and interactions between the cytoplasmic domain of CD44 and various cytoskeletal proteins in the regulation of hyaluronan binding (Kalomiris and Bourguignon, 1989; Uff *et al.*, 1995), could perhaps be explained simply if the cell lines employed in these various studies, differed in the relative contribution that this particular process made to the ligand binding activity of CD44. In cell lines in which CD44 can exist in a "functionally competent" state, simply overexpressing the protein (with or without a cytoplasmic domain) to a high enough level is likely to induce hyaluronan-binding activity. In cell lines where overall CD44 expression lies below the threshold level required for binding, aggregating the protein (e.g. by treatment with appropriate mAbs) will result in increased avidity which, if sufficiently high, may allow hyaluronan to be bound.

There is a consensus that CD44 can also be negatively regulated in a cell-specific fashion by post-translational mechanisms, most notably differential N-linked glycosylation (Katoh *et al.*, 1995; Lesley *et al.*, 1995; English *et al.*, 1998). It is noteworthy that aberrant glycosylation is commonly associated with malignant transformation perhaps explaining the high proportion of tumour cells that can constitutively bind hyaluronan (reviewed in Hakomori, 1996). Although the evidence presented in Chapter 2 demonstrates clearly that the major alternatively spliced CD44 isoforms expressed by normal and transformed hemopoietic cells (CD44H, CD44R1 and CD44R2) (Dougherty *et al.*, 1991) do not differ greatly in their hyaluronan binding function when expressed in K562 cells, it is conceivable that in other cell types, differential glycosylation events including the addition of chondroitin

sulfate side chains may result in the various isoforms having very different hyaluronan binding activities, as has been reported by others.

Although CD44H, CD44R1 and CD44R2 are all capable of binding hyaluronan, as described in Chapter 3, the three isoforms do differ greatly in their ability to homotypically and/or heterotypically interact with one another and promote cell-cell adhesion. The ability of exon v10 containing CD44 isoforms to promote cell-cell adhesion depends upon the presence of chondroitin sulfate moieties. It remains to be determined whether exon v10 simply serves as an additional site of chondroitin sulfate attachment or whether the additional [B(X_7)B] motif present in exon v10 increases the avidity of the CD44 protein for chondroitin sulfate moieties attached elsewhere on the CD44 molecule. To differentiate between these two possibilities, site directed mutagenesis could be used to functionally inactivate both the chondroitin sulfate attachment site and [B(X_7)B] hyaluronan/chondroitin sulfate-binding motif encoded by exon v10 in CD44R1 and CD44R2. The mutated cDNAs could be cloned into pCEP4 and transfected into K562 or another CD44-negative cell line which could then be tested for its ability to homotypically aggregate in culture and to bind to COS7 cells transfected with CD44H, CD44R1 or CD44R2, as we have previously described. The inhibitory effect of chondroitinase ABC and hyaluronidase on such cellular adhesion would also be determined.

In preliminary studies, a CD44R1 cDNA in which the putative chondroitin sulfate has been abolished using a site-directed mutagenesis approach, has been introduced and expressed in K562 cells using pCEP4. Although extensive cell binding studies have not yet

been undertaken, it is clear that preventing the attachment of chondroitin sulfate to exon v10 does not greatly impact on the ability of CD44R1 to induce the homotypic aggregation of K562 cells *in vitro* (data not shown). Thus, it seems logical that in future studies particular emphasis should be placed on determining the effect of targeting the [B(X_7)B] hyaluronan/chondroitin sulfate-binding motif encoded by exon v10 on the ability of CD44R1 and CD44R2 to interact with other CD44 isoforms.

The ability of exon v10 containing CD44 isoforms to interact with one another provides a possible explanation for the frequently observed association between the presence of such alternatively spliced CD44 isoforms and poor prognosis in a number of histologically distinct tumour types (East and Hart, 1993; Gunthert, 1993; Herrlich *et al.*, 1993; Tanabe and Saya, 1994; Sleeman *et al.*, 1995). Specifically, it is our hypothesis that signals transduced via the homotypic interaction of alternatively spliced CD44 isoforms can both promote the development of malignant disease and protect malignant cells from therapeutic agents by modulating the cellular response to pro-apoptotic stimuli. In support of this conclusion, signals transduced via the crosslinking of CD44 have recently been shown to block the induction of T cell apoptosis following stimulation with anti-CD3 or treatment with dexamethasone (Ayroldi *et al.*, 1995). In another recent study, Yu *et al* have similarly demonstrated that expression of a soluble CD44 cDNA within the highly metastatic murine mammary carcinoma TA3/St antagonized the ligand binding function of the corresponding cell surface protein and induced apoptosis and prevented tumour spread *in vivo* (Yu *et al.*, 1997). According to this model, the potent inhibitory effect that mAbs directed against CD44 have on both myelopoiesis and lymphopoiesis in murine and human long-term

cultures (Miyake *et al.*, 1990a; Miyake *et al.*, 1990b) can be explained if these antibodies block the delivery of anti-apoptotic signals transduced via CD44 upon the interaction of primitive hemopoietic cells with stromal elements.

REFERENCES

Ahrens, P. B. (1993). Enhancement of phorbol ester induced cell aggregation after alterations in asparagine-linked oligosaccharides. Biol. Cell *77*, 255-260.

Altieri, D. C., and Edgington, T. S. (1988). A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/CD18 (Mac-1). J. Immunol. *141*, 2656-2660.

Arceci, R. J. (1996). Tumour cell survival and resistance to therapy. Curr. Opin. Hematol. *3*, 279-287.

Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P., and Zoller, M. (1992). Participation in normal immune responses of a metastasis-inducing splice variant of CD44. Science *257*, 682-685.

Ariza, A., Mate, J. L., Isamat, M., Lopez, D., Von Uexkull-Guldeband, C., Rosell, R., Fernandez-Vasalo, A., and Navas-Palacios, J. J. (1995). Standard and variant CD44 isoforms are commonly expressed in lung cancer of the non-small cell type but not of the small cell type. J. Pathol. *177*, 363-368.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell *61*, 1303-1313.

Ayroldi, E., Cannarile, L., Migliorati, G., Bartoli, A., Nicoletti, I., and Riccardi, C. (1995). CD44 (Pgp-1) inhibits CD3 and dexamethasone-induced apoptosis. Blood *86*, 2672-2678.

Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J., and Aruffo, A. (1998). Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. J. Biol. Chem. *273*, 338-343.

Bartolazzi, A., Nocks, A., Aruffo, A., Spring, F., and Stamenkovic, I. (1996). Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. J. Cell Biol. *132*, 1199-1208.

Bartolazzi, A., Peach, R., Aruffo, A., and Stamenkovic, I. (1994). Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumour development. J. Exp. Med. *180*, 53-66.

Bates, R. C., Buret, A., van Helden, D. F., Horton, M. A., and Burns, G. F. (1994). Apoptosis induced by inhibition of intercellular contact. J. Cell Biol. *125*, 403-415.

Bates, R. C., Lincz, L. F., and Burns, G. F. (1995). Involvement of integrins in cell survival. Cancer Metastasis Rev. *14*, 191-203.

Bazil, V., Brandt, J., Chen, S., Roeding, M., Luens, K., Tsukamoto, A., and Hoffman, R. (1996). A monoclonal antibody recognizing CD43 (leukosialin) initiates apoptosis of human hematopoietic progenitor cells but not stem cells. Blood *87*, 1272-1281.

Bazil, V., and Horejsi, V. (1992). Shedding of the CD44 adhesion molecule from leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand. J. Immunol. *149*, 747-753.

Beller, D. I., Springer, T. A., and Schreiber, R. D. (1982). Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. J. Exp. Med. *156*, 1000-1009.

Bendall, L. J., Daniel, A., Kortlepel, K., and Gottlieb, D. J. (1994). Bone marrow adherent layers inhibit apoptosis of acute myeloid leukemia cells. Exp. Hematol. *22*, 1252-1260.

Bennett, K. L., Modrell, B., Greenfield, B., Bartolazzi, A., Stamenkovic, I., Peach, R., Jackson, D. G., Spring, F., and Aruffo, A. (1995). Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. J. Cell Biol. *131*, 1623-1633.

Berg, E. L., Magnani, J., Warnock, R. A., Robinson, M. K., and Butcher, E. C. (1992). Comparison of L-selectin and E-selectin ligand specificities: the L-selectin can bind the E-selectin ligands sialyl Le(x) and sialyl Le(a). Biochem. Biophys. Res. Commun. *184*, 1048-1055.

Birch, M., Mitchell, S., and Hart, I. R. (1991). Isolation and characterization of human melanoma cell variants expressing high and low levels of CD44. Cancer Res. *51*, 6660-6667.

Blood, C. H., and Zetter, B. R. (1990). Tumour interactions with the vasculature: angiogenesis and tumour metastasis. Biochim. Biophys. Acta *1032*, 89-118.

Bosworth, B. T., St John, T., Gallatin, W. M., and Harp, J. A. (1991). Sequence of the bovine CD44 cDNA: comparison with human and mouse sequences. Mol. Immunol.*28*, 1131-1135.

Bourguignon, L. Y., Kalomiris, E. L., and Lokeshwar, V. B. (1991). Acylation of the lymphoma transmembrane glycoprotein, GP85, may be required for GP85-ankyrin interaction. J. Biol. Chem. *266*, 11761-11765.

Bourguignon, L. Y., Lokeshwar, V. B., Chen, X., and Kerrick, W. G. (1993). Hyaluronic acid-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. J. Immunol. *151*, 6634-6644.

Bourguignon, L. Y., Lokeshwar, V. B., He, J., Chen, X., and Bourguignon, G. J. (1992). A CD44-like endothelial cell transmembrane glycoprotein (GP116) interacts with extracellular matrix and ankyrin. Mol. Cell. Biol. *12*, 4464-4471.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature *364*, 33-39.

Brown, T. A., Bouchard, T., St. John, T., Wayner, E., and Carter, W. G. (1991). Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. J. Cell Biol. *113*, 207-221.

Budd, R. C., Cerottini, J. C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C., and MacDonald, H. R. (1987a). Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. J. Immunol. *138*, 3120-3129.

Budd, R. C., Cerottini, J. C., and MacDonald, H. R. (1987b). Phenotypic identification of memory cytolytic T lymphocytes in a subset of Lyt-2+ cells. J. Immunol. *138*, 1009-1013.

Butcher, E. C., Scollay, R. G., and Weissman, I. L. (1980). Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. Eur. J. Immunol. *10*, 556-561.

Butterfield, K., Fathman, C. G., and Budd, R. C. (1989). A subset of memory CD4+ helper T lymphocytes identified by expression of Pgp-1. J. Exp. Med. *169*, 1461-1466.

Calvete, J. J., Solis, D., Sanz, L., Diaz-Maurino, T., Schafer, W., Mann, K., and Topfer-Petersen, E. (1993). Characterization of two glycosylated boar spermadhesins. Eur. J. Biochem. *218*, 719-725.

Camp, R. L., Kraus, T. A., and Pure, E. (1991). Variations in the cytoskeletal interaction and post-translational modification of the CD44 homing receptor in macrophages. J. Cell Biol. *115*, 1283-1292.

Camp, R. L., Scheynius, A., Johansson, C., and Pure, E. (1993). CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. J. Exp. Med. *178*, 497-507.

Campanero, M. R., Pulido, R., Alonso, J. L., Pivel, J. P., Pimentel-Muinos, F. X., Fresno, M., and Sanchez-Madrid, F. (1991). Down-regulation by tumour necrosis factor-alpha of neutrophil cell surface expression of the sialophorin CD43 and the hyaluronate receptor CD44 through a proteolytic mechanism. Eur. J. Immunol. *21*, 3045-3048.

Cannistra, S. A., Kansas, G. S., Niloff, J., DeFranzo, B., Kim, Y., and Ottensmeier, C. (1993). Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. Cancer Res. *53*, 3830-3838.

Cao, L., Yoshino, T., Kawasaki, N., Yanai, H., Kawahara, K., Kondo, E., Omonishi, K., Takahashi, K., and Akagi, T. (1996). Binding of human leukocytes to fibronectin is augmented by an anti-CD44 mAb (TL-1) and blocked by another anti-CD44 mAb (Hermes-3) but not by anti-VLA-4/VLA-5 mAbs. Immunobiology *196*, 504-512.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294-5299.

Chiu, R. K., Droll, A., Dougherty, S. T., Carpenito, C., Cooper, D. L., and Dougherty, G. J. (1998). Alternatively spliced CD44 isoforms containing exon v10 promote cellular adhesion through the recognition of chondroitin sulfate modified CD44. Exp. Cell Res. (in press).

Chiu, R. K., Droll, A., Cooper, D. L., Dougherty, S. T., Dirks, J. F., and Dougherty, G. J. (1995). Molecular mechanisms regulating the hyaluronan binding activity of the adhesion protein CD44. J. Neurooncol. *26*, 231-239.

Cobbold, S. (1987). Human leukocyte differentiation antigens: monoclonal antibody computer databases as a tool for the future. Mol. Cell. Probes *1*, 61-72.

Colombatti, A., Hughes, E. N., Taylor, B. A., and August, J. T. (1982). Gene for a major cell surface glycoprotein of mouse macrophages and other phagocytic cells is on chromosome 2. Proc. Natl. Acad. Sci. U S A *79*, 1926-1929.

Cooper, D. L., Dougherty, G., Harn, H. J., Jackson, S., Baptist, E. W., Byers, J., Datta, A., Phillips, G., and Isola, N. R. (1992). The complex CD44 transcriptional unit; alternative splicing of three internal exons generates the epithelial form of CD44. Biochem. Biophys. Res. Commun. *182*, 569-578.

Cooper, D. L., and Dougherty, G. J. (1995). To metastasize or not? Selection of CD44 splice sites. Nat. Med. *1*, 635-637.

Culty, M., Miyake, K., Kincade, P. W., Sikorski, E., Butcher, E. C., Underhill, C., and Sikorski, E. (1990). The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins [published erratum appears in J Cell Biol 1991 Feb;112(3):following 513]. J. Cell Biol. *111*, 2765-2774.

Culty, M., Nguyen, H. A., and Underhill, C. B. (1992). The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. J. Cell Biol. *116*, 1055-1062.

D'Amico, A. V., and McKenna, W. G. (1994). Apoptosis and a re-investigation of the biologic basis for cancer therapy. Radiother. Oncol. *33*, 3-10.

Dalchau, R., Kirkley, J., and Fabre, J. W. (1980). Monoclonal antibody to a human leukocyte-specific membrane glycoprotein probably homologous to the leukocyte-common (L-C) antigen of the rat. Eur. J. Immunol. *10*, 737-744.

de Fougerolles, A. R., and Springer, T. A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. *175*, 185-190.

De la Hera, A., Alvarez-Mon, M., Sanchez-Madrid, F., Martinez, C., and Durantez, A. (1988). Co-expression of Mac-1 and p150,95 on CD5+ B cells. Structural and functional characterization in a human chronic lymphocytic leukemia. Eur. J. Immunol. *18*, 1131-1134.

de los Toyos, J., Jalkanen, S., and Butcher, E. C. (1989). Flow cytometric analysis of the Hermes homing-associated antigen on human lymphocyte subsets. Blood *74*, 751-760.

De Rossi, G., Marroni, P., Paganuzzi, M., Mauro, F. R., Tenca, C., Zarcone, D., Velardi, A., Molica, S., and Grossi, C. E. (1997). Increased serum levels of soluble CD44 standard, but not of variant isoforms v5 and v6, in B cell chronic lymphocytic leukemia. Leukemia *11*, 134-141.

DeAngelis, P. L., Jing, W., Drake, R. R., and Achyuthan, A. M. (1998). Identification and molecular cloning of a unique hyaluronan synthase from Pasteurella multocida. J. Biol. Chem. *273*, 8454-8458.

DeAngelis, P. L., Papaconstantinou, J., and Weigel, P. H. (1993a). Isolation of a Streptococcus pyogenes gene locus that directs hyaluronan biosynthesis in acapsular mutants and in heterologous bacteria. J. Biol. Chem. *268*, 14568-14571.

DeAngelis, P. L., Papaconstantinou, J., and Weigel, P. H. (1993b). Molecular cloning, identification, and sequence of the hyaluronan synthase gene from group A Streptococcus pyogenes. J. Biol. Chem. *268*, 19181-19184.

Denning, S. M., Kurtzberg, J., Leslie, D. S., and Haynes, B. F. (1989). Human postnatal CD4- CD8- CD3- thymic T cell precursors differentiate in vitro into T cell receptor delta-bearing cells. J. Immunol. *142*, 2988-2997.

Denning, S. M., Le, P. T., Singer, K. H., and Haynes, B. F. (1990). Antibodies against the CD44 p80, lymphocyte homing receptor molecule augment human peripheral blood T cell activation. J. Immunol. *144*, 7-15.

Detmers, P. A., Wright, S. D., Olsen, E., Kimball, B., and Cohn, Z. A. (1987). Aggregation of complement receptors on human neutrophils in the absence of ligand. J. Cell Biol. *105*, 1137-1145.

Diamond, M. S., Staunton, D. E., Marlin, S. D., and Springer, T. A. (1991). Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell *65*, 961-971.

Docherty, R., Forrester, J. V., Lackie, J. M., and Gregory, D. W. (1989). Glycosaminoglycans facilitate the movement of fibroblasts through three-dimensional collagen matrices. J. Cell Sci. *92*, 263-270.

Doege, K. J., Sasaki, M., Kimura, T., and Yamada, Y. (1991). Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. J. Biol. Chem. *266*, 894-902.

Dougherty, G. J., Cooper, D. L., Memory, J. F., and Chiu, R. K. (1994). Ligand binding specificity of alternatively spliced CD44 isoforms. Recognition and binding of hyaluronan by CD44R1. J. Biol. Chem. *269*, 9074-9078.

Dougherty, G. J., Dougherty, S. T., Dirks, J. F., Chiu, R. K., Peters, C. E., and Droll, A. (1995). Regulation of the functional activity and ligand binding specificity of the adhesion protein CD44. Trends Glycosci. Glycotechnol. **7**, 45-56

Dougherty, G. J., Lansdorp, P. M., Cooper, D. L., and Humphries, R. K. (1991). Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. J. Exp. Med. *174*, 1-5.

Dougherty, G. J., Murdoch, S., and Hogg, N. (1988). The function of human intercellular adhesion molecule-1 (ICAM-1) in the generation of an immune response. Eur J. Immunol. *18*, 35-39.

Dransfield, I., and Hogg, N. (1989). Regulated expression of Mg2+ binding epitope on leukocyte integrin alpha subunits. EMBO J. *8*, 3759-3765.

Droll, A., Dougherty, S. T., Chiu, R. K., Dirks, J. F., McBride, W. H., Cooper, D. L., and Dougherty, G. J. (1995). Adhesive interactions between alternatively spliced CD44 isoforms. J. Biol. Chem. *270*, 11567-11573.

Du, X. P., Plow, E. F., Frelinger, A. L. d., O'Toole, T. E., Loftus, J. C., and Ginsberg, M. H. (1991). Ligands "activate" integrin alpha IIb beta 3 (platelet GPIIb-IIIa). Cell *65*, 409-416.

Dustin, M. L., Singer, K. H., Tuck, D. T., and Springer, T. A. (1988). Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by interferon gamma and is mediated by intercellular adhesion molecule 1 (ICAM-1). J. Exp. Med. *167*, 1323-1340.

Dustin, M. L., and Springer, T. A. (1988). Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. *107*, 321-331.

Dustin, M. L., and Springer, T. A. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Annu. Rev. Immunol. *9*, 27-66.

Dustin, M. L., and Springer, T. A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619-624.

East, J. A., and Hart, I. R. (1993). CD44 and its role in tumour progression and metastasis. Eur. J. Cancer *29A*, 1921-1922.

English, N. M., Lesley, J. F., and Hyman, R. (1998). Site-specific de-N-glycosylation of CD44 can activate hyaluronan binding, and CD44 activation states show distinct threshold densities for hyaluronan binding. Cancer Res. *58*, 3736-3742.

Esford, L. E., Maiti, A., Bader, S. A., Tufaro, F., and Johnson, P. (1998). Analysis of CD44 interactions with hyaluronan in murine L cell fibroblasts deficient in glycosaminoglycan synthesis: a role for chondroitin sulfate. J. Cell Sci. *111*, 1021-1029.

Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R., and McCarthy, J. B. (1992). A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J. Cell Biol. *116*, 521-531.

Fichtner, I., Dehmel, A., Naundorf, H., and Finke, L. H. (1997). Expression of CD44 standard and isoforms in human breast cancer xenografts and shedding of soluble forms into serum of nude mice. Anticancer Res. *17*, 3633-3645.

Fidler, I. J. (1990). Host and tumour factors in cancer metastasis. Eur. J. Clin. Invest. *20*, 481-486.

Fidler, I. J., and Hart, I. R. (1982). Biological diversity in metastatic neoplasms: origins and implications. Science *217*, 998-1003.

Figdor, C. G., van Kooyk, Y., and Keizer, G. D. (1990). On the mode of action of LFA-1. Immunol. Today *11*, 277-280.

Finn, L., Dougherty, G., Finley, G., Meisler, A., Becich, M., and Cooper, D. L. (1994). Alternative splicing of CD44 pre-mRNA in human colorectal tumours. Biochem. Biophys. Res. Commun. *200*, 1015-1022.

Forrester, J. V., and Balazs, E. A. (1980). Inhibition of phagocytosis by high molecular weight hyaluronate. Immunology *40*, 435-446.

Foxall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B. K. (1992). The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. J. Cell Biol. *117*, 895-902.

Fujita, N., Kato, Y., Naito, M., and Tsuruo, T. (1996). A novel anti-Thy-1 (CD90) monoclonal antibody induces apoptosis in mouse malignant T-lymphoma cells in spite of inducing bcl-2 expression. Int. J. Cancer *66*, 544-550.

Fulop, C., Salustri, A., and Hascall, V. C. (1997). Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex. Arch. Biochem. Biophys. *337*, 261-266.

Galandrini, R., Albi, N., Tripodi, G., Zarcone, D., Terenzi, A., Moretta, A., Grossi, C. E., and Velardi, A. (1993). Antibodies to CD44 trigger effector functions of human T cell clones. J. Immunol. *150*, 4225-4235.

Galandrini, R., De Maria, R., Piccoli, M., Frati, L., and Santoni, A. (1994). CD44 triggering enhances human NK cell cytotoxic functions. J. Immunol. *153*, 4399-4407.

Galandrini, R., Galluzzo, E., Albi, N., Grossi, C. E., and Velardi, A. (1994). Hyaluronate is costimulatory for human T cell effector functions and binds to CD44 on activated T cells. J. Immunol. *153*, 21-31.

Gallatin, W. M., Wayner, E. A., Hoffman, P. A., St. John, T., Butcher, E. C., and Carter, W. G. (1989). Structural homology between lymphocyte receptors for high endothelium and class III extracellular matrix receptor. Proc. Natl. Acad. Sci. U S A *86*, 4654-4658.

Gallatin, W. M., Weissman, I. L., and Butcher, E. C. (1983). A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature *304*, 30-34.

Galluzzo, E., Albi, N., Fiorucci, S., Merigiola, C., Ruggeri, L., Tosti, A., Grossi, C. E., and Velardi, A. (1995). Involvement of CD44 variant isoforms in hyaluronate adhesion by human activated T cells. Eur. J. Immunol. *25*, 2932-2939.

Ge, H., Zuo, P., and Manley, J. L. (1991). Primary structure of the human splicing factor ASF reveals similarities with Drosophila regulators. Cell *66*, 373-382.

Geppert, T. D., and Lipsky, P. E. (1991). Association of various T cell-surface molecules with the cytoskeleton. Effect of cross-linking and activation. J. Immunol. *146*, 3298-3305.

Ghaffari, S., Dougherty, G. J., Eaves, A. C., and Eaves, C. J. (1996). Altered patterns of CD44 epitope expression in human chronic and acute myeloid leukemia. Leukemia *10*, 1773-1781.

Ghaffari, S., Dougherty, G. J., Lansdorp, P. M., Eaves, A. C., and Eaves, C. J. (1995). Differentiation-associated changes in CD44 isoform expression during normal hematopoiesis and their alteration in chronic myeloid leukemia. Blood *86*, 2976-2985.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell *23*, 175-182.

Goldstein, L. A., Zhou, D. F., Picker, L. J., Minty, C. N., Bargatze, R. F., Ding, J. F., and Butcher, E. C. (1989). A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell *56*, 1063-1072.

Graham, I. L., and Brown, E. J. (1991). Extracellular calcium results in a conformational change in Mac-1 (CD11b/CD18) on neutrophils. Differentiation of adhesion and phagocytosis functions of Mac-1. J. Immunol. *146*, 685-691.

Grumont, R. J., and Gerondakis, S. (1994). Alternative splicing of RNA transcripts encoded by the murine p105 NF-kappa B gene generates I kappa B gamma isoforms with different inhibitory activities. Proc. Natl. Acad. Sci. U S A *91*, 4367-4371.

Gunthert, U. (1993). CD44: a multitude of isoforms with diverse functions. Curr. Top. Microbiol. Immunol. *184*, 47-63.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell *65*, 13-24.

Gunthert, U., Stauder, R., Mayer, B., Terpe, H. J., Finke, L., and Friedrichs, K. (1995). Are CD44 variant isoforms involved in human tumour progression? Cancer Surv. *24*, 19-42.

Guo, Y., Wu, Y., Shinde, S., Sy, M. S., Aruffo, A., and Liu, Y. (1996). Identification of a costimulatory molecule rapidly induced by CD40L as CD44H. J. Exp. Med. *184*, 955-961.

Guo, Y. J., Liu, G., Wang, X., Jin, D., Wu, M., Ma, J., and Sy, M. S. (1994). Potential use of soluble CD44 in serum as indicator of tumour burden and metastasis in patients with gastric or colon cancer. Cancer Res. *54*, 422-426.

Haegel, H., and Ceredig, R. (1991). Transcripts encoding mouse CD44 (Pgp-1, Ly-24) antigen: strain variation and induction by mitogen. Eur. J. Immunol. *21*, 1549-1553.

Hakomori, S. (1996). Tumour malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. Cancer Res. *56*, 5309-5318.

Hall, C. L., Wang, C., Lange, L. A., and Turley, E. A. (1994). Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. J. Cell Biol. *126*, 575-88.

Hanaoka, K., Fujita, N., Lee, S. H., Seimiya, H., Naito, M., and Tsuruo, T. (1995). Involvement of CD45 in adhesion and suppression of apoptosis of mouse malignant T-lymphoma cells. Cancer Res. *55*, 2186-2190.

Hardwick, C., Hoare, K., Owens, R., Hohn, H. P., Hook, M., Moore, D., Cripps, V., Austen, L., Nance, D. M., and Turley, E. A. (1992). Molecular cloning of a novel hyaluronan receptor that mediates tumour cell motility [published erratum appears in J Cell Biol 1992 Aug;118(3):753]. J. Cell Biol. *117*, 1343-1350.

Harn, H. J., Ho, L. I., Shyu, R. Y., Yuan, J. S., Lin, F. G., Young, T. H., Liu, C. A., Tang, H. S., and Lee, W. H. (1996). Soluble CD44 isoforms in serum as potential markers of metastatic gastric carcinoma. J. Clin. Gastroenterol. *22*, 107-110.

Hathcock, K. S., Hirano, H., Murakami, S., and Hodes, R. J. (1993). CD44 expression on activated B cells. Differential capacity for CD44-dependent binding to hyaluronic acid. J. Immunol. *151*, 6712-6722.

Haynes, B. F., Harden, E. A., Telen, M. J., Hemler, M. E., Strominger, J. L., Palker, T. J., Scearce, R. M., and Eisenbarth, G. S. (1983). Differentiation of human T lymphocytes. I. Acquisition of a novel human cell surface protein (p80) during normal intrathymic T cell maturation. J. Immunol. *131*, 1195-1200.

Haynes, B. F., Telen, M. J., Hale, L. P., and Denning, S. M. (1989). CD44--a molecule involved in leukocyte adherence and T-cell activation [published erratum appears in Immunol Today 1990 Mar;11(3):80]. Immunol. Today *10*, 423-428.

He, Q., Lesley, J., Hyman, R., Ishihara, K., and Kincade, P. W. (1992). Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. J. Cell Biol. *119*, 1711-1719.

Helander, T. S., Carpen, O., Turunen, O., Kovanen, P. E., Vaheri, A., and Timonen, T. (1996). ICAM-2 redistributed by ezrin as a target for killer cells. Nature *382*, 265-268.

Heldin, P., Laurent, T. C., and Heldin, C. H. (1989). Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. Biochem. J. *258*, 919-922.

Herrlich, P., Zoller, M., Pals, S. T., and Ponta, H. (1993). CD44 splice variants: metastases meet lymphocytes. Immunol. Today *14*, 395-399.

Hibbs, M. L., Xu, H., Stacker, S. A., and Springer, T. A. (1991). Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. Science *251*, 1611-1613.

Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., Tsukita, S., and Tsukita, S. (1996). Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. J. Cell Biol. *135*, 37-51.

Horst, E., Meijer, C. J., Duijvestijn, A. M., Hartwig, N., Van der Harten, H. J., and Pals, S. T. (1990). The ontogeny of human lymphocyte recirculation: high endothelial cell antigen (HECA-452) and CD44 homing receptor expression in the development of the immune system. Eur. J. Immunol. *20*, 1483-1489.

Horst, E., Meijer, C. J., Radaszkiewicz, T., Ossekoppele, G. J., Van Krieken, J. H., and Pals, S. T. (1990). Adhesion molecules in the prognosis of diffuse large-cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA-1 (CD11a/18), and ICAM-1 (CD54). Leukemia *4*, 595-599.

Huet, S., Groux, H., Caillou, B., Valentin, H., Prieur, A. M., and Bernard, A. (1989). CD44 contributes to T cell activation. J. Immunol. *143*, 798-801.

Hughes, P. F., Thacker, J. D., Hogge, D., Sutherland, H. J., Thomas, T. E., Lansdorp, P. M., Eaves, C. J., and Humphries, R. K. (1992). Retroviral gene transfer to primitive normal and leukemic hematopoietic cells using clinically applicable procedures. J. Clin. Invest. *89*, 1817-1824.

Hyman, R., Lesley, J., and Schulte, R. (1991). Somatic cell mutants distinguish CD44 expression and hyaluronic acid binding. Immunogenetics *33*, 392-395.

Hynes, R. O. (1991). The complexity of platelet adhesion to extracellular matrices. Throm. Haemost. *66*, 40-43.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Idzerda, R. L., Carter, W. G., Nottenburg, C., Wayner, E. A., Gallatin, W. M., and St. John, T. (1989). Isolation and DNA sequence of a cDNA clone encoding a lymphocyte adhesion receptor for high endothelium. Proc. Natl. Acad. Sci. U S A *86*, 4659-4663.

Itano, N., and Kimata, K. (1996). Expression cloning and molecular characterization of HAS protein, a eukaryotic hyaluronan synthase. J. Biol. Chem. *271*, 9875-9878.

Itano, N., and Kimata, K. (1996). Molecular cloning of human hyaluronan synthase. Biochem. Biophys. Res. Commun. *222*, 816-820.

Jackson, D. G., Buckley, J., and Bell, J. I. (1992). Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain. J. Biol. Chem. *267*, 4732-4739.

Jackson, J. A., Friberg, A. C., and Bahr, J. M. (1991). Preovulatory changes in glycosaminoglycans and collagen content in the stigma region of the follicle of the domestic hen. Biol. Reprod. *45*, 301-307.

Jalkanen, S., Bargatze, R. F., de los Toyos, J., and Butcher, E. C. (1987). Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. J. Cell Biol. *105*, 983-990.

Jalkanen, S., Jalkanen, M., Bargatze, R., Tammi, M., and Butcher, E. C. (1988). Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. J. Immunol. *141*, 1615-1623.

Jalkanen, S., Joensuu, H., Soderstrom, K. O., and Klemi, P. (1991). Lymphocyte homing and clinical behavior of non-Hodgkin's lymphoma. J. Clin. Invest. *87*, 1835-1840.

Jalkanen, S. T., Bargatze, R. F., Herron, L. R., and Butcher, E. C. (1986). A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. Eur. J. Immunol. *16*, 1195-1202.

Julius, M. H., Simpson, E., and Herzenberg, L. A. (1973). A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. *3*, 645-649.

Kalomiris, E. L., and Bourguignon, L. Y. (1989). Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85, and may function in GP85-ankyrin binding. J. Biol. Chem. *264*, 8113-8119.

Kalomiris, E. L., and Bourguignon, L. Y. (1988). Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. J. Cell Biol. *106*, 319-327.

Kan, M., Kanayama, H., Naruo, S., Tsuji, M., Kojima, K., Kurokawa, Y., and Kagawa, S. (1996). Serological evaluation of soluble CD44 in renal cancer. Jpn. J. Cancer Res. *87*, 1191-1194.

Kansas, G. S., and Dailey, M. O. (1989). Expression of adhesion structures during B cell development in man. J. Immunol. *142*, 3058-3062.

Kansas, G. S., Muirhead, M. J., and Dailey, M. O. (1990). Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. Blood *76*, 2483-2492.

Kansas, G. S., Wood, G. S., and Dailey, M. O. (1989). A family of cell-surface glycoproteins defined by a putative anti-endothelial cell receptor antibody in man. J. Immunol. *142*, 3050-3057.

Katoh, S., McCarthy, J. B., and Kincade, P. W. (1994). Characterization of soluble CD44 in the circulation of mice. Levels are affected by immune activity and tumour growth. J. Immunol. *153*, 3440-3449.

Katoh, S., Zheng, Z., Oritani, K., Shimozato, T., and Kincade, P. W. (1995). Glycosylation of CD44 negatively regulates its recognition of hyaluronan. J. Exp. Med. *182*, 419-429.

Keizer, G. D., Visser, W., Vliem, M., and Figdor, C. G. (1988). A monoclonal antibody (NKI-L16) directed against a unique epitope on the alpha-chain of human leukocyte function-associated antigen 1 induces homotypic cell-cell interactions. J. Immunol. *140*, 1393-1400.

Kerbel, R. S., Rak, J., Kobayashi, H., Man, M. S., St Croix, B., and Graham, C. H. (1994). Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumours. Cold Spring Harb. Symp. Quant. Biol. *59*, 661-672.

Kittl, E. M., Haberhauer, G., Ruckser, R., Selleny, S., Rech-Weichselbraun, I., Hinterberger, W., and Bauer, K. (1997). Serum levels of soluble CD44 variant isoforms are elevated in rheumatoid arthritis. Rheumatol. Int. *16*, 181-186.

Knudson, C. B. (1993). Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. J. Cell Biol. *120*, 825-834.

Knudson, C. B., and Knudson, W. (1990). Similar epithelial-stromal interactions in the regulation of hyaluronate production during limb morphogenesis and tumour invasion. Cancer Lett. *52*, 113-122.

Knudson, W., Bartnik, E., and Knudson, C. B. (1993). Assembly of pericellular matrices by COS-7 cells transfected with CD44 lymphocyte-homing receptor genes. Proc. Natl. Acad. Sci. U S A *90*, 4003-4007.

Knudson, W., and Knudson, C. B. (1991). Assembly of a chondrocyte-like pericellular matrix on non-chondrogenic cells. Role of the cell surface hyaluronan receptors in the assembly of a pericellular matrix. J. Cell Sci. *99*, 227-235.

Kohda, D., Morton, C. J., Parkar, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and Day, A. J. (1996). Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. Cell *86*, 767-775.

Koopman, G., Griffioen, A. W., Ponta, H., Herrlich, P., van den Berg, F., Manten-Horst, E., and Pals, S. T. (1993). CD44 splice variants; expression on lymphocytes and in neoplasia. Res. Immunol. *144*, 750-4; discussion 754-762.

Koopman, G., van Kooyk, Y., de Graaff, M., Meyer, C. J., Figdor, C. G., and Pals, S. T. (1990). Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through the LFA-1 pathway. J. Immunol. *145*, 3589-3593.

Kumari, K., and Weigel, P. H. (1997). Molecular cloning, expression, and characterization of the authentic hyaluronan synthase from group C Streptococcus equisimilis. J. Biol. Chem. *272*, 32539-32546.

Lacy, B. E., and Underhill, C. B. (1987). The hyaluronate receptor is associated with actin filaments. J. Cell Biol. *105*, 1395-1404.

Laker, C., Stocking, C., Bergholz, U., Hess, N., De Lamarter, J. F., and Ostertag, W. (1987). Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway. Proc Natl Acad Sci U S A *84*, 8458-8462.

Lamb, R. F., Hennigan, R. F., Turnbull, K., Katsanakis, K. D., MacKenzie, E. D., Birnie, G. D., and Ozanne, B. W. (1997). AP-1-mediated invasion requires increased expression of the hyaluronan receptor CD44. Mol. Cell. Biol. *17*, 963-976.

Lasky, L. A., Singer, M. S., Yednock, T. A., Dowbenko, D., Fennie, C., Rodriguez, H., Nguyen, T., Stachel, S., and Rosen, S. D. (1989). Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell *56*, 1045-1055.

Laurent, T. C., and Fraser, J. R. (1992). Hyaluronan. FASEB J. *6*, 2397-2404.

Lee, T. H., Klampfer, L., Shows, T. B., and Vilcek, J. (1993). Transcriptional regulation of TSG6, a tumour necrosis factor- and interleukin-1-inducible primary response gene coding for a secreted hyaluronan-binding protein. J. Biol. Chem. *268*, 6154-6160.

Legras, S., Levesque, J. P., Charrad, R., Morimoto, K., Le Bousse, C., Clay, D., Jasmin, C., and Smadja-Joffe, F. (1997). CD44-mediated adhesiveness of human hematopoietic progenitors to hyaluronan is modulated by cytokines. Blood *89*, 1905-1914.

Lesley, J., English, N., Perschl, A., Gregoroff, J., and Hyman, R. (1995). Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. J. Exp. Med. *182*, 431-437.

Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., and Kincade, P. W. (1992). Requirements for hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody. J. Exp. Med. *175*, 257-266.

Lesley, J., Howes, N., Perschl, A., and Hyman, R. (1994). Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. J. Exp. Med. *180*, 383-387.

Lesley, J., and Hyman, R. (1992). CD44 can be activated to function as an hyaluronic acid receptor in normal murine T cells. Eur. J. Immunol. *22*, 2719-2723.

Lesley, J., and Hyman, R. (1998). CD44 structure and function. Front. Biosci. *3*, D616-630.

Lesley, J., Hyman, R., and Kincade, P. W. (1993). CD44 and its interaction with extracellular matrix. Adv. Immunol. *54*, 271-335.

Lesley, J., Kincade, P. W., and Hyman, R. (1993). Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody. Eur. J. Immunol. *23*, 1902-1909.

Lesley, J., Schulte, R., and Hyman, R. (1990). Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. Exp. Cell Res. *187*, 224-233.

Liao, H. X., Levesque, M. C., Patton, K., Bergamo, B., Jones, D., Moody, M. A., Telen, M. J., and Haynes, B. F. (1993). Regulation of human CD44H and CD44E isoform binding to hyaluronan by phorbol myristate acetate and anti-CD44 monoclonal and polyclonal antibodies. J. Immunol. *151*, 6490-6499.

Lindahl, U., and Hook, M. (1978). Glycosaminoglycans and their binding to biological macromolecules. Annu. Rev. Biochem. *47*, 385-417.

Liu, D., and Sy, M. S. (1996). A cysteine residue located in the transmembrane domain of CD44 is important in binding of CD44 to hyaluronic acid. J. Exp. Med. *183*, 1987-1994.

Liu, D., and Sy, M. S. (1997). Phorbol myristate acetate stimulates the dimerization of CD44 involving a cysteine in the transmembrane domain. J. Immunol. *159*, 2702-2711.

Liu, D., Zhang, D., Mori, H., and Sy, M. S. (1996). Binding of CD44 to hyaluronic acid can be induced by multiple signals and requires the CD44 cytoplasmic domain. Cell. Immunol. *174*, 73-83.

Lokeshwar, V. B., and Bourguignon, L. Y. (1992). The lymphoma transmembrane glycoprotein GP85 (CD44) is a novel guanine nucleotide-binding protein which regulates GP85 (CD44)-ankyrin interaction. J. Biol. Chem. *267*, 22073-22078.

Lokeshwar, V. B., and Bourguignon, L. Y. (1991). Post-translational protein modification and expression of ankyrin-binding site(s) in GP85 (Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biosynthesis. J. Biol. Chem. *266*, 17983-17989.

Lokeshwar, V. B., Fregien, N., and Bourguignon, L. Y. (1994). Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J. Cell Biol. *126*, 1099-1109.

Lynch, F., and Ceredig, R. (1989). Mouse strain variation in Ly-24 (Pgp-1) expression by peripheral T cells and thymocytes: implications for T cell differentiation. Eur. J. Immunol. *19*, 223-229.

Mackay, C. R., Terpe, H. J., Stauder, R., Marston, W. L., Stark, H., and Gunthert, U. (1994). Expression and modulation of CD44 variant isoforms in humans. J. Cell Biol. *124*, 71-82.

Manabe, A., Murti, K. G., Coustan-Smith, E., Kumagai, M., Behm, F. G., Raimondi, S. C., and Campana, D. (1994). Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells. Blood *83*, 758-766.

Mann, D. M., Yamaguchi, Y., Bourdon, M. A., and Ruoslahti, E. (1990). Analysis of glycosaminoglycan substitution in decorin by site-directed mutagenesis. J. Biol. Chem. *265*, 5317-5323.

Markowitz, D., Goff, S., and Bank, A. (1988). A safe packaging line for gene transfer: separating viral genes on two different plasmids. J. Virol. *62*, 1120-1124.

Marlin, S. D., and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813-819.

Martin, S., Jansen, F., Bokelmann, J., and Kolb, H. (1997). Soluble CD44 splice variants in metastasizing human breast cancer. Int. J. Cancer *74*, 443-445.

Matsumura, Y., Sugiyama, M., Matsumura, S., Hayle, A. J., Robinson, P., Smith, J. C., and Tarin, D. (1995). Unusual retention of introns in CD44 gene transcripts in bladder cancer provides new diagnostic and clinical oncological opportunities. J. Pathol. *177*, 11-20.

McBride, W. H., Dougherty, G. D., Wallis, A. E., Economou, J. S., and Chiang, C. S. (1994). Interleukin-3 in gene therapy of cancer. Folia Biol. *40*, 62-73.

McBride, W. H., Thacker, J. D., Comora, S., Economou, J. S., Kelley, D., Hogge, D., Dubinett, S. M., and Dougherty, G. J. (1992). Genetic modification of a murine fibrosarcoma to produce interleukin 7 stimulates host cell infiltration and tumour immunity. Cancer Res. *52*, 3931-3937.

McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C., and Noble, P. W. (1996). Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. J. Clin. Invest. *98*, 2403-2413.

McNeil, J. D., Wiebkin, O. W., Betts, W. H., and Cleland, L. G. (1985). Depolymerisation products of hyaluronic acid after exposure to oxygen-derived free radicals. Ann. Rheum. Dis. *44*, 780-789.

Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. Mol. Biol. Cell *4*, 953-961.

Meyer, M. F., and Kreil, G. (1996). Cells expressing the DG42 gene from early Xenopus embryos synthesize hyaluronan. Proc. Natl. Acad. Sci. U S A *93*, 4543-4547.

Miller, J., Hatch, J. A., Simonis, S., and Cullen, S. E. (1988). Identification of the glycosaminoglycan-attachment site of mouse invariant-chain proteoglycan core protein by site-directed mutagenesis. Proc. Natl. Acad. Sci. U S A *85*, 1359-1363.

Miller, J., Knorr, R., Ferrone, M., Houdei, R., Carron, C. P., and Dustin, M. L. (1995). Intercellular adhesion molecule-1 dimerization and its consequences for adhesion mediated by lymphocyte function associated-1. J Exp Med *182*, 1231-1241.

Miyake, K., Medina, K. L., Hayashi, S., Ono, S., Hamaoka, T., and Kincade, P. W. (1990). Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. J. Exp. Med. *171*, 477-488.

Miyake, K., Underhill, C. B., Lesley, J., and Kincade, P. W. (1990). Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J. Exp. Med. *172*, 69-75.

Murakami, S., Shimabukuro, Y., Miki, Y., Saho, T., Hino, E., Kasai, D., Nozaki, T., Kusumoto, Y., and Okada, H. (1994). Inducible binding of human lymphocytes to hyaluronate via CD44 does not require cytoskeleton association but does require new protein synthesis. J. Immunol. *152*, 467-477.

Naor, D., Sionov, R. V., and Ish-Shalom, D. (1997). CD44: structure, function, and association with the malignant process. Adv. Cancer Res. *71*, 241-319.

Naujokas, M. F., Morin, M., Anderson, M. S., Peterson, M., and Miller, J. (1993). The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. Cell *74*, 257-268.

Neame, P. J., and Barry, F. P. (1993). The link proteins. Experientia *49*, 393-402.

Neame, S. J., and Isacke, C. M. (1992). Phosphorylation of CD44 in vivo requires both Ser323 and Ser325, but does not regulate membrane localization or cytoskeletal interaction in epithelial cells. EMBO J. *11*, 4733-4738.

Neame, S. J., Uff, C. R., Sheikh, H., Wheatley, S. C., and Isacke, C. M. (1995). CD44 exhibits a cell type dependent interaction with triton X-100 insoluble, lipid rich, plasma membrane domains. J. Cell Sci. *108*, 3127-3135.

Neugebauer, K. M., and Reichardt, L. F. (1991). Cell-surface regulation of beta 1-integrin activity on developing retinal neurons. Nature *350*, 68-71.

Ng, K. F., and Schwartz, N. B. (1989). Solubilization and partial purification of hyaluronate synthetase from oligodendroglioma cells. J. Biol. Chem. *264*, 11776-11783.

Nottenburg, C., Rees, G., and St. John, T. (1989). Isolation of mouse CD44 cDNA: structural features are distinct from the primate cDNA. Proc. Natl. Acad. Sci. U S A *86*, 8521-8525.

O'Toole, T. E., Loftus, J. C., Du, X. P., Glass, A. A., Ruggeri, Z. M., Shattil, S. J., Plow, E. F., and Ginsberg, M. H. (1990). Affinity modulation of the alpha IIb beta 3 integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. Cell Regul. *1*, 883-893.

O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F., and Ginsberg, M. H. (1991). Modulation of the affinity of integrin alpha IIb beta 3 (GPIIb-IIIa) by the cytoplasmic domain of alpha IIb. Science *254*, 845-847.

Oertli, B., Fan, X., and Wuthrich, R. P. (1998). Characterization of CD44-mediated hyaluronan binding by renal tubular epithelial cells. Nephrol. Dial. Transplant. *13*, 271-278.

Oppenheimer-Marks, N., Davis, L. S., and Lipsky, P. E. (1990). Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. J. Immunol. *145*, 140-148.

Pals, S. T., Hogervorst, F., Keizer, G. D., Thepen, T., Horst, E., and Figdor, C. C. (1989). Identification of a widely distributed 90-kDa glycoprotein that is homologous to the Hermes-1 human lymphocyte homing receptor. J. Immunol. *143*, 851-857.

Pals, S. T., Horst, E., Ossekoppele, G. J., Figdor, C. G., Scheper, R. J., and Meijer, C. J. (1989). Expression of lymphocyte homing receptor as a mechanism of dissemination in non-Hodgkin's lymphoma. Blood *73*, 885-888.

Pals, S. T., Horst, E., Scheper, R. J., and Meijer, C. J. (1989). Mechanisms of human lymphocyte migration and their role in the pathogenesis of disease. Immunol. Rev. *108*, 111-133.

Parish, C. R., Recny, M. A., Knoppers, M. H., Waldron, J. C., and Warren, H. S. (1993). Detection of a glycosylation-dependent ligand for the T lymphocyte cell adhesion molecule CD2 using a novel multimeric recombinant CD2-binding assay. J. Immunol. *150*, 4833-4843.

Park, J. R. (1996). Cytokine regulation of apoptosis in hematopoietic precursor cells. Curr. Opin. Hematol. *3*, 191-196.

Paul-Eugene, N., Kolb, J. P., Abadie, A., Gordon, J., Delespesse, G., Sarfati, M., Mencia-Huerta, J. M., Braquet, P., and Dugas, B. (1992). Ligation of CD23 triggers cAMP generation and release of inflammatory mediators in human monocytes. J. Immunol. *149*, 3066-3071.

Peach, R. J., Hollenbaugh, D., Stamenkovic, I., and Aruffo, A. (1993). Identification of hyaluronic acid binding sites in the extracellular domain of CD44. J. Cell Biol. *122*, 257-264.

Peck, D., and Isacke, C. M. (1996). CD44 phosphorylation regulates melanoma cell and fibroblast migration on, but not attachment to, a hyaluronan substratum. Curr. Biol. *6*, 884-890.

Peck, D., and Isacke, C. M. (1998). Hyaluronan-dependent cell migration can be blocked by a CD44 cytoplasmic domain peptide containing a phosphoserine at position 325. J. Cell Sci. *111*, 1595-1601.

Perschl, A., Lesley, J., English, N., Hyman, R., and Trowbridge, I. S. (1995). Transmembrane domain of CD44 is required for its detergent insolubility in fibroblasts. J. Cell Sci. *108*, 1033-1041.

Perschl, A., Lesley, J., English, N., Trowbridge, I., and Hyman, R. (1995). Role of CD44 cytoplasmic domain in hyaluronan binding. Eur. J. Immunol. *25*, 495-501.

Pesando, J. M., Hoffman, P., and Conrad, T. (1986). Malignant human B cells express two populations of p24 surface antigens. J. Immunol. *136*, 2709-2714.

Picker, L. J., and Butcher, E. C. (1992). Physiological and molecular mechanisms of lymphocyte homing. Annu. Rev. Immunol. *10*, 561-591.

Picker, L. J., Nakache, M., and Butcher, E. C. (1989). Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. J. Cell Biol. *109*, 927-937.

Pierres, A., Lipcey, C., Mawas, C., and Olive, D. (1992). A unique CD44 monoclonal antibody identifies a new T cell activation pathway. Eur. J. Immunol. *22*, 413-417.

Poenie, M., Tsien, R. Y., and Schmitt-Verhulst, A. M. (1987). Sequential activation and lethal hit measured by [Ca2+]i in individual cytolytic T cells and targets. EMBO J. *6*, 2223-2232.

Prehm, P. (1989). Identification and regulation of the eukaryotic hyaluronate synthase. Ciba Found. Symp. *143*, 21-30; discussion 30-40, 281-285.

Pullman, W. E., and Bodmer, W. F. (1993). Cloning and characterization of a gene that regulates cell adhesion. Nature *361*, 564.

Pullman, W. E., and Bodmer, W. F. (1992). Cloning and characterization of a gene that regulates cell adhesion [published erratum appears in Nature 1993 Feb 11;361(6412):564]. Nature *356*, 529-532.

Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995). Alternative reading frames of the INK4a tumour suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell *83*, 993-1000.

Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. (1994). Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J. Cell Biol. *127*, 537-546.

Recny, M. A., Luther, M. A., Knoppers, M. H., Neidhardt, E. A., Khandekar, S. S., Concino, M. F., Schimke, P. A., Francis, M. A., Moebius, U., Reinhold, B. B., and *et al.* (1992). N-glycosylation is required for human CD2 immunoadhesion functions. J. Biol. Chem. *267*, 22428-22434.

Ristamaki, R., Joensuu, H., Gron-Virta, K., Salmi, M., and Jalkanen, S. (1997). Origin and function of circulating CD44 in non-Hodgkin's lymphoma. J. Immunol. *158*, 3000-3008.

Ristamaki, R., Joensuu, H., Salmi, M., and Jalkanen, S. (1994). Serum CD44 in malignant lymphoma: an association with treatment response. Blood *84*, 238-243.

Roden, L., Campbell, P., Fraser, J. R., Laurent, T. C., Pertoft, H., and Thompson, J. N. (1989). Enzymic pathways of hyaluronan catabolism. Ciba Found. Symp. *143*, 60-76; discussion 76-86, 281-285.

Rothman, B. L., Blue, M. L., Kelley, K. A., Wunderlich, D., Mierz, D. V., and Aune, T. M. (1991). Human T cell activation by OKT3 is inhibited by a monoclonal antibody to CD44. J. Immunol. *147*, 2493-2499.

Rothman, B. L., Kennure, N., Kelley, K. A., Katz, M., and Aune, T. M. (1993). Elevation of intracellular cAMP in human T lymphocytes by an anti-CD44 mAb. J. Immunol. *151*, 6036-60342.

Saari, H., Konttinen, Y. T., and Nordstrom, D. (1991). Effect of joint mobilization on serum hyaluronate. Ann. Med. *23*, 29-32.

Sachs, L. (1996). The control of hematopoiesis and leukemia: from basic biology to the clinic. Proc. Natl. Acad. Sci. U S A *93*, 4742-4749.

Salles, G., Zain, M., Jiang, W. M., Boussiotis, V. A., and Shipp, M. A. (1993). Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas: characterization and comparison with normal activated B cells and epithelial malignancies. Blood *82*, 3539-3547.

Sampson, P. M., Rochester, C. L., Freundlich, B., and Elias, J. A. (1992). Cytokine regulation of human lung fibroblast hyaluronan (hyaluronic acid) production. Evidence for cytokine-regulated hyaluronan (hyaluronic acid) degradation and human lung fibroblast-derived hyaluronidase. J. Clin. Invest. *90*, 1492-1503.

Sant, A. J., Cullen, S. E., Giacoletto, K. S., and Schwartz, B. D. (1985). Invariant chain is the core protein of the Ia-associated chondroitin sulfate proteoglycan. J. Exp. Med. *162*, 1916-1934.

Sant, A. J., Cullen, S. E., and Schwartz, B. D. (1985). Biosynthetic relationships of the chondroitin sulfate proteoglycan with Ia and invariant chain glycoproteins. J. Immunol. *135*, 416-422.

Schirrmacher, V. (1985). Cancer metastasis: experimental approaches, theoretical concepts, and impacts for treatment strategies. Adv. Cancer Res. *43*, 1-73.

Schmits, R., Filmmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., Furlonger, C., Zakarian, A., Simard, J. J. L., Ohashi, P. S., Paige, C. J., Gutierrez-Ramos, J. C., Mak, T. W. (1997). CD44 Regulates Hematopoietic Progenitor Distribution, Granuloma Formation, and tumorigenicity. Blood *90*, 2217-2233.

Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc. Natl. Acad. Sci. U S A *89*, 12160-12164.

Seiter, S., Arch, R., Reber, S., Komitowski, D., Hofmann, M., Ponta, H., Herrlich, P., Matzku, S., and Zoller, M. (1993). Prevention of tumour metastasis formation by anti-variant CD44. J. Exp. Med. *177*, 443-455.

Seth, A., Gote, L., Nagarkatti, M., and Nagarkatti, P. S. (1991). T-cell-receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp90MEL-14. Proc. Natl. Acad. Sci. U S A *88*, 7877-7881.

Sham, M. H., Hunt, P., Nonchev, S., Papalopulu, N., Graham, A., Boncinelli, E., and Krumlauf, R. (1992). Analysis of the murine Hox-2.7 gene: conserved alternative transcripts with differential distributions in the nervous system and the potential for shared regulatory regions. EMBO J. *11*, 1825-1836.

Shattil, S. J., and Brass, L. F. (1987). Induction of the fibrinogen receptor on human platelets by intracellular mediators. J. Biol. Chem. *262*, 992-1000.

Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985). Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. J. Biol. Chem. *260*, 11107-11114.

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. Immunol. Today *13*, 106-112.

Shimizu, Y., Van Seventer, G. A., Siraganian, R., Wahl, L., and Shaw, S. (1989). Dual role of the CD44 molecule in T cell adhesion and activation. J. Immunol. *143*, 2457-2463.

Shtivelman, E., and Bishop, J. M. (1991). Expression of CD44 is repressed in neuroblastoma cells. Mol. Cell. Biol. *11*, 5446-5453.

Shyjan, A. M., Heldin, P., Butcher, E. C., Yoshino, T., and Briskin, M. J. (1996). Functional cloning of the cDNA for a human hyaluronan synthase. J. Biol. Chem. *271*, 23395-23399.

Skelton, T. P., Zeng, C., Nocks, A., and Stamenkovic, I. (1998). Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. J. Cell Biol. *140*, 431-446.

Sleeman, J., Moll, J., Sherman, L., Dall, P., Pals, S. T., Ponta, H., and Herrlich, P. (1995). The role of CD44 splice variants in human metastatic cancer. Ciba Found. Symp. *189*, 142-151; discussion 151-156, 174-176.

Sleeman, J., Rudy, W., Hofmann, M., Moll, J., Herrlich, P., and Ponta, H. (1996). Regulated clustering of variant CD44 proteins increases their hyaluronate binding capacity. J. Cell Biol. *135*, 1139-1150.

Sleeman, J. P., Rahmsdorf, U., Steffen, A., Ponta, H., and Herrlich, P. (1998). CD44 variant exon v5 encodes a tyrosine that is sulphated. Eur. J. Biochem. *255*, 74-80.

Spangrude, G. J. (1989). Enrichment of murine haemopoietic stem cells: diverging roads. Immunol. Today *10*, 344-350.

Spertini, O., Kansas, G. S., Munro, J. M., Griffin, J. D., and Tedder, T. F. (1991). Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. Nature *349*, 691-694.

Spicer, A. P., Augustine, M. L., and McDonald, J. A. (1996). Molecular cloning and characterization of a putative mouse hyaluronan synthase. J. Biol. Chem. *271*, 23400-23406.

Spicer, A. P., Olson, J. S., and McDonald, J. A. (1997). Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. J. Biol. Chem. *272*, 8957-8961.

Springer, T. A. (1990). Adhesion receptors of the immune system. Nature *346*, 425-434.

St. John, T., Meyer, J., Idzerda, R., and Gallatin, W. M. (1990). Expression of CD44 confers a new adhesive phenotype on transfected cells. Cell *60*, 45-52.

Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1989). A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell *56*, 1057-1062.

Stamenkovic, I., Aruffo, A., Amiot, M., and Seed, B. (1991). The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J. *10*, 343-348.

Stamper, H. B., Jr., and Woodruff, J. J. (1976). Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. J. Exp. Med. *144*, 828-833.

Staunton, D. E., Dustin, M. L., and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61-64.

Stoolman, L. M. (1989). Adhesion molecules controlling lymphocyte migration. Cell *56*, 907-910.

Sugimoto, K., Tsurumaki, Y., Hoshi, H., Kadowaki, S., LeBousse-Kerdiles, M. C., Smadja-Joffe, F., and Mori, K. J. (1994). Effects of anti-CD44 monoclonal antibody on adhesion of erythroid leukemic cells (ELM-I-1) to hematopoietic supportive cells (MS-5): CD44, but not hyaluronate-mediated, cell-cell adhesion. Exp. Hematol. *22*, 488-494.

Swain, S. L., Weinberg, A. D., and English, M. (1990). CD4+ T cell subsets. Lymphokine secretion of memory cells and of effector cells that develop from precursors in vitro. J. Immunol. *144*, 1788-1799.

Sy, M. S., Guo, Y. J., and Stamenkovic, I. (1991). Distinct effects of two CD44 isoforms on tumour growth in vivo. J. Exp. Med. *174*, 859-866.

Sy, M. S., Guo, Y. J., and Stamenkovic, I. (1992). Inhibition of tumour growth in vivo with a soluble CD44-immunoglobulin fusion protein. J. Exp. Med. *176*, 623-627.

Takahashi, K., Stamenkovic, I., Cutler, M., Dasgupta, A., and Tanabe, K. K. (1996). Keratan sulfate modification of CD44 modulates adhesion to hyaluronate. J. Biol. Chem. *271*, 9490-9496.

Takeuchi, K., Yamaguchi, A., Urano, T., Goi, T., Nakagawara, G., and Shiku, H. (1995). Expression of CD44 variant exons 8-10 in colorectal cancer and its relationship to metastasis. Jpn. J. Cancer Res. *86*, 292-297.

Tammi, R., Saamanen, A. M., Maibach, H. I., and Tammi, M. (1991). Degradation of newly synthesized high molecular mass hyaluronan in the epidermal and dermal compartments of human skin in organ culture. J. Invest. Dermatol. *97*, 126-130.

Tan, P. H., Santos, E. B., Rossbach, H. C., and Sandmaier, B. M. (1993). Enhancement of natural killer activity by an antibody to CD44. J. Immunol. *150*, 812-820.

Tanabe, K. K., and Saya, H. (1994). The CD44 adhesion molecule and metastasis. Crit. Rev. Oncog. *5*, 201-212.

Thomas, L., Byers, H. R., Vink, J., and Stamenkovic, I. (1992). CD44H regulates tumour cell migration on hyaluronate-coated substrate. J. Cell Biol. *118*, 971-977.

Toole, B. P. (1990). Hyaluronan and its binding proteins, the hyaladherins. Curr. Opin. Cell Biol. *2*, 839-844.

Toyama-Sorimachi, N., Miyake, K., and Miyasaka, M. (1993). Activation of CD44 induces ICAM-1/LFA-1-independent, Ca2+, Mg(2+)-independent adhesion pathway in lymphocyte-endothelial cell interaction. Eur. J. Immunol. *23*, 439-446.

Toyama-Sorimachi, N., and Miyasaka, M. (1994). A novel ligand for CD44 is sulfated proteoglycan. Int. Immunol. *6*, 655-660.

Toyama-Sorimachi, N., and Miyasaka, M. (1994). A sulfated proteoglycan as a novel ligand for CD44. J. Dermatol. *21*, 795-801.

Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K., and Miyasaka, M. (1995). A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. J. Biol. Chem. *270*, 7437-7444.

Trowbridge, I. S., Lesley, J., Schulte, R., Hyman, R., and Trotter, J. (1982). Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glycoprotein expressed on lymphoid tissues. Immunogenetics *15*, 299-312.

Trowbridge, I. S., Lesley, J., Trotter, J., and Hyman, R. (1985). Thymocyte subpopulation enriched for progenitors with an unrearranged T-cell receptor beta-chain gene. Nature *315*, 666-669.

Tsukita, S., Oishi, K., Sato, N., Sagara, J., Kawai, A., and Tsukita, S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. *126*, 391-401.

Uff, C. R., Neame, S. J., and Isacke, C. M. (1995). Hyaluronan binding by CD44 is regulated by a phosphorylation-independent mechanism. Eur. J. Immunol. *25*, 1883-1887.

van der Voort, R., Manten-Horst, E., Smit, L., Ostermann, E., van den Berg, F., and Pals, S. T. (1995). Binding of cell-surface expressed CD44 to hyaluronate is dependent on splicing and cell type. Biochem. Biophys. Res. Commun. *214*, 137-144.

van Kooyk, Y., van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T. W., and Figdor, C. G. (1989). Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature *342*, 811-813.

von Andrian, U. H., Hasslen, S. R., Nelson, R. D., Erlandsen, S. L., and Butcher, E. C. (1995). A central role for microvillous receptor presentation in leukocyte adhesion under flow. Cell *82*, 989-999.

Watanabe, K., and Yamaguchi, Y. (1996). Molecular identification of a putative human hyaluronan synthase. J. Biol. Chem. *271*, 22945-22948.

Webb, D. S., Shimizu, Y., Van Seventer, G. A., Shaw, S., and Gerrard, T. L. (1990). LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. Science *249*, 1295-1297.

Weber, G. F., Ashkar, S., Glimcher, M. J., and Cantor, H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science *271*, 509-512.

Weigel, P. H., Hascall, V. C., and Tammi, M. (1997). Hyaluronan synthases. J. Biol. Chem. *272*, 13997-14000.

West, D. C., Hampson, I. N., Arnold, F., and Kumar, S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. Science *228*, 1324-1326.

West, D. C., and Kumar, S. (1989). The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. Exp. Cell Res. *183*, 179-196.

West, D. C., and Kumar, S. (1989). Hyaluronan and angiogenesis. Ciba Found. Symp. *143*, 187-201; discussion 201-207, 281-285.

Wolffe, E. J., Gause, W. C., Pelfrey, C. M., Holland, S. M., Steinberg, A. D., and August, J. T. (1990). The cDNA sequence of mouse Pgp-1 and homology to human CD44 cell surface antigen and proteoglycan core/link proteins. J. Biol. Chem. *265*, 341-347.

Woodruff, J. J., Clarke, L. M., and Chin, Y. H. (1987). Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. Annu. Rev. Immunol. *5*, 201-222.

Wright, S. D., and Silverstein, S. C. (1982). Tumour-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. *156*, 1149-1164.

Yang, B., Yang, B. L., Savani, R. C., and Turley, E. A. (1994). Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. EMBO J. *13*, 286-296.

Yednock, T. A., and Rosen, S. D. (1989). Lymphocyte homing. Adv. Immunol. *44*, 313-378.

Yoneda, M., Shimizu, S., Nishi, Y., Yamagata, M., Suzuki, S., and Kimata, K. (1988). Hyaluronic acid-dependent change in the extracellular matrix of mouse dermal fibroblasts that is conducive to cell proliferation. J. Cell Sci. *90*, 275-286.

Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., and Tsukita, S. (1998). Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J. Cell Biol. *140*, 885-895.

Yoshida, K., Bolodeoku, J., Sugino, T., Goodison, S., Matsumura, Y., Warren, B. F., Toge, T., Tahara, E., and Tarin, D. (1995). Abnormal retention of intron 9 in CD44 gene transcripts in human gastrointestinal tumours. Cancer Res. *55*, 4273-4277.

Yoshida, K., Sugino, T., Bolodeoku, J., Warren, B. F., Goodison, S., Woodman, A., Toge, T., Tahara, E., and Tarin, D. (1996). Detection of exfoliated carcinoma cells in colonic luminal washings by identification of deranged patterns of expression of the CD44 gene. J. Clin. Pathol. *49*, 300-305.

Yu, Q., Toole, B. P., and Stamenkovic, I. (1997). Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumour cell surface CD44 function. J. Exp. Med. *186*, 1985-1996.

Zeimet, A. G., Widschwendter, M., Uhl-Steidl, M., Muller-Holzner, E., Daxenbichler, G., Marth, C., and Dapunt, O. (1997). High serum levels of soluble CD44 variant isoform v5 are associated with favourable clinical outcome in ovarian cancer. Br. J. Cancer *76*, 1646-1651.

Zhang, K., Saxon, A., and Max, E. E. (1992). Two unusual forms of human immunoglobulin E encoded by alternative RNA splicing of epsilon heavy chain membrane exons. J. Exp. Med. *176*, 233-243.

Zhang, M., Wang, M. H., Singh, R. K., Wells, A., and Siegal, G. P. (1997). Epidermal growth factor induces CD44 gene expression through a novel regulatory element in mouse fibroblasts. J. Biol. Chem. *272*, 14139-14146.

Zhou, D. F., Ding, J. F., Picker, L. J., Bargatze, R. F., Butcher, E. C., and Goeddel, D. V. (1989). Molecular cloning and expression of Pgp-1. The mouse homolog of the human H-CAM (Hermes) lymphocyte homing receptor. J. Immunol. *143*, 3390-3395.