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Compensatory mechanisms in CD44-deficient mice

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Zusammenfassung
Kompensatorische Mechanismen in CD44-defizienten Mäusen.

CD44 ist eine Familie transmembraner Glycoproteine, denen eine Rolle bei verschiedenen physiologischen und pathologischen Prozessen, wie zum Beispiel bei der Embryonalentwicklung, der Hämatopoese, in der Immunantwort, bei Tumorwachstum und der Bildung von Metastasen zugeschrieben wurde. Interessanterweise zeigen Mäuse, bei denen alle Isoformen von CD44 durch gezielte Mutagenese ausgeschaltet worden sind, keine offensichtlichen embryonalen oder immunologischen Abnormalitäten; lediglich ein geringfügiger Defekt in der Hämatopoese wurde beobachtet (Schmits et al., 1997). Daraus wurde geschlussfolgert, dass Mäuse, bei denen CD44 genetisch defekt ist, einen kompensatorischen Mechanismus benutzen, der einen funktionellen Ausgleich schafft. Das Ziel der vorliegenden Arbeit war es, die Veränderungen zu untersuchen, die in CD44-defekten Mäusen für den Mangel an CD44 kompensieren. Die Suche nach einem kompensatorischen Molekül basierte auf den Bindungseigenschaften von CD44 für spezifische Liganden, die potenzielle CD44-substituierende Moleküle definieren könnten.

Da Hyaluronsäure als einer der Hauptliganden fur CD44 bekannt ist, wurde die Hyaluronsäure-Bindekapazität von verschiedenen primären Mauszellinien untersucht: Primäre, embryonale Fibroblasten von Wildtyp-Mäusen banden weder an lösliche noch an immobilisierte Hyaluronsäure, solange sie nicht in geringer Dichte kultiviert wurden. Sowohl die Stärke der CD44-Expression als auch die CD44-abhängige Hyaluronsäure-Bindung durch embryonale Fibroblasten von Wildtyp-Mäusen verhielt sich umgekehrt proportional zur Zelldichte. Im Gegensatz zum Wildtyp hatten embryonale Fibroblasten von CD44-defekten Mäusen überhaupt keine messbare Affinität zu Hyaluronsäure, ganz gleich unter welchen Bedingungen oder welcher Dichte sie kultiviert worden waren. Auch onkogene Transformation stimulierte die Hyaluronsäure-Bindung von embryonalen Fibroblasten nicht. Von den anderen untersuchten Zellinien banden ruhende und Thioglykolat-induzierte peritoneale Makrophagen der Wildtyp-Mäuse an Hyaluronsäure. Allerdings war diese Affinität CD44-unabhängig, da sie nicht durch den Antikörper KM81 blockiert wurde, der mit der Bindung von CD44 an Hyaluronsäure interferiert. Die bereits beschriebene CD44-abhängige Hyaluronsäure-Bindung von Interleukin 5-stimulierten B-Zellen aus DBA/2-Mäusen (Murakami et al., 1990) war offenbar spezifisch für diesen Stamm. Dieser Befund machte es unmöglich, nach kompensatorischen Molekülen in B-Zellen zu suchen, denn die CD44 defekten Mäuse gehören der C57Bl6/J-Linie an, die diese Affinität nicht zeigt. T-Zellen, die aus Lymphknoten von C57Bl6/J und Balb/c Mäusen isoliert worden waren, entwickelten nach Stimulation mit einem Anti-CD3 Antikörper eine Subpopulation von Zellen, die CD44-abhängig an Hyaluronsäure banden. Im Gegensatz dazu

entwickelten T-Zellen aus Lymphknoten von CD44-defekten C57Bl6/J-Mäusen keine Subpopulation, die eine CD-3-vermittelte Affinität zu Hyaluronsäure aufwies.

Diese Befunde lassen vermuten, dass in den untersuchten Zelltypen von CD44-defekten C57Bl6/J-Mäusen kein Ersatz für die CD44 abhängige Hyaluronsäure-Bindung besteht. In Sherman et al., 1998 wurde gezeigt, dass eine Heparan-Sulfat modifizierte Proteoglykan-Form von CD44 dazu diente, Fibroblasten Wachstumsfaktor 4 (FGF-4) und FGF-8 zu präsentieren und somit das Wachstum der sich entwickelnden Gliedmassenknospe zu regulieren. Um ein Molekül zu identifizieren, das in CD44-defekten Mäusen diese Rolle übernimmt, wurden Menge und Muster mesenchymaler Heparan-Sulfat modifizierter Proteoglykane in CD44-positiven und CD44-negativen embryonalen Gliedmassenknospen verglichen. Die Gesamtmenge an Heparan-Sulfat an der äusseren Zellmembran mesenchymaler Zellen war gleich, jedoch wurden in den embryonalen Gliedmassenknospen von CD44-defekten Mäusen drei Heparan-Sulfat modifizierte Proteoglykane mit niedrigem Molekulargewicht verstärkt exprimiert. Diese Proteoglykane könnten als Ersatz fur CD44 dienen und FGF in CD44 defizienten Gliedmassenknospen präsentieren.

Abstract

CD44 is a closely related family of transmembrane glycoproteins, which have been implicated in a number of physiological and pathological phenomena such as embryonic development, hemopoiesis, immune response, tumour growth and metastasis formation. However, mice with a gene-targeted disruption of all CD44 isoforms do not display any overt embryological or immune abnormalities, having only a minor defect in the hemopoietic system (Schmits et al., 1997). It was therefore concluded that the CD44-deficient mice utilize compensatory mechanisms in order to overcome the need for CD44. The goal of the work in this thesis was to investigate the changes in CD44-mutant mice which compensate for the lack of CD44. The search was based on the ligand binding properties of CD44 which may define the potential CD44 -substitutive molecule or molecules.

Since hyaluronic acid (HA) is known to be a major CD44 ligand, the hyaluronic acid binding capacity of different primary mouse cell types was examined. Primary embryonic fibroblasts from wild-type mice did not bind to soluble and immobilized hyaluronic acid, unless they were cultured at low confluency. The level of CD44 expression and the CD44-dependent hyaluronic acid binding by embryonic fibroblasts from the wild-type mice was found to inversly correlate with cell density. In contrast to the wild-type, embryonic fibroblasts obtained from the CD44-deficient mice did not show any hyaluronic acid binding under any culturing conditions, including a low confluency. Oncogenic transformation did not stimulate hyaluronic acid binding in CD44-deficient embryonic fibroblasts. As to the other cell types tested, resident and thioglycollate-elicited peritoneal macrophages obtained from the wild-type mice bound hyaluronic acid. However this binding was CD44-independent, since it was not blockable by KM81, the anti-CD44 antibody interferring with hyaluronic acid binding of CD44. The previously described CD44-dependent HA binding of a subpopulation of interleukin-5 stimulated B-cells derived from DBA/2 mice (Murakami et al., 1990) appeared to be strain specific. This made it impossible to look for a substitution of CD44-dependent HA binding in the B-cells derived from CD44-deficient mice generated on the C57Bl6/J background. Lymph node T-cells derived from both C57Bl6/J and Balb/c mice developed a subpopulation of cells binding HA in a CD44-dependent manner upon stimulation with anti-CD3 antibody, although the hyaluronic acid binding subpopulation was somewhat smaller in the C57Bl6/J derived cells. In contrast, lymph node-derived T-cells from CD44-deficient mice did not develop a hyaluronic acid binding subpopulation upon treatment with anti-CD3 antibody. Together, this suggests that CD44-dependent hyaluronic acid binding is not substituted in the cell types tested in CD44-deficient C57Bl6/J mice.

A heparan sulfated proteoglycan form of CD44 has been previously shown to serve as a FGF4 and FGF8-presenting molecule in the developing limb bud, thus regulating limb outgrowth (Sherman et al., 1998). To find the substitutive molecule(s) taking over this function of CD44 in CD44-deficient developing limb buds, the amount and pattern of mesenchymal heparan sulfated proteoglycans from CD44-positive and CD44-negative embryonic limb buds were compared. The total level of surface heparan sulfate was equivalent in both wild-type and CD44-deficient limb bud mesenchymal cells. However, in the CD44-negative limb bud mesenchyme, 3 low molecular weight heparan sulfated proteoglycans were significantly upregulated compared to the wild-type cells. These are possible candidates for CD44 substitutive molecules which present FGFs in CD44-deficient embryonic limb buds.

Contents

List of abbreviations.

A	Ampere
ab	Antibody
abs	Antibodies
AER	Apical Ectodermal Ridge
APC	Antigen-Presenting Cell
Bq	Bequerel
bp	base pair
BSA	Bovine Serum Albumin
^{0}C	degrees Celsius
cAMP	cyclic AMP, Adenosine-3',5'-monophosphate
CD	Claster of Differentiation
CD40L	CD40 ligand
CD44E	Epithelial CD44
CD44H	Hemopoietic CD44
CD44 Rg	CD44 Receptor globuline
CD44s	CD44 standard protein
CDK	Cyclin-Dependent Kinase
Ci	Curi
cm	Centimeter
ConA	Concanavalin A
cpm	counts per minute
CPC	Cetylpyridinium Chloride
CRIII	Class III Collagen Receptor
CS	Chondroitin Sulfate
CTLA	Cytotoxic T-Lymphocyte Antigen
Cys	Cysteine
dCTP	2'-deoxycytidine-5'-triphosphate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNFB	2,4 dinitro-1-fluorobenzene
DOC	Deoxicholate
DTH	Delayed-Type Hypersensitivity
DTT	Dithiothreitol
g	gram
x g	fold gravity
GAG	Glycosaminoglycan
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
gp	glycoprotein
GPI	Glycosylphosphatidylinositol
E	day of embryonic development
ECL	Enchanced Chemi-Luminescence

ECM	Extracellular Matrix
ECMRIII	Extracellular Matrix Receptor Type III
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
eds	editors
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
emb.	embryo
ERM	Ezrin-Radixim-Moesin
et al.	and others
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocianate
h	hour
HA	Hyaluronic Acid
HABP	Hyaluronic Acid Binding Protein
H'ase	Heparinase
HB-EGF	Heparin-Binding Epidermal Growth Factor
HEV	High Endothelial Venules
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HS	Heparan Sulfate
HSPG	Heparan Sulfated Proteoglycan
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IGF	Insulin-Like Growth Factor
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneally
K	Kilo-
kb	kilobase
kDa	kilodalton
l	liter
LAK	Lymphokine-Activated Killer
LFA	Leukocyte Function Associated Antigen
LMW	Low Molecular Weight
LN	Lymph Node
LPA	L-α-Lysophosphatidic acid
LPS	Lipopolysaccharide
M	Molar
m	milli-
µ	micro-
mAb	monoclonal antibody
MAd	Mucosal Vascular aAddressin
MES	2-N-morpholino ethanesulfonic acid
MHC	Major Histocompatibility Complex
min	minute

MIP	Macrophage Inflammatory Protein
mlc	Myosin Light Chain
mm	millimeter
µm	micrometer
mRNA	messenger RNA
MW	Molecular Weight
n	nano-
N	number
nm	nanometer
NK	Natural Killer
NP-40	Nonidet P-40
OD	Optical Density
OX40L	OX40 ligand
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PG	Proteoglycan
Pgp-1	Phagocytic glycoprotein-1
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PP	Peyer's Patches
RANTES	regulated upon activation, normally T cell expressed and secreted
Rb	Retinoblastoma
SDS	Sodium Dodecyl Sulfate
SEB	Staphylococcal Enterotoxin B
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethyldiamine
TGF	Transforming Growth Factor
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TPA	12-O-tetradecanoylphorbol-13-acetate
v	variant
V	Volt
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen
VLS	Vascular Leak Syndrome
vol	volume
U	Unit
UV	Ultraviolet

Chapter 1. Introduction

The term CD44 encompasses a family of cell surface glycoproteins which differ due to alternatve splicing and glycosylation pattern. The members of the CD44 family are able to mediate cell-cell adhesion and cell-extracellular matrix attachment, cell activation, differentiation and movement. They are implicated in embryogenesis, hemopoiesis, the immune response, tumorigenesis and metastasis formation.

During discovery of CD44, different names were used by different groups for some particular CD44 family members. One was called p80, or In(Lu)-related p80, by Haynes and colleagues, when found in human thymocytes (Haynes et al., 1983) and, later, in human erythrocytes and leukocytes (Telen et al., 1986). A CD44 member which mediated adhesion of human fibrosarcoma cells to collagen and fibronectin was named as extracellular matrix receptor type III (ECMR III) or class III collagen receptor (CRIII) by Carter and Wayner (Wayner and Carter, 1987; Carter and Wayner, 1988). The lymphocyte homing receptor, gp90, recognised by the Hermes-1 antibody (Jalkanen et al., 1986a) was later found to be structurally homologous to CRIII (Gallatin et al., 1989). In mice, CD44 has been long known as Ly-24, a marker for *in vivo* primed T-lymphocytes (reviewed in Lynch and Ceredig, 1989). Another name, Pgp-1 (phagocytic glycoprotein -1) was given by Hughes and colleagues, who characterised this protein isolated from murine NIH/3T3 (Hughes and August, 1981; Hughes et al., 1983) and mapped it on mouse chromosome 2 (Colombatti et al., 1982). Separately, Trowbridge and colleagues described a 95 kDa murine leukocyte surface glycoprotein; its genetic mapping suggested the identity of this protein to Pgp-1 (Lesley and Trowbridge, 1982). Finally, it turned out that all these proteins are identical or closely related members of the CD44 family (Zhou et al., 1989; Picker et al., 1989a), encoded by a single gene in both mouse and humans, which participate in an astonishing variety of cellular events.

The discovery of CD44 functions usually began from the development of a specific antibody able to modulate some defined cellular phenomenon, such as cell-cell attachment, binding of particular matrix components or some other cellular function. The identity of CD44 family member bound by these antibodies was achieved by cross-immunoprecipitation or

molecular cloning. The investigation of the expression pattern of CD44 in different tissues and cells lines permitted a correlative analysis, which suggested the possible involvement of CD44 in process such as tumour progression, lymphocyte activation or embryonic development. CD44 functions have been demonstrated by the examination of the effect of previously characterised anti-CD44 antibodies on cellular properties *in vitro.* The anti-CD44 antibody administration *in vivo* helped to detect the role of CD44 during various physiological and pathological processes. As a next step in the discovery of CD44 functions, gene targeting technology was used to generate mice with total (Schmits et al.,1997) or tissue-specific (Kaya et al., 1997) disruption of the CD44 gene. Transgenic mice overexpressing a particular CD44 isoform on T-cells have also been used (Moll et al., 1996). In order to analyse the information obtained from one of these animal models, the CD44-null mutant mouse (Schmits et al., 1997), I would first like to bring together what is known about the structure, ligands and functions of CD44.

1.1 CD44 structure

The CD44 gene is located on the short arm of chromosome 11 in humans (Goodfellow et al., 1982) and on chromosome 2 in mice (Colombatti et al., 1982; Lesley and Trowbridge, 1982). Original genomic cloning studies (Screaton et al., 1992) identified 19 exons, spanning 50-60 kb of genomic DNA in humans. An additonal exon, 6a, was later located between exon 5 and 6 (Screaton et al., 1993; Tölg et al., 1993). While exons 1 - 5 and 16 - 17 are constant in both species, exons 6a - 14, encoding the membrane-proximal extracellular domain of CD44, and exons 18 - 19, encoding the cytoplasmic tail, are subject to alternative splicing. Exons 6a - 14 are called variable exons and have their own nomenclature from v1 to v10. The shortest version of CD44 (standard, CD44s, or hemopoietic, CD44H) includes none of the variable exons. Insertion of variant exons into the CD44 sequence generates higher molecular weight CD44 isoforms, named by the exon used, like CD44v4-v7 or CD44v3,8-10 and so on. Inclusion of exon 19 instead of exon 18 results in a the short-tailed CD44 version (Screaton et al., 1992). A new exon named v9a was discovered recently between v9 and v10, whose inclusion may result in a small soluble CD44 isoform (Yu and Toole, 1996).

Beside alternative splicing, the huge diversity of CD44 isoforms arises from variable post-transcriptional modifications, such as glycosylation and glycanation of the molecule. CD44s is extensively glycosylated at several sites, so that approximately half of the molecular size on the SDS-polyacrylamide gel electrophoresis (85-95 kDa) appears to be due to the N- and O-linked carbohydrates decorating the extracellular region. (Jalkanen et al., 1988, Zhou et al., 1989; Lokeshwar and Bourguignon, 1991). The consensus motif for chondroitin sulfate (CS) assembly, which is comprised of a serine-glycine motif followed by one or more proximal acidic amino acids, was found in exons 5, 15 and 16 in CD44s. The utilisation of these sites for CS modification is likely dependent on the cell type. Namalwa cells did not support the chondroitination of transfected CD44s isoforms (Jackson et al. 1995), while in a study done on COS cells the serine-glycine site in exon 5 of a transfected CD44-immunoglobulin fusion protein was modified by CS (Greenfield et al., 1999). In addition, CD44s was found to be modified by keratan sulfate in a cell type specific manner (Takahashi et al., 1996), though the attachment site for keratan sulfate is not known. The insertion of variant exons endows CD44 with additional possibilities for the glycosylation and for glycosaminoglycan assembly. Of two additional sites for CS modification encoded by the variable exons v3 and v10, at least one (in exon v3) is found to support chondroitination of CD44 (Greenfield et al, 1999). Exon v3 is also decorated with heparan sulfate (HS), attached at a serine-glycine-serine-glycine motif (Brown et al., 1991; Bennet et al., 1995a; Jackson et al., 1995; Greenfield et al. 1999). In addition, inclusion of exons v4-v7, lacking the sites for glycosaminoglycans assembly, exerts a distal effect on the composition of heparan and chondroitin sulfate moieties at the other sites of CD44 protein (Piepcorn et al., 1999). Both alternative splicing and post-transcriptional modifications of CD44 strongly depend on the tissue/cell type expressing CD44 and on the activation status of the cells.

Of the other posttranscriptional modifications, CD44 can be phosphorylated at several cytoplasmic serine and threonine residues (Kalomiris and Bourguignon, 1989; Bourguignon et al., 1992; Camp et al., 1991; Isacke et al., 1986; Carter and Wayner, 1988). Palmitoylation of CD44 upon treatment of T-lymphocytes with anti-CD44 antibodies has also been described (Guo et al., 1994).

1.2 Pattern of CD44 expression

Although CD44 is expressed in many tissues, the expression follows a strictly restricted pattern with respect to cell type and stage of differentiation. During mouse embryogenesis, CD44 is highly expressed in the embryonic heart at day E8,25-E12,5, but is confined mostly to the myocardium at later stages (Wheatley et al., 1993). It is also abundant in the arterial system at day E9,5-E12,5, in contrast to the venous system where it was not found at any stage of development. CD44 is temporarily expressed in the developing somites, and in the tail part of the notochord after day E10,5. It has a specific pattern of expression in developing craniofacial tissue. In the developing limb bud CD44 is localised in the ectoderm and, after formation of the apical ectodermal ridge (AER), high CD44 expression is restricted to this tissue. At later stages, CD44 is also found in condensing limb bud mesenchyme prior to chondrogenesis. CD44 is also strongly expressed in the branchial arches beginning from day E8,5. It is observed there only on endodermal and, later, on ectodermal tissues, while the branchial arch mesenchyme is CD44-negative. The expression of CD44 variant isoforms during embryogenesis shows even more restricted distribution. In contrast to CD44s which is most abundant at the stage after day E9,5, CD44 variants appear as early as at day E6,5 in the egg cylinder (Ruiz et al., 1995). CD44v3-v10, CD44v4-v10 and CD44v6-v10 are the most prominant CD44 variants, expressed on the actively proliferating and invading epithelia at sites of epithelial-mesemchymal interaction, such as developing teeth, nose and the hair follicules (Yu and Toole, 1997). The expression of several high molecular weight CD44 isoforms including CD44v3-v10, CD44v4-v10 and CD44v6-v10 was demonstrated on AER (Yu et al., 1996; Wainwright, 1998).

In adults, CD44 is expressed in the epithelium of different tissues, including skin, cheek, tongue, esophagus, vagina, intestines, oviduct and bladder (Alho and Underhill, 1989), but not in cornea and stomach epithelial cells. It is mostly localized on proliferating keratinocytes in the basal and spinous layers in skin, and in the basal layer of other types of epithelium (Alho and Underhill, 1989; Wang et al., 1992). Epidermal skin keratinocytes express both CD44s and CD44 variant isoforms, including CD44v2-v10, CD44v3-v10, CD44v4-v10, CD44v6-v10 and CD44v8-

v10 whereas dermal fibroblasts carry only the CD44s form (Hudson et al. 1995). In human skin melanocytes two CD44s isoforms are predominant, which differ only in chondroitin sulfatation (Herbold et al., 1996). CD44 is expressed in the adult hamster lung, mostly on the basolateral surface of epithelium lining bronchi and larger bronchioles, as well as on pulmonary macrophages (Green et al., 1988).

The expression of CD44 on T- and B-cells during lymphoid ontogeny and in the immune response is quite complex. The very early hematopoietic progenitors, the pluripotent bone marrow stem cells are CD44 positive (reviewed in Lesley et al., 1993a). In thymus, the earliest thymocytes ($CD4^-$, $CD8^-$, $CD3^-$, $Il\text{-}2^-$) express CD44s (Horst et al., 1990). CD44 is absent at an intermediate stage of thymocyte maturation, characterized by co-expression of both CD4 and CD8. CD44 appears again on mature thymocytes, selectively expressing CD4 or CD8, after they have passed the TCR rearrangment and became retricted to a certain MHC class (Husmann et al., 1988; de los Toyos et al., 1989; Penit et al., 1989; Lesley et al., 1990). Mature human peripheral T-cells express CD44 (de los Toyos et al. 1989). In mice CD44 expression depends on the mouse strain. Those mice which carry the CD44.1 allele have a higher percentage of CD44 positive T-cells and thymocytes compared to CD44.2 mice (Lesley et al., 1988; Lynch et al., 1989). CD44 is highly upregulated on activated and memory T-lymphocytes (MacDonald et al., 1990; Budd et al., 1997a,b), which allows it to be used as a specific marker for antigen-primed and memory T-cells. Activated T-cells in addition to CD44s express CD44v3, v6, v7 and v9 variants (Seiter et al., 1999; Wittig et al., 1997; Galluzzo et al., 1995). Bone marrow B-cell progenitors are mostly CD44-negative (Deguchi et al., 1999; Ryan et al., 1990), but CD44 is detected on mature blood B-lymphocytes. It is still expressed following antigenic activation of B-cells, but only until their blast-transformation. CD44 dissappears at the blast and centroblast stages of B-cell differentiation in lymph node germinal centers and is re-expressed again at the centrocyte stage (Kremmidiotis and Zola, 1995; Collado et al., 1991). CD44 remains expressed on memory B-cells and plasma cells.

CD44 expression is found also on granulocytes, monocytes, macrophages, dendritic cells of different origin, and erythrocytes. CD44 is expressed on all early $CD34^+$ myeloid progenitors.

In granulocytes, it is down-redulated during the intermediate stage of maturation and is re-expressed on mature granulocytes (Lund-Johansen and Terstappen, 1993). During the inflammatory response, the CD44 level on neutrophils is decreased (Humbria et al., 1994). An inflammatory cytokine, tumor necrosis factor-α (TNF-α), was shown to down-modulate CD44 on neutrophils via CD44 shedding (Campanero et al., 1991). In contrast, *in vitro* stimulation of eosinophils with Il-3, Il-5 or GM-CSF, but not with interferon-γ, resulted in up-regulation of CD44 on these cells (Matsumoto et al., 1998b). The level of CD44 on eosinophils was increased upon cytokine-mediated activation and was higher in the blood eosinophils from bronchial asthma patients than in those of healthy donors (Sano et al., 1997). Human peripheral blood monocytes express predominantly the standard CD44 isoform, but upon stimulation either by *in vitro* culturing or by IFN-γ or LPS treatment they upregulate several high molecular weight CD44 variants, including CD44 containing sequences encoding exons v3, v4, v5, v6 and v9 (Culty et al., 1994; Weiss et al., 1998). Langerhans cells and skin dendritic cells normally expressing CD44s, upregulate the expression of CD44 variants following antigenic stimuli. These include sequences of exons v4, v5, v6 and v9 (Weiss et al., 1997). Maturation of Langerhans cells and skin dendritic cells in vitro also increases their expression of v3, v6 and v9 exons of CD44 (Haegel-Kronenberger et al., 1998). In macrophages, the major CD44 isoform detected has a MW of 95-97 kDa, corresponding to CD44s (Camp et al., 1991).

1.3 CD44 ligands

1.3.1 Hyaluronan

When human CD44 and its mouse homologue Pgp-1 were cloned (Goldstein et al., 1989; Stamenkovic et al., 1989; Zhou et al., 1989), it became clear that CD44 possesses a significant homology with the hyaluronic binding proteins, cartilage core and link proteins, suggesting that hyaluronate can be a ligand for CD44. Indeed, CD44 can serve as a receptor for hyaluronic acid (HA) (Miyake et al., 1990; Aruffo et al., 1990).

Hyaluronic acid, or hyaluronan (HA), is a large polymer consisting of repeating disaccharides, D-glucuronic acid / N-acetyl-D-glucosamine, linked by β(1->3) and β(1->4) glucosidic linkages. It is characterised by an enormous viscosity, resulting from both huge molecular weight (Mr≥ 10^6) and from the stiffness of the polysaccharide chain due to hydrogen bonding between the sugar residues (Laurent and Fraser, 1986). HA is a major component of extracellular matrix (ECM), where it is associated with other ECM components such as proteoglycans in highly organized structures. It is abundant in connective tissues, but also found on cell surfaces and in body fluids such as lymph and serum. HA has been implicated in maintaining the structural integrity of tissues, in wound healing, cell motility, proliferation and differentiation, angiogenesis, inflammation and embryogenesis (reviewed in Evered and Whelan (eds), 1989). Most of its functions cannot be ascribed entirely to its biochemical properties, but obviously are based on specific HA-cell interactions.

The receptor-mediated cellular binding to HA was demonstrated long before the discovery of CD44 (Turley and Torrance, 1985; Green et al., 1988; Underhill et al., 1985, 1987; Knudson and Knudson, 1991). With the use of an anti-CD44 antibody which blocks HA binding by mouse SV-3T3 fibroblasts and specifically depletes the HA-binding activity from the detergent cell lysates, the molecule previously referred to as "fibroblast HA-receptor" was identified as CD44 (Culty et al., 1990).

CD44 binds HA most likely via the region at the N-terminus homologous to the B-loop of cartilage link protein. It was found that all HA-binding proteins share the common HA- binding motif containing two basic amino acids, flanking a seven amino acid strech (B(X_7)B) (Yang et al., 1994). CD44 possesses 3 such motives, one is located on the N-terminus, homologous to the HA binding domain in cartilage link protein, another is in the membrane proximal extracellular domain and the third is in the cytoplasmic tail. Site-directed mutagenesis revealed that the most distal N-terminal and central domains are both responsible for HA binding by CD44 (Peach et al., 1993). However, naturally occuring CD44 mutants with substitutions at the HA-binding motifs do not display any defect in HA binding (reviewed in Borland et al., 1998).

Not all CD44-expressing cells are able to bind HA (Stamenkovic et al., 1991; Lesly, 1993b; Galluzzio et al., 1995). It was postulated that CD44 may be in 3 functional states as far as its HA binding capacity is concerned (Lesley and Hyman, 1992). In some cells CD44 constitutively binds HA. In others it may be induced to do so upon activation, either with "inducing" anti-CD44 antibody, such as IRAWB 14 in mouse and F10.44.2 in human systems, or, alternatively, with phorbol esters. Later it was found that beside the inducing antibodies and phorbol esters other stimuli are also able to convert CD44 into an HA-binding stage, such as IL-5 for B-cells (Murakami et al, 1990), the TCR triggering for T-cells (DeGrendele et al., 1997), *in vivo* activation by inducing the graft-versus-host reaction (Murakami et al., 1991) or an allogenic immune response (Lesley et al., 1994), or *in vitro* culturing manipulation including adhesion-dependent priming of monocytes (Culty et al., 1994) or serum conditions for fibroblasts (Kogerman et al., 1996). The third state of CD44 is represented by CD44-positive cells, in which HA binding cannot be induced (Lesley and Hyman, 1992).

Major efforts were directed to find out what structural properties discriminate the HA-binding form of CD44 from the non-binding one. The influence of alternative splicing, glycosylation, glycanation, phosphorylation, binding to the cytoskeleton and CD44 clustering on the ability of CD44 to bind HA was examined.

Since only CD44s but not the CD44v8-v10 (CD44E) isoform transfected into the CD44-negative Namalwa cell line conferred on these cells an HA-dependent adhesion to the endothelial cells, it was concluded that the higher molecular weight CD44 isoforms are not able to bind HA (Stamenkovic et al., 1991). In accordance, a CD44E-immunoglobulin fusion protein bound immobilized HA much weaker than did a CD44s-immunoglobulin fusion (Bennett et al., 1995b). However the opposite was observed in rat pancreatic carcinoma cell line transfected with different CD44 isoforms: only CD44v4-v7 but not CD44s overexpression endowed these cells with increased binding to both soluble and immobilized HA (Sleeman et al., 1996a). Gain of HA binding by differentiating KG-1 and K562 hemopoetic cell lines was associated with expression of CD44v4-v10 by these cells, and the HA binding by this CD44 isoform was directly demonstrated by CPC precipitation of CD44v4-v10 with HA (Moll et al., 1998). Other authors

showed that CD44 variants bind to HA when transfected into COS cells (Dougherty et al., 1994) or T-lymphoma cells (He et al. 1992). The different capacity of CD44 variants to bind to HA was explained by the influence of the cell type on the HA binding capacity of the same CD44 constructs (van der Voort et al., 1995).

Glycosylation of the CD44 molecule was found to regulate HA binding both positively and negatively. The inhibition of O-linked glycosylation in CD44s-transfected colon carcinoma cells resulted in enhanced binding to HA without increasing CD44 expression (Dasgupta et al., 1996), while the inhibition of N-linked glycosylation did not significantly alter HA binding of the cells. In IL-5 stimulated B-lymphocytes, the reduction in N-glycosylation of CD44 was correlated with its increased HA-binding capacity (Hathcock et al., 1993). The impaired glycosylation in some subclones of Chinese hamster ovary (CHO) cells correlated with an increased HA binding, and removal of sugar residues with tunicamicin or deglycosylating enzymes enchanced HA binding in wild-type CHO (Katoh et al., 1995). However in other cell line such as differentiating K562 cells the inhibition of N-linked glycosylation with tunicamicin treatment reduced HA binding without affecting the overall level of CD44 (Moll et al., 1998). The same was observed in CD44v4-v7 transfected rat pancreatic tumour cell line, where binding to soluble and immobilized HA was inhibited by tunicamicin (Sleeman et al., 1996b). The affinity of a CD44-immunoglobulin fusion protein to HA in a capillary electrophoresis assay was dependent on the glycosylation pattern of the molecule, namely it was inhibited by terminal sialic acid on N-linked oligosaccharides, enhanced by the presence of the first N-linked N-acetylglucosamine residue and further modulated by O-linked glycans (Skelton et al., 1998). The attachment of glycosaminoglycan was also found to be important for HA binding by CD44. Of two clones of the KM12 colon carcinoma cell line one was shown to support the modification of CD44 with keratan sulfate. This clone bound HA more poorly than another one, and the CD44-dependent HA binding was increased in this clone by removal of keratan sulfate, suggesting an inhibitory role of keratan sulfatation on HA-binding by CD44 (Takahashi et al., 1996). Since both glycosylation and glycanation are tissue/cell type -specific modifications, it can in part explain the difference in HA binding capacity of different cell types expressing the same core CD44 protein.

The cytoplasmic tail of CD44 is important for CD44-mediated HA binding. CD44-negative cells transfected with CD44 which lacks the cytoplasmic domain, did not bind to soluble HA, in contrast to the wild-type CD44 transfectants, though binding to immobilised HA was still detectable in cells transfected with the truncated version of CD44 (Lesley et al, 1992). The importance of phosphorylation at cytoplasmic residues for the competence of CD44 for HA binding was investigated on different phosphorylation mutants of CD44. The defect in phosphorylation at serines 325 and 327 in mouse CD44 resulted in impaired HA binding (Pure et al., 1995). However other reports indicate that phosphorylation-incompetent human CD44 are still able to confer CD44-dependent soluble and immobilised HA binding on transfected AKR1 cells (Uff et al., 1995). It is possible therefore that human and mouse CD44 differ in their requirement of phosphorylation for HA binding. The phosphorylation state of the cytoplasmic part of CD44 correlates, in some cell types, with CD44 interaction with the cytoskeleton. For example, in resident murine macrophages only the non-phosphorylated CD44 molecules were associated with cytoskeleton. In thioglycollate-elicited macrophages all CD44 forms were in phosphorylated state and were found in the NP-40 - soluble fraction, meaning that they were not bound to the cytoskeleton (Camp et al., 1991).

CD44 has been described to interact with cytoskeletal elements such as ankyrin (Bourguignon et al, 1992, 1993), actin (Lacy and Underhill, 1987) and the members of the ezrin-radixin-moesin family (Hirao et al., 1996). The importance of CD44 interaction with the cytoskeleton is still under discussion. A mutation in ankyrin-binding site abolished HA binding and HA-mediated adhesion (Lokeshwar et al., 1994). Furthermore, this HA binding was inhibited by cytochalasin-B treatement blocking actin polymerisation, or by W-7 treatment preventing actomyosin contraction (Galluzo et al., 1995). However, other data exist showing that microfilament blockage does not influence CD44-dependant HA binding (Murakami et al., 1994).

One of the important mechanisms for activation of CD44-dependent HA binding is a dimerisation of the CD44 receptor. The ability of CD44v4-v7 to form homodimers was shown to be associated with HA binding conferred by this CD44 isoform on rat pancreatic carcinoma cell lines. The homodimerisation and oligomerisation occured only in nonreducing conditions,

required N-linked glycosylation of CD44v4-v7 but appeared to be independent of cytoplasmic tail of the molecule (Sleeman et al., 1996b). CD44 homodimerisation was found to be stimulated in human Jurkat leukemia cells by a phorbol ester, which is known to induice HA binding in these cells. This process involves the bridging at Cys 286 in transmembrane domain of CD44 (Liu and Sy, 1997). The same residue is required for the anti-CD3 antibody induced binding of HA in these cells (Borland et al., 1998). The interaction of CD44 with cytoskeletal elements may facilitate the HA dimerisation, and this could be one of the reasons for the importance of interaction of CD44 with the cytoskeleton.

Thus, in contrast to the wide-spread CD44 tissue distribution, the CD44 function as an HA receptor is tightly regulated.

1.3.2 Glycosaminoglycans

The binding of CD44s to chondroitin sulfates (CS), preferentially to chondroitin-4-sulfate, was demonstrated using a soluble chimeric protein consisting of the extracellular portion of CD44s and human IgG1, named CD44 Receptor globulin, CD44 Rg (Aruffo et al., 1990; Peach et al., 1993). CD44 Rg binding to its ligand(s) on the high endothelial cells was inhibited by treatment with chondroitin AC lyase or by incubation of the cells with an excess of chondroitin 4- and 6- sulfates (Aruffo et al., 1990). CD44 Rg also bound to plastic-immobilized chondroitin-4-sulfate (Peach et al., 1993). In all these cases the concentration of CS was 100 fold higher than the concentration of HA required to reach a similar effect. However, other authors failed to detect the binding of CS to CD44s (Miake et al., 1990; Toyama-Sorimachi et al., 1995), which may be partially due to the particular cellular enviroment. The binding of CD44 variants to a range of glycosaminoglycans (GAGs) including heparin, heparan sulfate, chondroitin sulfate A, B and C was demonstrated in CPC precipitation experiments with CD44v4-v7 expressed in the rat pancreatic carcinoma cells (Sleeman et al., 1997). The binding of CD44 variant to these GAGs required that both v6 and v7 epitopes of CD44 were expressed, since CD44v6-7 but not CD44v6 or CD44v7 displayed the glycosaminoglycan binding capacity. The CD44 variants encoding both

v6 and v7 exons were able to confer the CSA binding on transfected cells of different origin. CSA binding was abolished by mutation in a N-terminal domain of CD44 interfering with HA binding, indicating that GAGs bind to CD44 at the same or overlapping site as HA (Sleeman et al, 1997). The aquisition of CSA binding by differentiating hemopoietic cell lines KG-1 and K562 correlated with the expression of CD44v4-v10 by these cells, and almost exclusive binding of CSA by high molecular weight CD44 isoforms was confirmed by CPC precipitation (Moll et al., 1998). CSA binding by CD44 variants showed similar characteristics to HA binding: it was induced during differentiation of the hemopoietic cell lines, was blockable by Hermes-1, and was dependent on N-glycosylation of the CD44 molecule. However, CSA binding by CD44 seemed to mediate different cellular events compared to CD44-mediated HA binding (Moll et al., 1998). Chondroitin sulfate binding by CD44 was shown to trigger proliferation of B-cells (Rachmilewitz and Tykocinski, 1998) and mediate the adhesion of glioma cells to the extracellular matrix (Radotra et al., 1994). The GAG binding ability of CD44 further widens the list of CD44 ligands, since it allows the GAG-modified proteoglycans to interact with CD44. Amongst these, invariant chain (li) and serglycin have been reported to bind CD44 via their CS chains (Naujokas et al., 1993; Toyama-Sorimachi et al., 1995)

1.3.3 Other ECM components: fibronectin, collagen and laminin

In 1984, it was reported that the fibroblast HA receptor, later identified as CD44, was also able to bind fibronectin, collagen and laminin (Turley and Moore, 1984). The nature of CD44 binding to fibronectin is relatively well investigated. Fibronectin appears to interact with chondroitin sulfate (CS) chains on glycanated CD44 proteins. A CD44 binding site is located on a COOH-terminal heparin binding domain of fibronectin. The same site is involved in interaction of fibronectin with syndecan, and therefore the fibronectin binding of CD44 can be competed by syndecan (Jalkanen and Jalkanen, 1992).

The fibronectin binding by CD44 can be regulated. Stimulation of human lung fibroblasts with TGF-β 1and bFGF, separately and in concert, increased the length of CS chains of CD44,

which led to the 2-fold enhancement in the affinity of CD44 to fibronectin (Romaris et al., 1995). In addition, a stimulating anti-CD44 antibody (TL-1) was raised which specifically induces CD44 binding to fibronectin and facilitates a homotypic aggregation of lymphoid cells (Cao et al., 1996). This speaks for the inducibility of fibronectin binding of CD44, similar to the phenomenon observed for CD44-dependent HA binding.

In addition to fibronectin and laminin, different types of collagen are described to be ligands for CD44. CD44 was found to be involved in melanoma cell motility and invasion on type I collagen matrix, and the binding of CD44 to type I collagen was dependent on chondroitin sulfate chain on CD44, similar to fibronectin binding. (Faassen et al., 1992). A chondroitin/dermatan sulfate form of CD44 was shown to be a receptor for type XIV collagen (Ehnis et al, 1996).The binding of a CD44E-bearing human colorectal carcinoma cell line to laminin and type IV collagen was blocked by the anti-CD44 antibodies Hermes-1, Hermes-3 and J173, indicating the involvment of CD44 in cell adhesion to these components of ECM. The CD44 binding site for laminin and type IV collagen appears to be different to that for HA (Ishii et al., 1993).

1.3.4 Mucosal vascular addressin

CD44 expressed on human lymphocytes binds to the mucosal vascular addressin (MAd), an endothelial-specific carbohydrate molecule, which is likely to direct lymphocyte homing to the high endothelial venules (Picker et al., 1989b). CD44 isolated from other human tissues varied for its capacity to bind MAd: glial and fibroblast CD44 was able to bind this ligand, whereas CD44 derived from sqamous epithelial cells lacked this capability (Picker er al., 1989b). Whether mouse CD44 under any circumstances bind to MAd is not known.

1.3.5 Osteopontin

Osteopontin, a chemokine secreted by activated T-cells, macrophages (Weber and Cantor, 1996) and osteoblasts (Oldberg et al., 1986) is known to mediate attachment and motility of different cell types *in vitro* (Liaw et al., 1995), as well as macrophage recruitment to the inflammatory tissue *in vivo* (Weber and Cantor, 1996; Singh et al., 1990). Osteopontin was found to be a ligand for CD44 (Weber et al., 1996). Osteopontin can be secreated in two forms - phosphorylated and non-phosphorylated. The association of osteopontin with extracellular matrix as well as engagement of the other osteopontin ligand, $\alpha_v\beta_3$ integrin, was shown to be dependent on osteopontin phosphorylation (Nemir et al., 1989; Ek-Rylander et al., 1994). The CD44 binding site on osteopontin differs from the binding site for integrins, which is an Arg-Gly-Asp domain (RGD) (Oldberg et al., 1986; Weber et al.,1996). CD44 variants, but not CD44s was shown to interact with both amino- and COOH-terminl portions of osteopontin. This binding was inhibited by antibody to β1 integrin, indicating the cooperation of CD44 and integrins in their binding to osteopontin (Katagiri et al, 1999). The osteopontin binding site on CD44 is not known. It has also been reported that osteopontin was not able interact with various CD44-immunoglobulin fusion proteins (Smith et al., 1999), indicating that perhaps an intact CD44 molucule or the correct cellular context is required for the osteopontin-CD44 interaction.

1.3.6 Serglycin

A novel ligand for CD44, gp600, was identified as serglycin. Serglycins belong to the family of small proteoglycans, with a short (16-18 kDa) core protein composed mostly of serine-glycine repeats and the glycosaminoglycan side chains varing in length and amounts (Ruoslahti, 1989). Their expression is restricted to yolk sac, hemopoietic cells and some tumor cells. gp600 is a chondroitin-sulfated type of serglycin, and chondroitin-4-sulfate, its major glycosaminoglycan chain, is involved in binding to CD44. It was shown that it binds to CD44 at the site identical to, or overlapping with the hyaluronic binding site of CD44, since serglycin and HA competed for

the binding to CD44. Furthermore, the "inducible" antibody, IRAWB14, activating the HA-binding potency of CD44 in T-cells also increased the binding of CD44 to serglycin (Toyama-Sorimachi et al., 1995). The ability of serglycin to exert the costimulatory signal on the CD3-activated cytotoxic T-cells resulting in the release of granzyme A (Toyama-Sorimachi et al., 1995) suggests the potential role for CD44-sergycin interaction in T-cell activation.

1.4 CD44 functions

1.4.1 Role of CD44 in pericellular matrix assembly

Several cell types such as chondrocyte (Knudson et al., 1996), fibroblasts (Hedman et al., 1979; Messadi and Bertolami, 1993; Gallivan et al., 1996; Yamagata et al., 1993) and different cells of embryonic origin (Deyst and Toole, 1995; Knudson and Toole, 1985) are able to assemble a hyaluronate-rich pericellular matrix. For chondrocytes, the main cartilage cell type, a pericellular matrix is necessary for organizing the structure of cartilage. For fibroblasts, a hyaluronate-rich pericellular matrix may be important for their locomotion (Turley, 1989). Many other cells, for example tumour cells, do not have an endogenous pericellular matrix but are able to form one in the presence of exogenously added hyaluronan and aggregating proteoglycans (Knudson and Knudson, 1991). CD44 was found to be the main anchoring molecule, organizing and retaining pericellular matrix around these cells. An anti-CD44 antibody blocked the pericellular matrix assembly by chondrocytes (Knudson et al., 1996) and the transfection of COS-7 cells with CD44 (a standard as well as an epithelial form) conferred to these cells the capacity to organze a hyaluronan-dependent pericellular matrix in presense of exogenous HA and proteoglycan (Knudson et al., 1993). The function of CD44 in pericellular matrix assembly does not require any intracellular signalling, since both alive and glutaraldehyde fixed CD44-transfected cells showed the ability to organize the matrix (Knudson et al., 1991).

1.4.2 Role of CD44 in hemopoiesis

CD44 is abundantly expressed on all bone marrow hematopoietic progenitor cells of all cell lineages (Kansas et al., 1990; Lewinsohn et al., 1990), which suggests a role for CD44 in hemopoiesis. The first functional evidence for involvement of CD44 in hemopoiesis came from experiments with an antibody that inhibits binding of B-lineage precursors to stromal cells and which was found to recognize CD44. This antibody was able to block myelo- and lymphopoiesis *in vitro* in the long term bone marrow cultures (Miyake et al., 1990). The ligand for CD44 in the interaction between hematopoietic cell and stromal cells is not yet identified. Despite high CD44 expression, only a minor (13,3%) subpopulation of $CD34^+$ hematopoietic progenitors was able to bind HA, though it was possible to augment the HA binding capacity with PMA, cytokines or "inducing" antibody (Legras et al., 1997). On the model of erythroid leukemic cell line, the rossete formation between the undifferentiated erythroid progenitors with hematopoietic stromal cells was shown to be mediated by CD44 but not by CD44-dependent HA binding (Sugimoto et al., 1994). One possible candidate CD44 ligand in the adhesion of hematopoietic progenitors to stroma is fibronectin. Human hematopoietic colony-forming cells bound to fibronectin present in stromal extracellular matrix via both $\alpha_1\beta_4$ integrin and CD44. The synergistic effect of anti-CD44 and anti-integrin antibodies in inhibiting this adhesion suggested cooperation between these receptors (Verfaillie et al., 1994). Furthermore, VLA-4 and VLA-5 integrins and CD44 in B-lineage lymphoblastic leukemia cells grown on a bone marrow fibroblast layer were situated in the lymphoblast-fibroblast attachment sites in a close proximity with fibronectin (Murti et al., 1996).

From the experiments of Miyake and collegues, described above (Miyake et al., 1990) it follows that the major reason for the inhibiting effect of anti-CD44 antibody on hemopoiesis *in vitro* might be the impaired attachment of hematopoietic progenitors to the bone marrow stromal cells. However, further investigations revealed that the function of CD44 in hemopoiesis is not limited only by cell adhesion, and that different isoforms of CD44 could be responsible for different steps in the development of hematopoietic progenitors. In the rat system, anti-CD44s but

not anti-CD44v6 antibodies inhibited the settlement of hematopoietic precursor cells on bone marrow stromal elements, whereas both antibodies interferred with progenitor maturation (Khaldoyanidi et al. 1997). This suggests that CD44v6 has a function which is different from cell-cell attachment. Similarly, in human long term bone marrow culture both anti-CD44v6 antibodies and an antibody against the N-terminal part of CD44 inhibited myelopoiesis, but only the latter antibody affected myeloid colony formation (Moll et al., 1998). In addition, anti-CD44 antibodies which enhance the adherance of progenitors to stromal cells (Oostendorp et al., 1998) or which increase the number of colony-forming bone marrow cells in long term culture (Ghaffari et al., 1997; Rossbach et al., 1996) have been described. It is therefore not surprising that application of various anti-CD44 antibodies in *in vitro* studies resulted in completly different effects, from depressing the hemopoiesis (Zöller et al., 1998) to its facilitation (Rösel et al., 1999; Rossbach et al., 1996).

1.4.3 Role of CD44 in the immune system

1.4.3.1 CD44 in the homing of naive lymphocytes to the peripheral lymphoid tissue

Mature lymphocytes in the absence of antigenic challenge continuously recirculate between the blood stream and secondary lymphoid tissues such as peripheral lymph nodes, Peyer's patches, tonsil and spleen. This trafficking of the naive lymphocytes is different from the memory and effector lymphocytes paths (Mackay et al., 1990), since the memory and effector lymphocytes also extravasate into extralymphoid tissues at the site of inflammation in addition to the secondary lymphoid organs, with a restricted tissue-specific pattern of recirculation (Butcher and Picker, 1996). Both naive and memory/effector lymphocyte immigration from blood is tightly regulated at the level of their interaction with high endothelial venules (HEV) of either secondary lymphoid organs or inflamed tissues. The successful lymphocyte - HEV interaction, so-called homing, discriminates between different lymphocyte subsets, allowing some of them to pass the blood vessel barrier and enter the particular tissue.

Differential lymphocyte homing to high endothelial venules (HEV) of lymph node (LN) or Peyer's patches (PP) has been detected in mice (Butcher et al., 1980), rat (Chin et al., 1986) and in humans (Jalkanen et al., 1986a). It was shown by selective inhibition of lymphocyte attachment to frozen HEV sections from various lymphoid tissue by different subsets of antibodies. In addition, a distinct mechanism for human lymphocyte interaction with HEV at the inflammatory site has been suggested (Jalkanen et al., 1986b). A cross-immunoprecipitation study with antibodies or antiserum inhibiting different types of lymphocyte homing in humans revealed that they recognise distinct epitopes of the same Hermes-1 antigen (Jalkanen et al., 1987), later identified as CD44 (Picker et al., 1989a). However, antibodies interfering with HA binding by CD44 (Miyake et al., 1990) failed to inhibit mouse and hamster lymphocyte homing to lymph node and mucosal HEV (Culty et al., 1990). Therefore another CD44 ligand might be invoved in interaction of lymphocytes to LN and PP HEVs.

There is an obvious difference in the molecules involved in naive lymphocyte homing between humans and mice. An antibody which selectively blocked mouse lymphocyte homing to LN HEV, MEL-14 (Gallatin et al., 1983) crossreacted with human CD44 and partially blocked human lymphocyte homing to LN but not to PP HEV (Jalkanen et al., 1986b; Jalkanen et al., 1987). However, it turned out to recognise not a mouse CD44 homologue, but mouse L-selectin (Stamenkovic et al., 1989; Siegelman and Weissman, 1989), suggesting that in mice L-selectin plays a role of „homing receptor" for naive lymphocytes.

More direct evidence that CD44 is not involved in mouse naive lymphocyte homing to HEV came from *in vivo* experiments (Camp et al., 1993), where the lymphocytes which have lost their surface CD44 as a result of anti-CD44 ab administration *in vivo*, homed normally to the peripheral and mesenteric lymphoid organs and spleen upon injection into healthy mice.

Thus, although CD44 was initially described as a homing receptor, normal lymphocyte homeostasis in healthy mice does not seem to be dependent on CD44.

1.4.3.2 CD44 in the inflammation process

In a range of experiments employing anti-CD44 antibody administration *in vivo*, evidence is accumulating that CD44 plays a role in inflammatory processes. 2,4 dinitro-1-fluorobenzene (DNFB) - induced Delayed-Type Hypersensitivity (DTH), measured by the degree of swelling of the inflamed ear, was inhibited in the early phase of DTH by two different anti-CD44 antibodies, both inducing a transient loss of CD44 from the lymphocytes due to shedding. The anti-CD44 antibody treatment reduced both edema and lymphocyte infiltration at the DTH site. Strikingly, the few cells infiltrating the inflamed tissue appeared to re-express CD44 (Camp et al., 1993). The inhibition of both edema and leukocyte infiltration in the DNFB-induced delayed-type hypersensitivity, as well as an inhibition of Bacillus Calmette Guerin (BCG)-induced granuloma formation was observed in mice treated with either anti-CD44s or anti-CD44v10 antibodies (Rösel et al., 1997). Treatment of arthritic mice with anti-CD44 antibodies resulted in a similar picture: anti-CD44 antibody administration induced the loss of CD44 from both leukocytes and synovial cells and abrogated tissue swelling and leukocyte infiltration (Mikecz et al., 1995). Furthermore, injection of anti-CD44 antibody inhibited the migration of fluorescently-labelled inflammatory T-lymphocytes into the site of SEB-induced inflammation, in a short-term *in vivo* homing experiment (DeGrendele et al., 1997a). All these data show that CD44 is important for leukocyte emigration into inflammatory tissue and for the progression of the immune response.

Many *in vivo* studies suggest the involvement of CD44-dependent HA binding in the inflammatory process. Several anti-CD44 antibodies had different effects on the severity of proteoglycan-induced mouse arthritis. The administration of the mice with KM81, an antibody which blocks the interaction of CD44 with HA, reduced the inflammatory symptoms, whereas the treatment with IRAWB14 antibody, which enhances HA binding, aggravated the disease (Mikecz et al., 1999). In short-term homing experiments, the influx of fluorescently labelled inflammatory lymphocytes into an inflamed peritoneum was inhibited both by hyaluronidase treatment and by administration of KM81 but not KM703 anti-CD44 antibody (the latter does not affect the CD44-

HA interaction). In addition, the depletion from the T-lymphocyte pool of HA-binding T-lymphocytes abrogated a T-cell homing to the inflamed peritoneum (DeGrendele et al., 1997a).

1.4.3.3 CD44-hyaluronic acid interaction in leukocyte extravasation into inflamed tissues

The *in vivo* data suggest that CD44 and its binding to HA are required at some stage of development of imflammation. Perhaps one of the most investigated processes which might involve CD44-HA interaction in inflammation is the transendothelial extravasation of T-lymphocytes into inflammatory tissues.

Leukocyte extravasation through the endothelium of the blood vessel includes several steps: primary adhesion (rolling), activation, secondary (firm) adhesion and transendothelial migration. Primary adhesion is performed under the flow stress from the blood stream and is known to be mediated by selectins and their carbohydrate ligands. Generally, expression of L-selectins and E- and P-selectin ligands on leukocytes are constant, but their counterpartners on endothelial cells are upregulated during inflammation. This transient, unstable but repeatable interaction, visualised as a rolling of the leukocyte along the endothelial surface, slows down the leukocyte motion at the site of inflammation and allows it to receive the activation and chemotactic signals produced by the surroundings. The subsequent activation of the leukocytes leads to an alteration in the pattern of leukocyte adhesive molecules. Integrins, the main players of the secondary adhesion, become upregulated and activated to bind their couterpartners, members of the Ig-like superfamily. As the result, cells attach firmly to the endothelium. Finally, the co-operative interaction between leukocytes and endothelial cells leads to the migration of the leukocytes across the endothelium (Butcher, 1991; Mackay and Imhof, 1993).

The CD44-HA interaction was shown to support rolling of some lymphoid cell lines and activated T-lymphocytes on HA-coated surfaces and stromal cell monolayers under shear stress, resembling the physiologic flow conditions in blood vessels (DeGrendele et al., 1996; DeGrendele et al., 1997b; Clark et al., 1996; Lesley et al., 1997). Induction of rolling on HA coated plates correlated with induction of soluble HA binding in both antigen-specific and

nonspecific (PMA, anti-CD3 ab) stimulated T-cells, while the unstimulated T-cells showed none of these effects, in agreement with previous findings that CD44-dependent HA binding of primary T-lymphocytes requires stimulation (Lesley and Hyman, 1992; Galandrini et al., 1994; Galluzzo et al., 1995). Importantly, during an *in vivo* immune response, activated T cells acquire the ability to bind HA (Lesley et al., 1994). On the other hand, endothelial cells are also affected by inflammation, as both TNFα, a potent mediator of inflammatory process and Il-1β, a cytokine produced in a Th1- type of immune response, are able to induce synthesis of HA in some endothelial cell lines and in primary microvascular endothelial cultures, increasing their ability to support lymphocyte rolling and adhesion under shear stress conditions (Mohamadzadeh et al., 1998). Taken together, these data suggest that the CD44-HA interaction is an adhesion event which may be involved in leukocyte extravasation through the microvascular endothelium at the inflammatory site.

1.4.3.4 CD44 in T-lymphocyte activation

Another thoroughly studied function of CD44 which may be important for inflammatory reactions is the costimulatory role of this molecule in T-lymphocyte activation. The triggering of TCR by antigen/MHC complex or by TCR complex-crosslinking agents alone was shown to be insufficient to stimulate the full activation of resting T cells, and the need for a costimulatory signal, for example via CD28 or IL-1 receptor stimulation, is widely appreciated (Weiss A., 1993). CD44 was shown to exert or enhance such a costimulatory signal in some cell models. A panel of different anti-CD44 antibodies was able to enhance CD3 or CD2 mediated induction of proliferation in human peripheral T-cells (Shimizu et al., 1989). Furthermore, a proliferation of CD3-triggered human peripheral T-lymphocytes as well as IL-2 production by human T-cell clones was augmented by the CD44 ligand, HA. In some of these clones, treatment with HA alone resulted in T-cell proliferation (Galandrini et al., 1994). In a mouse system, an anti-CD44 antibody was costimulatory for CD3-induced IL-2 production and proliferation of freshly purified splenic $CD4^+$ T-cells and in addition showed a synergistic effect with an anti-CD28 antibody

(Sommer et al., 1995). On other hand, there are the data about an inhibiting effect of anti-CD44 antibodies on T-cell activation. The anti-CD44 antibody 212.3 inhibited the anti-CD3 antibody-induced T-cell proliferation but augmented the proliferation of T-cells induced by antibody against different CD2 epitopes (Rothman et al., 1991). Similarly, of several different anti-CD44 antibody which enhance the proliferative response of human peripheral blood lymphocytes on anti-CD2 antibody, some showed an inhibitory effect on anti-CD3 induced T-cell proliferation (Guo et al., 1994).

The presumable costimulatory signal for the TCR by CD44 on lymphocytes must occur naturally during antigen presentation process through CD44 crosslinking by some surface molecule on the antigen-presenting cell (APC). Alternatively, during APC driven T-cell activation CD44 can be triggered by HA fragments which are accumulated under inflammatory conditions (Sampson et al., 1992; Evered and Whelan (eds), 1989). However the significance of CD44-mediated costimulatory or inhibitory events for T-cell proliferation and cytokine production during an *in vivo* immune response is not known.

1.4.3.5 CD44 and hyaluronic acid in macrophage stimulation

An important role in a progression of inflammation belongs to macrophages. They not only unspecifically eliminate antigens by phagocytosis but also produce a range of inflammatory cytokines. Among those are mediators of inflammation such as interferon γ (INF γ), interleukin-1 β (IL-1β), tumour necrosis factor α (TNFα), as well as chemotactic proteins interleukin-8 (IL-8), intereukin-12 (IL-12) and others. HA, in particular its low molecular weight fragments (LMW-HA) was able to induce TNFα, IL-1β and insulin-like growth factor-1 (IGF-1) production in bone marrow-derived murine macrophages, and this effect was blocked by anti-CD44 antibody KM201 (Noble et al., 1993). The list of HA-dependent cytokines was further extended by MIP-1α, MIP-1β and RANTES, whose production in a murine alveolar macrophage cell line MH-S was induced by LMW-HA. Both HA binding and, partially, the HA-induced cytokine synthesis in MH-S cells was inhibited by an anti-CD44 antibody (McKee et al., 1996). In thioglycollate-

elicited but not resident peritoneal murine macrophages LMW-HA stimulated the production of MIP-1α, MIP-1β, RANTES and IL-12. The induction of IL-12 could be blocked with KM81, an anti-CD44 antibody interfering with HA binding, but not with the anti-CD44 antibody IM7 (Hodge-Dufour et al., 1997). Together with the above mentioned accumulation of small HA fragments at the inflammation site, these data suggest that interaction of low molecular weight HA with CD44 in macrophages can serve as a potent regulatory signal in inflammation.

1.4.4 Role of CD44 in cytokine and growth factor presentation

Many cytokines and growth factors in addition to their cognate receptors display affinity to the various heparan sulfated and condroitin sulfated proteoglycans. Proteoglycans immobilised on endothelial cell surface are thought to capture the inflammatory cytokines, localising them despite the blood flow. Proteoglycans may present the concentrated cytokines to immune cells, thus facilitating a local immune response. Furthermore, proteoglycans are proposed to activate bound cytokines and protect them from degradation (Tanaka et al, 1998). The role of proteoglycans in regulating growth factor action is even more intriguing. It is well established that many growth factors require binding to heparin or a heparan sufated proteoglycan (HSPG) in order to exert their biological activity on the responsive cells (Rapraeger, 1993; Vlodavsky et al., 1996). Besides presentation of growth factors to their high affinity receptors, HSPGs have been implicated in internalization, intracellular distribution and turnover of growth factors, as well as in the protection of growth factors from protease cleavage in the extracellular enviroment (Taipale and Keski-Oja, 1997; Vlodavsky et al., 1996).

Heparan sulfate chains present on proteoglycans are highly heterogeneous in respect to their sugar combination and level of sulfatation (Lindahl et al., 1998). This results in the specificity of different cytokines and growth factors in binding to some particular heparan sulfate-containing proteoglycans. As mentioned before, the variant of CD44 containing sequences encoded by exon v3 can be modified by heparan sulfate, in addition to the chondroitin sulfate chains found on different CD44 isoforms (Jackson et al., 1995; Brown et al. 1991). This

proteoglycan form of CD44 was shown to bind MIP-1β, the chemotactic and activating agent for T-lymphocytes which is produced by inflammatory macrophages, and to present the MIP-1β to T-lymphocytes. MIP-1β, immobilised on CD44-coated plastic was able to stimulate T-cell adhesion to VCAM-1 (Tanaka et al., 1993). Interferon-γ was found to be associated with a chondroitin sulfated CD44 isoform, and this association was necessary for the normal response of human arterial smooth muscle cells to this cytokine (Hurt-Camejo et al., 1999). In another study, an artificial proteoglycan composed of the extracellular domain of LFA-3 combined with the CD44 motif modified with CS and HS bound the inflammatory cytokine RANTES, and the binding was mediated by both HS and CS chains (Wolff et al., 1999). These data suggest a potential role for CD44 in cytokine presentation in inflammatory tissues.

The suggested role for CD44 in the presentation of inflammatory cytokines would require the expression of glycosaminoglycan-modified CD44v3 on the inflammatory endothelium. The data about CD44v3 expression in this tissue are controversial. Bennett and collegues found CD44v3 neither in cultured activated endothelial cells nor in the inflamed skin vascular endothelium on tissue sections (Bennett et al., 1995a). However other authors reported endothelial CD44v3 expression (Koopman et al., 1998).

Fibroblast growth factors (FGFs) require the interaction with HSPGs for their successfull signalling via the high affinity tyrosine kinase receptors (Szebenyi and Fallon, 1999). The heparan sulfated form of CD44 was reported to bind basic fibroblasts growth factor (bFGF) (Bennett et al., 1995a). This brings the v3-containing proteoglycan form of CD44 into the family of low affinity receptors for the fibroblast growth factors. CD44 was also found to interact with heparin-binding epidermal growth factor (HB-EGF) (Bennett et al., 1995a), and hepatocyte growth factor/scatter factor (HGF/SF) (van der Voort et al., 1999). HGF/SF association with CD44 was implicated in a c-met mediated signalling (van der Voort et al., 1999), which is proposed to be responsible for the propagation of some tumours such as multiple myeloma (Seidel et al., 1998). The interaction of CD44 with bFGF or HB-EGF is likely to be involved in the regulation of keratinocyte growth. Both bFGF and HB-EGF-induced proliferation of keratinocytes was impaired in transgenic mice which expressed bearing antisense CD44 under the control of a

keratinocyte-specific promoter. Furthermore, both TPA and carcinogen application failed to stimulate the keratinocyte proliferation in these mice, in contrast to the wild-type controls, indicating that CD44 is important for pathological reactions in the mouse skin (Kaya et al., 1997).

1.4.4.1 Role of CD44 in limb development

A new role for CD44 splice variants containing exon v3 in limb morphogenesis was recently proposed (Sherman et al., 1998). This form of CD44 was shown to interact with FGF4 and FGF8 via its heparan sulfate chain and present these growth factors to the mesenchymal cells in thereby inducing their FGF-dependent proliferation (Sherman et al., 1998).

In the developing mouse and rat embryo CD44 is expressed in a restricted pattern (Wheatley et al., 1993; Wainwright D., 1998). In the limb bud of day 10,5-11,5 mouse embryos the high CD44v3 expression is co-localized with the area of expression of FGF4 and FGF8 in the apical ectodermal ridge (AER). (Niswander and Martin, 1992; Heikinheimo et al., 1994; Wainwright D., 1998). The AER is of particular importance for the limb development. This tiny tissue promotes the proliferation of the underlying mesenchyme and maintains the activity of polarizing zone, thus playing a crucial role in limb patterning and outgrowth (Kosher et al, 1979; Saunders J.W., 1972). While removal of AER results in the cessation of the limb outgrowth (Rubin and Saunders et al., 1972), ectopic application of either FGF4 or FGF8 is able to compensate for the absence of AER both *in vivo* and *in vitro* (Niswander et al., 1993; Vogel et al., 1996), indicating that the FGFs are key factors in the cross-talk between AER and mesenchymal cells.

The interaction between FGFs and FGFRs and consequently the specific cell response to the growth factors is regulated by the HSPGs (reviewed in Szebenyi and Fallon, 1999). The role of CD44 in the FGF-responsiveness of limb bud mesenchymal cells was shown in an *in vitro* coculture assay, where an anti-CD44v6 antibody inhibited the AER-induced proliferation of cultured limb bud mesenchymal cells. More directly, CD44v3-10 but not CD44s transfected Namalwa cells were able to support the FGF4 or FGF8-induced proliferation of mesenchymal

cells, and this effect was abolished by anti-CD44v3 and anti-CD44v6 antibodies (Sherman et al., 1998). The importance of CD44 for the FGF/FGFR interaction in limb development was further demonstrated, as an antibody specific for the epitope v6 of CD44, but not an anti-pan CD44 antibody blocked limb outgrowth when applied to the AER *in vivo.*

1.5 Generation and characterisation of CD44-deficient mice

To study the multifunctional role of CD44 *in vivo*, gene-targeting technology was used to disrupt the single gene coding for all possible isoforms of CD44 (Schmits et al., 1997). In this approach, a „neo“ cassete in antisense orientation was inserted between a 5’ Nco-I fragment of exon 2 and the 3’ intronic region of exon 3, replacing a 3’-part of exon 2 and the complete exon 3 sequence of CD44. The construct was introduced into the E14 embryonic stem cell line, and recombinant clones were injected into C57Bl/6J blastocysts and transferred into CD1 foster mother. The progenies of the chimeras were later backcrossed into C57Bl/6J mice. This strategy abolished the expression of virtually all CD44 isoforms in homozygous offspring (Schmits at al., 1997).

Homozygous CD44-mutant mice developed normally, without any overt abnormalities in embryos or adult animals. They exhibited a normal T- and B-cell subset distribution. There was no alteration in serum immunoglobulin level, irrespective of subclasses. The IgM and IgG level in the anti-virus response was also identical to that of wild-type controls, suggesting that the B-cell functions are normal. The examination of T-cell reactions, such as proliferation *in vitro* upon treatment with anti-CD3 antibody, ConA or superantigen stimulation, the allogenic response in a mixed lymphocyte reaction and virus-directed NK and and T-cell mediated cytotoxicity did not reveal any defect in the CD44-deficient mice. Furthermore, the *in vivo* inflammatory reaction of the CD44 mutant mice after 2,4 dinitro-1-fluorobenzene (DNFB) application did not differ from control mice. The CD44-deficient mice displayed an increased number and size of granulomas in response to challenge with intracellular bacteria *C. parvum* compared to wild -type mice. Thus the immune system function was largely unimpaired in CD44-negative mice.

A defect in CD44-mutant mice was observed in the hemopoietic system, as evidenced by the reduced egress of hematopoietic progenitors from bone marrow and diminished mobilisation of these cells in the spleen as well as in peripheral blood. This was especially apparent after stimulation of the animals with granulocyte colony-stimulating factor (G-CSF), which may reflect impaired cytokine production or presentation during hematopoietic progenitor emigration from the bone marrow in CD44-deficient mice.

Enhanced tumorigenicity was found in SV40 large T antigen-transformed embryonic fibroblasts from CD44-negative animals compared with the same cells with reconstituted CD44 expression. When injected subcutanuosly in BALB/c nude mice, the CD44-negative transfected fibroblasts gave a rise to much bigger tumours than did CD44-reconstituted cells over the same period of time.

The paradoxically mild phenotype of mice lacking CD44, together with the previousely described multiple roles of this molecule in various systems suggest that CD44-mutant mice may develop one or several compensatory mechanisms for the lack of CD44 which take over CD44 functions during embryonic development and immunity. To determine if it is the case, a detailed examination of how normally CD44-dependent functions are carried out in CD44-deficient mice is required.

1.6 Aims of the PhD thesis

The aim of this work was to investigate of whether mechanisms exist in CD44-mutant mice which compensate for the lack of CD44. Specifically, two normally CD44-dependent functions - HA binding of various cell types and growth factor presentation during limb development - were examined in CD44-deficient mice.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 General chemicals

Agarose	Peqlab, Erlangen
Alcian blue 8 GX	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
Biotin-LC-Hydraside	Pierce, Sankt Augustin
2-β Mercaptoethanol	Roth, Karlsruhe
Brewer thioglycollate medium	Difco, Hamburg
Bromphenol blue	Serva, Heidelberg
BSA (Bovine Serum Albumin)	Serva, Heidelberg
Carbozole	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
Coomassie brilliant blue G-250	Serva, Heidelberg
Crystal violet	Sigma, Deisenhofen
Dibutyltin dialurate	Fluka, Neu-Ulm
DMSO (Dimethyl sulfoxide)	Fluka, Neu-Ulm
DOC (Deoxycholate)	Sigma, Deisenhofen
DTT (Dithiothreitol)	Sigma, Deisenhofen
EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodi-imide hydrochloride)	Pierce, Sankt Augustin
EDTA	Merck, Darmstadt
EGF, human, recombinant	Sigma, Deisenhofen
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Deisenhofen
FGF8b, mouse, recombinant	R&D system
Fluorescein isothiocyanate isomer I	Fluka, Neu-Ulm
Folin-Ciocalteu reagent	Merck, Darmstadt
Formamid	Merck, Darmstadt
G418	Gibco, Eggenstein
Glycerol	Merck, Darmstadt
Heparan sulfate	Sigma, Deisenhofen
HEPES	Roth, Karlsruhe
Hyaluronic acid, from Rooster Comb	Sigma, Deisenhofen
Interleukin-1	Sigma, Deisenhofen
Interleukin-5, mouse, recombinant	Calbiochem, Bad Soden
Isopropanol	Merck, Darmstadt
Leupeptin	Sigma, Deisenhofen
L-Lysine	Boehringer, Mannheim
LPA (L-α-Lysophosphatidic acid)	Sigma, Deisenhofen
Lipopolysacharide (LPS)	Sigma, Deisenhofen
Methanol	Roth, Karlsruhe
2-N-Morpholino ethanesulfonic acid	Fluka, Neu-Ulm
Nonidet P-40	Boehringer, Mannheim
Phenol red	Merck, Darmstadt
PMSF	Sigma, Deisenhofen
Polibrene	Sigma, Deisenhofen
Ponceau S	Sigma, Deisenhofen

Roti®Phenol	Roth, Karlsruhe
Rotiphorese® Gel 30: Acrylamide/*bis*-acrylamide (30% / 0,8%)	Roth, Karlsruhe
TEMED	Sigma, Deisenhofen
TNFα	Calbiochem, Bad Soden
TPA	Sigma, Deisenhofen
Tris	Roth, Karlsruhe
Triton X-100	BioRad, München
Tween-20	Serva, Heidelberg
Xylene Cyanol	Serva, Heidelberg

All other general chemicals were purchased from Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) or Sigma (Deisenhofen).

2.1.2 Enzymes

Dispase I	Boehringer, Mannheim
Heparinase II	Sigma, Deisenhofen
Hyaluronidase, from Bovine Testis, type VI	Sigma, Deisenhofen
Proteinase K	Merck, Darmstadt
Restriction endonucleases	New England Biolabs, U.K.
RNase A	Sigma, Deisenhofen

2.1.3 Radiochemicals

[α^{32}P]-dCTP	Amersham Buchler GmbH, Braunschweig
[6-^{3}H]-thymidine	Amersham Buchler GmbH, Braunschweig
L-[^{35}S]-methionine/cysteine mix	Amersham Buchler GmbH, Braunschweig

2.1.4 Other materials

DE81 DAE-cellulose membrane	Schleicher & Schuell, Dassel
3MM Whatman paper	Whatman, Maidstone, U.K.
GeneScreen Plus® hybridisation transfer membrane	NEN® Research Product, USA Amersham Life Science, Braunschweig
RediPrime DNA labelling system	Schleicher & Schuell, Dassel
Elutip-d Minicolumns	Millipore, Eschborn
Immobilon-P membrane	Amersham Life Science, Braunschweig
ECL western blotting detection reagents	Polygram® Sil G
Thin Layer Chromatography Plates, 0,25 mm	Miltenyi Biotech, Bergisch Gladbach
MS^{+} magnetic separation columns	Falcon,USA
Plastic sieves, 70 μm pore size	

2.1.5 Solutions

YENB	0,75% 0,8%	Bacto yeast extract Bacto nutrient broth
SOC	20 g/l 5 g/l 0,5 g/l 2,5 mM 10 mM 10 mM 2 mM	Bacto trypton Yeast extract NaCl KCl $MgCl_2$ $MgSO_4$ Glucose
LB	10 g/l 5 g/l 10 g/l 1 g/l	Bacto trypton Yeast extract NaCl Glucose
2 x TY	20 g/l 10 g/l 5 g/l	Bacto trypton Yeast extract NaCl
LB agar (for bacterial plates)	1% 0,5% 1% 1%	Bacto trypton Yeast extract NaCl Agar
1 x TE	10 mM 1 mM	Tris HCl, pH=8,0 EDTA
1 x TAE	20 mM 10 mM 1 mM	Tris HCl Acetic acid EDTA pH=8,3
10 x TBE	0,9 M 0,9 M 24 mM	Tris Boric acid EDTA pH=8,3
20 x SSC	3 M 0,3 M	NaCl Na_3citrate pH=7,0
Church-Gilbert buffer	1 mM 7% 0,5 M	EDTA SDS Na_2HPO_4 pH=7,2, ajusted with H_3PO_4
2 x protein sample buffer	160 mM 4% 16% 0,57 M 0,01%	Tris HCl, pH=6,8 SDS Glycerol 2-β mercaptoethanol Bromphenol blue

RIPA buffer	50 mM	Tris HCl, pH=7,2-8,0
	150 mM	NaCl
	1 %	Nonidet P-40
	0,5 %	DOC
PBS	8 g/l	Na Cl
	0,2 g/l	KCl
	0,1 g/l	$CaCl_2$ x 2 H_20
	0,13 g/l	$MgCl_2$ x 6 H_20
	1,15 g/l	Na_2HPO_4 x 2 H_20
	0,2 g/l	KH_2PO_4
2 x HBS	280 mM	NaCl
	1,5 mM	Na_2HPO^4
	50 mM	HEPES, pH=7,1
ACK buffer	0,15 M	NH_4Cl
	1 mM	$KHCO_3$
	0,1 mM	EDTA
		pH=7,2-7,4

2.1.6 Cell culture reagents

Trypsin, 0,25%	Difco, Detroit
DMEM	GibcoBRL Life Technologies, Scotland
RPMI 1640	GibcoBRL Life Technologies, Scotland
AlfaMEM	GibcoBRL Life Technologies, Scotland
Methionin-free DMEM	Sigma, Deisenhofen
FCS	Bio-Whittaker, Belgium
Penicillin	GibcoBRL Life Technologies, Scotland
Streptomycin	GibcoBRL Life Technologies, Scotland
Glutamax	GibcoBRL Life Technologies, Scotland
Sodium pyruvate	GibcoBRL Life Technologies, Scotland

2.1.7 Cell lines

cell line	source and property	growth medium
HA9	a clone from a mouse T-cell lymphoma cell line (LB), constitutively binding hyaluronic acid (a kind gift of D.Naor)	RPMI 1640, 10%FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (pH=7,4), 1% non-essential amino acids, 1 mM sodium pyruvate, 1 µg/ml insulin, 0,05 mM 2-β mercaptoethanol

MS5	a preadipocyte hemopoietic stromal cell line (Itoh et al., 1989), known to bind HA via CD44	alfa MEM, 10% FCS, 2 mM L-glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin
10AS	a cell line derived from the fast growing non-metastasizing variant of spontaneous rat tumour (BSp73) tissue (Matzku et al., 1983)	RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin
ASML	a cell line derived from the slow growing metastasising variant of spontaneous rat tumour (BSp73) tissue (Matzku et al., 1983)	RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin
NIH3T3	Mouse embryonic fibroblast cell line (ATCC CRL 1658)	DMEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin
Ras-NIH3T3	transformed NIH3T3, a result of stable transfection of NIH3T3 with Ras^{leu-61} (a kind gift of H.Morrison)	DMEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml puromycin
ωE	Helper-free mouse ecotropic packaging cell line for the retrovirus production (Morgenstern and Land, 1990).	DMEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin

2.1.8 Antibodies

CD44-FITC	IM7, rat IgG_{2b}, against pan-CD44 epitope	PharMingen, Hamburg
CD44-PE	IM7, rat IgG_{2b}, against pan-CD44 epitope	PharMingen, Hamburg
CD44-Cy-Chrome®	IM7, rat IgG_{2b}, against pan-CD44 epitope	PharMingen, Hamburg
CD69-PE	H1.2F3, armenian hamster IgG, against mouse CD69	PharMingen, Hamburg
CD45R/B220-PE	RA3-6B2, rat IgG_{2a}, against mouse CD45R	PharMingen, Hamburg
CD4-PE	RM4-5, rat IgG_{2a}, against mouse CD4	PharMingen, Hamburg
CD8-FITC	53-6.7, rat IgG_{2a}, against α chain of mouse CD8	PharMingen, Hamburg
Mac-1/CD11b-PE	M1/70, rat IgG_{2b}, against mouse α_M subunit of Mac-1, crossreacts with human.	PharMingen, Hamburg
Thy 1.2 coupled to		Miltenyi Biotech,

the magnetic beads		USA
KM81	KM81, against pan-CD44 epitope, interfering with HA binding by CD44	from J. Moll
CD44	IM7, rat IgG_{2b}, against pan-CD44 epitope	from J. Moll
CD3ε	145-2C11, Armenian hamster IgG	PharMingen, Hamburg
anti-Δ HS (3G10 epitope)	F69-3G10, mouse IgG_{2b}, against a heparan sulfate neo-epitope, generated by digesting heparan sulfate with heparinase	Seikagaku corporation, Japan
anti-HS	HepSS-1, mouse IgM, against heparan sulfate	Seikagaku corporation, Japan
anti-Erk-1,2	K-23, a rabbit polyclonal IgG against the 305-327 amino acids of rat Erk-1	Santa Cruz, Kalifornia
anti-β tubulin	MAB380, mouse IgM	Chemicon, CA
rabbit anti - mouse IgM, biotinylated		DACO, Denmark
goat anti-mouse F‘(ab)-HRP	goat polyclonal, against F'(ab) fragment of mouse IgG; adsorbed with human IgG	Sigma, Deisenhofen
hamster γ-globulin		Dianova, Hamburg

2.1.9 Plasmids

CD44-probe containing plasmid: bluescript 129 with a cloned CD44 probe, corresponding to the flanking 500 bp Kpn-I fragment upstream of exon 2 of CD44 (a kind gift of R. Schmits)

pcDNA3.1+ , Invitrogen

pZip *ras-myc* plasmid: a murine retrovirus shuttle vector containing the v-*Ha-ras* and avian myelocytomatosis virus-derived *gag-myc* oncogens (a kind gift of H. Land)

2.2 Methods

2.2.1 General methods

2.2.1.1 Preparation of electrocompetent bacteria

E.Coli DH5α bacteria were grown overnight in 10 ml of YENB (0,75% Bacto yeast extract, 0,8% Bacto Nutrient Broth). One liter of fresh YENB was inoculated with 5-10 ml of the overnight bacterial culture and grown at 37^0C with shaking until the OD_{600} was between 0,5 and 0,9. The flask was chilled for 5 min on ice and the culture was harvested by centrifugation at 4000 x g for 10 min at 4^0C. Pellet was washed twice in 100 ml of cold water and resuspended in 20 ml of cold 10% glycerol. After another centrifugation the supernatant was discarded and the bacteria were resuspended in a final volume of 2-3 ml of cold 10% glycerol, aliquoted into Eppendorf tubes, 40 µl per tube, and placed over dry ice until frozen. Electrocompetent bacteria were stored at -80^0C.

2.2.1.2 Transformation of electrocompetent bacteria

Before use, the aliquot of electrocompetent DH5α bacteria was thawed slowly on ice. 1 µl of supercoiled plasmid DNA (10-100 ng) was mixed on ice with 40 µl of the bacteria, incubated 1 min on ice, transferred into the pre-chilled electroporation cuvette (BioRad, gap 0,1 cm) and pulsed at 1,8 KV in the Bio-Rad E coli Pulser. Immediately after that 1 ml of SOC medium (20 g/l bacto trypton, 5 g/l yeast extract 0,5 g/l NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2 mM glucose) was added to the cuvette, mixed and together with bacteria tranferred into a 15 ml blue-cap tube. The cells in SOC were shaken at 37^0C for 30 min, concentrated by centrifugation for 2 min at 300 x g and plated on the LB-agar plates with antibiotics. Plates were incubated overnight at 37^0C and the bacterial colonies were detected on the next day.

2.2.1.3 Small scale plasmid DNA preparation (miniprep)

Individual colonies from the plated transfected bacteria were picked and grown in 2 ml of 2 x TY (10 g/l yeast extract, 20 g/l bacto tryptone, 5 g/l NaCl), or LB (10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/ml NaCl, 1 g/l glucose) medium containing 100 mg/ml of ampicillin for 8-12 h at 37^0C, with shaking. 1,5 ml of bacteria culture was pelleted at 4000 x g for 5 min. The pellets were resuspended in 100 µl of solution I (50 mM Glucose, 25 mM Tris-HCl (pH=8,0), 10 mM EDTA with 300 mg/ml RNase A) and incubated for 5 min at room temperature. 200 µl of solution II (0,2 M NaOH, 1% SDS) was added to each tube and mixed gently. 150 µl of solution II (3M sodium acetate, pH=5,2) was added to each mixture, mixed by gentle inversion of the tubes and left on ice for 15 min. The precipitated proteins were removed by centrifugation at 10000 x g for 15 min. Supernatants were collected in fresh tubes. DNA was extracted sequentially with phenol, phenol/chloroform (ratio 1:1) and chloroform and precipitated with 2,5 volumes of ethanol in the presence of 1/10 volume of 3,3 M sodium acetate, pH=5,2. Precipitates were washed with 70% ethanol, dried completely and dissolved in 50 µl of sterile water or 1 x TE buffer (10 mM Tris HCl, pH=8,0, 1 mM EDTA).

2.2.1.4 Large scale plasmid DNA preparation (maxiprep)

The large scale DNA preparation was done according to the Qiagen protocol and using the Qiagen solutions and columns. 500 ml of LB or 2 x TY medium containing 100 mg/ml ampicillin were inoculated with 0,5-2 ml of culture of transformed bacteria clone and allowed to grow for another 12-16 h at 37^0C, with shaking. Bacteria were harvested by centrifugation at 6000 x g for 15 min at 4^0C and resuspended in 10 ml of buffer P1 (50 mM Tris HCl, pH=8,0; 10 mM EDTA) containing 100 µg/ml RNase A. 10 ml of buffer P2 (200 mM NaOH, 1% SDS) was added to the resuspended cells and mixed gently, inverting the tube several times. After 5 min incubation at room temperature, 10 ml of cold buffer P3 (3,0 M potassium acetate, pH=5,5) was added, gently mixed and the reaction mixture was allowed to precipitate for 20 min. The sample was centrifuged at ≥20000 x g for 30 min, and the supernatant was loaded on a Qiagen-tip 500 column, equilibrated before with 10 ml of buffer QBT (750 mM NaCl, 50 mM 3-[N-Morpholino]propanesulfonic acid (MOPS), pH=7,0, 15% isopropanol, 0,15% Triton X-100). The

column was washed twice with 30 ml of buffer QC (1,0 M NaCl,50 mM3-[N-Morpholino]propanesulfonic acid, pH=7,0, 15 ml isopropanol) and DNA was eluted with 15% of buffer QF (1,25 M NaCl, 50 mM Tris HCl, pH=8,5, 15% isopropanol). DNA was precipitated at room temperature with 0,7 volume (10,5 ml) of isopropanol, mixed and centrifuged at ≥15000 x g for 30 min at 4^0C. The pellet was washed with 70% ethanol, air dried and dissolved in sterile water or 1 x TE (10 mM Tris HCl, pH=8,0, 1 mM EDTA).

2.2.1.5 Isolation of genomic DNA from tissue (for the genotyping)

A piece of tissue, e.g. the 2 mm piece of a mouse tail, was incubated overnight in 500 µl of lysis buffer (50 mM Tris HCl, pH=8,0, 100 mM NaCl, 100 mM EDTA, 1% SDS) containing 0,5 mg/ml of proteinase K, with shaking at 55^0C. The lysate was mixed for 5 min on the Eppendorf mixer, 167 µl of saturated NaCl solution (approximately 6 M) was added and the sample was mixed for 5 more min. The debries were pelleted by 7 min centrifugation at 15000 x g. The clear supernatant was transferred to the new tube and DNA was precipitated with 500 µl of isopropanol, washed with 70% ethanol, dried completely and dissolved in 50 µl of distilled water or 1 x TE (10 mM Tris HCl, pH=8,0, 1 mM EDTA).

2.2.1.6 Determination of DNA concentration in solution

The DNA concentration in the aqueous solutions was measured spectroscopically as an extinction at 260 nm wave length. OD_{260}=1 corresponds to 50 mg/ml double stranded DNA, 40 mg/ml of RNA or 20 mg/ml single stranded oligonucleotides. To estimate the purity of DNA or RNA preparation, the ratio OD_{260}/OD_{280} was calculated. Pure DNA solution have an OD_{260}/OD_{280} ratio of approximately 1,8; pure RNA solution - about 2,0.

2.2.1.7 Digestion of DNA with restriction endonucleases

Unless indicated otherwise, 1 µg DNA was digested with 2-3 units of the enzyme in the presence of an appropriate buffer, recommended by the supplier. The reaction was carried out at 37^{O}C for more than 1 h and terminated either by heat inactivating the enzyme for 10 min at 65^{0}C or by phenol/chloroform extraction.

2.2.1.8 Resolution of DNA on the agarose gel

A 0,75-2% agarose solution in TAE buffer (20 mM TrisHCl, 10 mM acetic acid, 1 mM EDTA, pH=8,3-8,5) containing 0,5 µg/ml of ethidium bromide was melted and allowed to harden in a horizontal gel chamber with the comb forming the appropriate loading wells. The gel was covered with TAE buffer and the comb was removed carefully. DNA samples were supplemented with 1/5 volume of 5 x DNA sample buffer (50% (vol/vol) glycerol, 25 mM EDTA, 0,25% bromphenol blue, 0,25% Xylene Cyanol) and loaded into the wells. DNA fragments were separated in the gel at 60-80 mA and visualised under UV light.

2.2.1.9 Isolation of DNA fragments from the agarose gel

After DNA fragments were electrophoretically separated in the agarose gel, a strip of DE81 DEAE-cellulose membrane (Schleicher & Schuell, Dassel) was inserted into the gel below desired DNA fragment, and the electrophoresis was continued until the chosen DNA fragment migrated quantitatively onto the membrane. The membrane was then carefully removed with forceps, rinsed briefly with distilled water and cut into pieces. They were placed in an Eppendorf tube and incubated with 400 µl of DEAE elution buffer (10 mM Tris HCl, pH=8,0, 1 mM EDTA, 1M NaCl) for more than 1 h at 65^{0}C, with shaking. The eluted DNA was then purified by phenol/chloroform extraction, precipitated in ethanol, washed twice with 70% ethanol and air dried. The pellet was dissolved in a small amount of water or TE buffer.

2.2.1.10 Southern blotting of the large DNA fragments onto membrane

The DNA was resolved on a 0,75% agarose gel under TAE buffer at 40-80 V. The gel was then removed from the elecrophoretic chamber and gently agitated with 0,25 M HCl for 20 min. Thereafter it was rinsed in deionised water and incubated with 0,5 M NaOH, 1,5 M NaCl for 30 min, agitating. Two sheets of 3MM Whatman paper soaked in 10 x SSC were carefully laid down in a flat chamber. The gel was soaked in 10 x SSC and placed on top of the paper. The GeneScreen Plus® hybridisation transfer membrane (NEN® Research Product, USA) equilibrated in 10 x SSC was put on top of the gel and covered with another two sheets of 3MM Whatman paper soaked in 10 x SSC. Care was taken that there are no air bubbles between the paper, gel and the membrane layers. A stack of paper towels was placed on top, followed by a glass plate, and all was pressed down with a suitable weight. After overnight blotting the membrane was removed, rinsed in distilled water and the DNA was fixed on the membrane by UV crosslinking in a UV stratalinker 2400 (Stratagene, La Jolla, CA).

2.2.1.11 Radioactive labelling of the DNA probe for hybridisation

A double stranded DNA probe for hybridisation was labelled according the Rediprime kit instructions (Rediprime DNA labelling system, Amersham Life Science). 25 ng of DNA in a volume of 45 µl was denatured at 95^0C for 5 min and added to the labelling mix provided in the kit. The reconstituted mixture was incubated together with 5 µl of [α^{32}P]-dCTP (370 MBq/ml, 10 mCi/ml, Amersham Buchler GmbH, Braunschweig) for 20-30 min at 37^0C. The labelled probe was then purified following the Elutip purification protocol (Schleicher & Schuell, Dassel, Germany). Briefly, the labelled mixture in 5 ml of low salt buffer (0,2 M NaCl, 20 mM Tris HCl, 1 mM EDTA, pH=7,4) was applied on the Elutip-d Minicolumn equilibrated before with this buffer, and the liquid was allowed to pass trough the column. The bound probe was washed twice with the low salt buffer and eluted with 500 µl of the high salt buffer (1 M NaCl, 20 mM Tris HCl, 1 mM EDTA, pH=7,4). The specific activity of the probe was measured on a Minibeta liquid scintillation counter. Before use, the probe was denatured at 95^0C for 5 min.

2.2.1.12 Hybridisation of Southern blot with a radiolabelled DNA probe

The membranes with the immobilized DNA were incubated for 1 h at 65^0C with 1 x SSC, 1% SDS and pre-hybridised in 10 ml of Church-Gilbert buffer (1 mM EDTA, 7% SDS, 0,5 M Na_2HPO_4, pH=7,2, adjusted with H_3PO_4) for 1 h, rotating at 65^0C. Hybridisation was carried out rotating the membranes overnight at 65^0C in a minimal sufficient amount of fresh Church-Gilbert buffer with 2 x 10^6 cpm of denatured labelled probe per every ml of buffer. The membranes were then washed at 55^0C in several changes of 1-2 x SSC, 1% SDS and in several changes of 0,1-0,2 x SSC, 0,1% SDS, dried and exposed to the X-ray film or to the Imaging Plate (Fuji).

2.2.1.13 Preparation of protein lysates

The cellular protein lysates were prepared in two ways.

a) Cell pellets or cells on the plates were placed on ice and directly lysed with 50-400 µl of a 2 x protein sample buffer (160 mM Tris HCl, pH=6,8, 4% SDS, 16% glycerol, 0, 57 M 2-β mercaptoethanol, 0,01% bromphenol blue) and the genomic DNA was disrupted by sonification (Branson cell disruptor B15).

b) If the amount of protein had to be measured, cell pellets were placed on ice and lysed in a small amount of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0,5 % DOC, 0, 1% SDS, 50 mM Tris HCl, pH=7,2-8,0) containing 10 µg/ml leupeptin, 10 µg/ml aprotinin and 0,4 mM PMSF. After 15 min incubation on ice the samples were pushed several times trough a 27G needle in order to share genomic DNA. The nuclei were removed by centrifugation at $+2^0$C for 15 min at 11000 x g and the clear supernatants were collected.

2.2.1.14 Determination of the protein concentration

a) The Lowry method.

Lowry-I reagent (2% Na_2CO_3 in 0,1 N NaOH) was added to 5 µl of the measured protein in 250 mM Tris HCl (pH=7,5), or to the various amounts (from 0 to 60 µl) of 0,5 mg/ml BSA supplemented with 5 µl of 250 mM Tris HCl (pH=7,5) up to a total volume of 150 µl. 250 µl of freshly prepared Lowry-IV reagent (Lowry-I with 1/100 vol of 2% K-Na tartate and 1/100 vol of 1% $CuSO_4$) was added to the above solution and mixture was left for 5 min at room temperature. Then 30 µl of 50% Folin-Ciocalteu reagent was added, mixed and the colour reaction was developed for 30-90 min in the dark. 100 µl of each sample was transferred into a separate well of 96-well ELISA plate and the OD_{660} was measured on the Titertek Multiskan® MKII. A standard curve was made by plotting the absorbance versus concentration of the BSA solutions. The protein concentration in the experimental sample was determined by fitting its OD_{660} value into the standard curve.

b) The Bradford method.

Various amounts (from 0 to 30 µl) of 0,5 mg/ml BSA solution and the experimental protein solutions were diluted with 0,15 M NaCl up to final volume of 100 µl. One ml of Coomassie brilliant blue solution (see below) was added to each tube and vortexed. The probes were incubated for 2 min at room temperature and the absorbance was read at 595 nm. The standard curve was made by plotting the adsorbance versus concentration of the BSA solutions, and the concentration of the investigated protein was determined from this curve. The Coomassie brilliant blue solution was prepared as followed: 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol and supplemented with 100 ml of 85% phosphoric acid. The volume was brought to 1 liter with water. The solution was filtered and stored at $+4^0$C.

2.2.1.15 Separation of proteins on reducing SDS-polyacrylamid gel electrophoresis

The proteins were separated on the basis of their molecular size in the discontinuous denaturing one-dimentional gel electrophoresis according to the Laemmli method. The separating gel mix (7,5-10% acrylamide, 0,16-0,27% N,N'-methylbisacrylamide, 375 mM Tris, pH=8,8,

0,1% SDS, 0,1% ammonium persulfate, 0,1% TEMED) was poured between two vertical glass plates separated by 0,75-1,5 mm spacers and allowed to polymerize. The stacking gel mixture (3-5% acrylamide, 0,075-0,125% N,N'-methylbisacrylamide, 124 mM Tris HCl, pH=6,8), 0,1% SDS, 0,1% ammonium persulfate, 0,1% TEMED) was poured on the top of the separating gel and the comb was placed in the gel to form the wells. After polymerization, the gel sandwich was fixed in the electrophoresis chamber and the chamber was filled up with the running buffer (25 mM Tris, 192 mM glycine, 0,1% SDS, pH=8,3). The samples containing usually 10-25 μg of protein lysate diluted 1:1 in a 2 x protein sample buffer (160 mM Tris HCl, pH=6,8, 4% SDS, 16% glycerol, 0, 57 M 2-β mercaptoethanol, 0,01% bromphenol blue) were boiled at 95^0C for 5 min and loaded into the wells. The electrophoresis was run at 25-40 mA until the tracking dye passed the desired distance. The proteins in the gel were detected by Coomassie brilliant blue staining or/and were subjected to Western blotting followed by immunodetection of proteins transferred on the membrane.

2.2.1.16 Staining of proteins with Coomassie brilliant blue

The gel was gently agitated overnight with a Coomassie solution (0,125% Coomassie brilliant blue R250, 10% acetic acid, 50% methanol), then destained in several changes of washing solution I (50% methanol, 10% acetic acid) and brought to the original size by incubation with washing solution II (5% methanol, 7% acetic acid). The gel was then placed on the 3MM Watman paper and vacuum dried.

2.2.1.17 Western blotting

Prior immunodetection, the proteins from the gel were electrophoretically transferred onto an Immobilon-P (Millipore) membrane in the Trans-Blot chamber (BioRad). For this a transfer sandwich was assembled under transfer buffer (25 mM Tris HCl, pH=8,3, 250 mM glycine, 20% methanol) to minimize the trapping of air bubbles; each component of the sandwich cassette was prewet in the transfer buffer and assembled in the following order: a sponge, two pieces of 3MM

Whatman paper, gel, the transfer membrane (activated with methanol and rinsed with water and transfer buffer), two pieces of 3MM Whatman paper, a sponge. The transfer stack was then fixed in the tank filled with transfer buffer and the proteins migrated onto membrane at 300 mA overnight at $+4^0$C. The membrane was rinsed in PBS and used for the immunodetection of the proteins.

2.2.1.18 Ponceau S staining of the proteins on the membrane

In order to control the transfer of proteins onto the membrane, fast and reversible staining with Porceau-S Red was used. The membrane was incubated for 1 min with 3% Ponceau S solution in 1% acetic acid and destained with several changes of sterile water, until the proteins were visible. Subsequent incubation with 0,1 N NaOH removed the protein staining.

2.2.1.19 Immunodetection of proteins on the membrane

The membrane was blocked with 10% non-fat milk in TPBS (PBS containing 0,2% Tween-20) for 1 h at room temperature and incubated with a primary antibody diluted in 10% non-fat milk in TPBS to the appropriate concentration for 0,5-3 h, with shaking at room temperature. The anti-ΔHS antibody was used at the concentration of 8 μg/ml for 3 h, anti-Erk1,2 antibody - at concentration 0,05 μg/ml for 1 h. The anti-β tubulin antibody was diluted 1:500 from the solution supplied by the manufacturer and incubated for 2 h. The membrane was washed 3 times for 7 min in TPBS and incubated with an appropriate peroxidase-coupled secondary antibody diluted usually 1:2000 in 10% non-fat milk in TPBS. The membrane was washed again 3 times in TPBS and the protein staining was visualized using the Amersham ECL western blotting detection reagents as recommended by the supplier. The membrane was exposed to the X-ray film for 10-60 sec or longer, if necessary. When the membrane had to be used again for the hybridization with another antibody, it was stripped by the agitation in the stripping buffer (62,5 mM Tris HCl, pH=6,8, 2% SDS, 50 mM DTT) at 60^0C for 20 min and washed several times in TPBS before being used in a new immunodetection analysis.

2.2.2 Carbohydrate methods

2.2.2.1 Preparation of biotin-labelled hyaluronic acid

HA was biotinylated by Biotin-LC-Hydraside (Pierce) as described by Yu and Toole (1995). HA was dissolved in PBS up to 3 mg/ml and dialyzed against MES buffer (0,1 M 2-N-morpholino ethanesulfonic acid, pH=5,5). 20 µl of 50 mM Biotin-LC-Hydrazide in DMSO (Fluka) was added to 1 ml of dialyzed HA solution, mixed and supplemented with 13 µl of freshly prepared EDC buffer (100 mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Pierce) in 0,1 M MES buffer, pH=5,5). The mixture was rotated overnight at room temperature, the possible precipitates were removed by 3 min centrifugation at 300 x g and the solution was dialyzed at $+4^0$C against PBS.

2.2.2.2 Preparation of fluorescein-labelled hyaluronic acid

The labelling of HA was performed according to the method described by de Belder and Wik (1975). 0,2 g of hyaluronic acid was dissolved in 40 ml of formamid (Merck) rotating slowly for 12-24 h. 50 ml of DMSO (Fluka), 95 µl (0,1 g) of dibutyltin dilaurate (Fluka), and 0,3 g of fluorescein isothiocyanate isomer I (Fluka) were added sequentially to the HA solution. The mixture was shaken for 30 min in the water bath at 100^0C. The product was diluted with 50 ml of water and poured into 2 liters of absolute ethanol, containing 100-200 µl of saturated NaCl. A gel-like precipitate was transferred to the fresh ethanol-NaCl solution. The washing was repeated several times until all non-bound fluorescein was washed away. This was detected by running the sample of the last ethanol wash on the Thin Layer Chromatography (TLC) plate as described below. The clean fluorescein-labelled HA was dried under a laminar flow in the dark, diluted in PBS and stored at $+4^0$C.

2.2.2.3 Control of the purity of fluorescein-labelled HA from non-bound fluorescein

The chromatographic chamber was filled on 2 cm with a mixture of chloroform and methanol (3:1) and was saturated with the chloroform-methanol vapour. The samples were loaded on the TLC plate (0,25 mm, Polygram® Sil G) on the distance of 2 cm from the plate edge and as far from each other as possible. The plate with the samples was placed vertically into the chromatographic chamber so that the samples were at the line of a liquid/gas interphase, and the samples were allowed to migrate up together with the chloroform/methanol mixture. The presence of fast migrating free fluorescent substances was examined under UV light.

2.2.2.4 Digest of hyaluronic acid with hyaluronidase

Hyaluronic acid (Sigma) was dissolved in PBS up to 8-10 mg/ml. Bovine testicular hyaluronidase (Sigma) was diluted up to 2 μg/μl (6,12 units/μl) in PBS and was added to the HA solution in an amount approximately 1,125 units per each mg of hyaluronic acid. Digest was carried out by rotating the mixture at 37^0C for 1, 6, 10 and 24 h. The reaction was terminated by heating for 5 min at 95-100^0C.

2.2.2.5 Digest of heparan sulfate with heparanidase

Heparan sulfate was dissolved in PBS up to 10 mg/ml. Heparinase II (Sigma) was routinely taken up in PBS up to 100 units/ml and stored at -20^0C. 25 μg of heparan sulfate was digested in PBS with 0,05 units of Heparinase II in final volume of 25 μl, for 3 h at room temperature. The reaction was terminated by heating for 5 min at 95-100^0C.

2.2.2.6 Determination of HA concentration

The concentration of HA was quantified by the Modified Carbazole Reaction (Bitter and Muir, 1962). HA sample in a volume of 150 μl was added to 0,9 ml of cold 0,025 M sodium

tetraborate in concentrated sulfuric acid and thoroughly mixed at $+4^0$C. The solution was heated for 10 min at 100^0C and allowed to cool down to the room temperature. 30 µl of 0,125% carbazole in absolute ethanol was added to the mixture, the resulting solution was boiled for 15 min and chilled to the room temperature. The optical density was then measured at 530 nm. Samples containing a known amount of HA, from 15 ng to 30 µg, were used for calibration. The concentration of hyaluronic acid in the experimental sample was found by fitting its OD_{530} value into the calibration curve.

2.2.2.7 Analysis of glycosaminoglycans on gel electrophoresis

The method was adopted from "Carbohydrate analysis. A practical approach" (Chaplin and Kennedy (Eds), 1994). Polysaccharides or glycosaminoglycans were electrolytically separated on the 15% polyacrylamid gel. Freshly prepared gel mix consisting of 6 ml of 10 x TBE (0,9 M Tris, 0, 9 M Boric acid, 24 mM EDTA, pH=8,3), 30 ml of acrylamide/*bis*-acrylamid (30%/0,8%, Carl Roth GmbH & Co, Karlsruhe), 2 ml of 10% ammonium persulfate, 22 ml of distilled water and 50 µl TEMED was poured between 20 x 12 cm glass plates separated by 2 mm spaces. The comb with the appropriate theeth size was set in the gel and the gel was allowed to polymerize. In some cases a small amount (3 ml) of the mix was firstly prepared and poored into the chamber to form the plug, to prevent the gel leakage during polymerisation. The samples containing 15-25 µg of glycosaminoglycan supplemented with 1 volume of 2 x loading buffer (2 x TBE, 10% sugrose, 0,001% bromphenol blue, 0, 001% phenol red) were loaded into the wells and allowed to run under 1 x TBE at 120-160 V for approximately 4-5 h. The gel was removed and stained for more than 45 min in 2% acetic acid containing 0, 5% alcian blue 8GX. The background staining was removed by several washings with 0,2% acetic acid.

2.2.3 Cell culture handling

Cells were routinely grown under an appropriate cell culture medium (sm. paragraph 2.1.7) at 37^0C in a humid atmosphere containing 5% CO_2. When the desired confluency was reached, cells were detached from the culture plates by trypsinization (5-15 min incubation in

small amount of 0, 25% trypsin after double washing in Ca^{2+}/Mg^{2+} -free PBS) or by removal of Ca^{2+} and Mg^{2+} with EDTA (10-20 min incubation with 5 mM EDTA in Ca^{2+}/Mg^{2+} -free PBS after double washing in Ca^{2+}/Mg^{2+} -free PBS) and replated at 1:10 -1:2 dilution, dependent on the cell type. The nonadherent cells were splitted by addition of 3 ml of dense cell suspension into 20 ml of fresh cell culture medium. For some experiments cells were counted in the counter chamber to determine the cell concentration and then diluted as required in the experimental protocol. For storage, the cells were collected, washed twice by repeated resuspension in the cell culture medium and centrifugation at 300 x g for 4 min, and the cell pellet was resuspended in a small amount of ice-cold cell culture medium containing 50% FCS and 10% DMSO. The cell suspension was transferred into pre-chilled cryo-vial, incubated on ice for 30-60 min and frozen at -80^{0}C. For the long term storage, the frozen cells were kept in liquid nitrogen. To re-propagate cells, the vial content (usually 0,5 ml) was thawed fast in 37^{0}C water bath and transferred into a blue-cup tube. The cell suspension was carefully overlayed with 9,5 ml of culture medium, incubated for 5 min at room temperature, then mixed and centrifuged at 300 x g for 4 min. Cell pellet was washed once more and plated in a 5 ml culture flask.

2.2.3.1 Transfection of cells using calcium phosphate method

One day before transfection cells were splitted 1:10 from nearly confluent culture and plated into 10 cm dish. 3,5 hours before the transfection the culture medium was changed on fresh one. 5-10 µg of plasmid DNA at room temperature was mixed with sterile pre-heated to 37^{0}C water and 250 µl of pre-heated to 37^{0}C 2 x HBS solution (280 mM NaCl, 1,5 mM Na_2HPO_4, 50 mM HEPES, pH=7,1) in a final volume of 475 µl. 25 µl of sterile 2,5 M $CaCl_2$ pre-heated to 37^{0}C was added dropwise into the above mixture and incubated for 30 min at room temperature. The resulting precipitate was added to the cell culture and cells were allowed to grow for the next 5 h. After that the medium was discarded and 4 ml of 15% glycerol in PBS was added for exactly 2 min, followed by two washing with PBS. The fresh cell culture medium was added and cells were grown for at least 36-48 h before any further manipulations.

2.2.3.2 Production of stable transfected clones

When a transfected DNA included a selection marker, such as "neo" cassette, cells were grown under cell culture medium containing 1 mg/ml G418 for 2-3 week until defined colonies were formed. The colonies were transferred separately into a 24-well culture plate and propagated further under the selective medium.

2.2.3.3 Assaying the proliferation rate by thymidine incorporation

The cells grown in a 96-well plates were pulsed by adding 1 µCi of [6-^{3}H] thymidine per well containing 100 µl medium. Cells were then incubated for 4-6 h at 37^{0}C in a standard conditions, harvested in Tomtec cell harvester according to the instructions of the manufacturer. The incorporated radioactivity was measured in a Microbeta liquid scintillation & luminescence counter (EG&G WALLAC, Finland)

2.2.4 Special methods

2.2.4.1 Genotyping of mice: detecion of mutant or wild-type CD44 allele in Southern blot analysis (the "tail assay")

20-25 µl of mouse genomic DNA, corresponding to 1/2 to 1/3 of the total amount of DNA isolated from the 2-3 mm mouse tail tissue, was digested overnight at 37^{0}C with 6 µl of Kpn I in an appropriate buffer containing 0,1 µg/µl BSA and 1 µl of 1 µg/µl RNase A in a total volume of 35-40 µl. The digestion mixture was loaded on a 23 cm long 0,75% agarose gel and the DNA fragments were resolved under 1 x TAE buffer at 40-60 V for 8-10 h. The DNA was blotted onto a GeneScreen Plus® hybridisation transfer membrane (NEN® Research Product, USA) using the normal Southern blot technique, and hybridized with 10-15 x 10^{6} cpm of a [α^{32}P]-dCTP - labelled CD44 probe in 5 ml of the Church-Gilbert buffer at 65^{0}C overnight. The hybridisation membrane was then washed in SSC/SDS buffer as above, sealed into the plastic bag, exposed for 2 days to the Imager Plate and analysed in the Bio-Imaging analyzer (Fuji). The CD44 probe, corresponding to the flanking 500 bp Kpn-I fragment upstream of exon 2 of CD44 cloned into a

bluescript 129 vector (a kind gift of R. Schmits) was prepared as follows. 20 µg of the CD44-probe containing plasmid was digested overnight with 2 µl of Kpn-I in an appropriate buffer conditions, including 0,1 µg/µl BSA. The digested DNA was resolved on the 1% agarose gel with 2 cm wide wells and the 600 bp DNA fragment was allowed to run on the DE81 DEAE-cellulose membrane (Schleicher & Schuell, Dassel) inserted into the gel. The 600 bp Kpn-I fragment was eluted from the membrane, purified by phenol-chloroform extraction, precipitated in ethanol, washed in 70% ethanol and dried. The pellet was dissolved in 30 µl of water and the DNA concentration was measured. 25 ng of the probe prepared in this way was labelled with [α^{32}P]-dCTP using RediPrime kit as described above.

2.2.4.2 Isolation and culturing of primary mouse cells

2.2.4.2.1 Mouse embryonic fibroblasts

Primary fibroblasts cultures were established from the 14,5 day mouse embryos. Embryos obtained from timed pregnant mice were placed on a cold dry petri dish and processed separately. The heads, hearts, livers and intestines were removed with a scalpel. When embryos were descendent from a heterozygous mating, the heads were taken for the genotyping. The rest of the tissues was chopped finely with a scalpel, transferred into a 15 ml blue-cap tube, washed twice in cold sterile PBS and disaggregated in 100-200 µl of 0,25% trypsin. The trypsinization was proceeded for 30 min at 37^0C, with occasional agitating. The bigger pieces were allowed to settle down, suspension cells were collected, washed in DMEM containing 10% FCS, 100U/ml penicillin and 100 µg/ml streptomycin and stored on ice. The pieces were trypsinized once more in the same way, and collected cell suspensions from the same embryo were pooled together. The cells were counted and plated at a concentration of 1 x 10^7 cells per 10 cm petri dish under DMEM containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture was maintained in a standard conditions and splitted when confluent. For spontaneous immortalisation, the subculturing was continued for more then 2 months until the cells stopped growing and begun to die out. The cells which overcame the crisis and started to proliferate again were pooled together.

2.2.4.2.2 Mouse embryonic limb bud mesenchymal cells

The limb bud mesenchyme was isolated from the 10,5 day mouse embryos. The excised embryos were placed into the ice-cold PBS and the forelimbs were cut out with electrolytically sharpened tungsten needles. Forelimbs were rinsed and incubated in 3 U/ml dispase I solution in Ca/Mg-free PBS for 10-15 min. The ectodermal cell layer, including AER (apical ectodermal ridge), was removed completely by the tungsten needles. The distal third portions of the mesenchymal tissues were collected together, washed once with ice-cold PBS and agitated with 0,25% trypsin for 5 min at room temperature. The suspension cells were collected, washed in DMEM supplemented with 15% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Trypsinization was repeated until all tissue was disaggregated. The cells were plated in 6 well plates (Falcon) under the complete medium and cultured at high density in a standard incubator conditions.

2.2.4.2.3 Resident and thioglycollate-elicited peritoneal macrophages

To obtain the thioglycollate-elicited peritoneal macrophages, mice were injected intraperitoneally with 1 ml of sterile 3% Brewer thioglycollate medium and the inflammatory response was allowed to proceed for 7 days. Resident macrophages were obtained from untreated animals. To collect peritoneal macrophages, alive mice were injected intraperitoneally with 5 ml of DMEM, 5% endotoxin-free FCS. In 10 min mice were sacrificed and peritoneal fluid was collected with a syringe, transferred into a 50 ml blue-cap Falcon tube and kept on ice during all further steps. The cells were collected by centrifugation, washed in ice-cold DMEM, 5% FCS and used immediately.

2.2.4.2.4 Splenocytes and spleen B-lymphocytes

The mouse spleen was homogenized by smashing it through sterile cotton tissue wet in the RPMI 1640 medium. Nonadherent spleen cells were collected and washed in RPMI. The

erythrocytes were eliminated by ACK lysis: the cell pellet was taken up in 2 ml of growth medium (RPMI 1640, 10% FCS, 10 mM HEPES (pH=7,2), 1 x Glutamax, 0,05 mM 2-β mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin) and mixed with 6 ml of room temperature ACK buffer (0,15 M NH_4Cl, 1 mM $KHCO_3$, 0,1 mM Na_2EDTA, pH=7,2-7,4). The suspension was left for 5 min at room temperature and then filled up with another 20 ml of growth medium, followed by centrifugation for 5 min at 200 x g. Cell pellet was washed twice in growth medium or PBS containing 3% FCS, depending on further use. To separate B cells from the total splenocyte population, splenocytes depleted of adherent cells and erythrocytes as described above were resuspended in 300 µl of PBS, 3% FCS, supplemented with 100 µl of anti-Thy-1.2 antibody (all mice strain used were of the Thy-1.2 haplotype) coupled to the magnetic MicroBeads (Miltenyi Biotech) and incubated for 30 min at 4^0C. The cells were then washed three times in PBS with 3% FCS, resuspended in 500 µl PBS, 3% FCS and applied on the pre-washed MS^+ separation column (Miltenyi Biotech) fixed on the magnet. The cells which flew through the column (Thy-1.2 negative, B-cells) were collected. The column was washed with 0,5 ml of PBS, 3% FCS and the washed out cells were added to the Thy-1.2 negative fraction. To collect T-cells, the column was removed from the magnet, placed on a new collection tube and flushed with PBS, 3% FCS to wash out the Thy-1.2 positive cells, T-cells. The purity of B- and T-cell fractions was checked by the flow cytometry after staining either with anti-CD4 and anti-CD8 antibody (for T-cells) or with B220 antibody (for B-cells). Cells were washed in PBS, 3% FCS and maintained in RPMI 1640, 10% FCS, 10 mM HEPES (pH=7,2), 1 x Glutamax, 0,05 mM 2-β mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin in a small cell culture flasks in standard conditions.

2.2.4.2.5 Lymph node cells

Collected facial, jugular, cervical, brachial, axillary, inguinal, popliteal, caudal and mesenteric mouse lymph nodes were pooled together and homogenized by smashing through the 70 µm pore size plastic sieve. Resulting cell suspension was passed through the syringe with 21G needle, washed twice and resuspended in full medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0,05 mM 2-β mercaptoethanol, 10 mM HEPES (pH=7,2),

100 U/ml penicillin, 100 μg/ml streptomycin). ACK lysis was performed if necessary as described above. Cells were grown under standard incubator conditions in the full medium.

2.2.4.3 Stimulation of fibroblasts with mitogens

Fibroblasts were plated at a concentration of 4 x 10^5 cell per 6 cm petri dish under DMEM, 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin and incubated in a standard conditions overnight. Cells were starved for 10 h in DMEM containing 0,5%FCS and the medium was replaced on the 0,5% FCS in DMEM with one of the mitogens: 25 μM LPA, 10 ng/ml TNFα, 10 ng/ml interleukin-1β, 10 ng/ml EGF or 100 ng/ml TPA in duplicates. 15 min later the cells were assayed on the Erk 1,2 phosphorylation, and 14 h later the binding of fibroblasts to soluble HA was measured. In the experiments with fibroblasts binding to immobilized HA, they were stimulated overnight with 10 ng/ml TPA, without serum starvation.

2.2.3.4 Stimulation of B-cells and splenocytes with interleukin-5

Splenocytes and B-cell stimulation was done as described (Murakami et al, 1990). Isolated splenocytes or splenic B-lymphocytes at 2 x 10^6 cell/ml in freshly prepared RPMI containing 10% FCS, 10 mM HEPES, pH=7,2, 2 mM L-glutamine, 0,05 mM 2-β mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin were stimulated with either 15 μg/ml LPS or various (1-100 ng/ml) amounts of interleukin-5 for the indicated time. Unstimulated cells were set up at 4 x 10^6 cell/ml in the same medium.

2.2.3.5 Stimulation of lymph node T-cells with anti-CD3 antibody

The stimulation of T-cells was done as described (DeGrendele et al., 1997b). Anti-CD3 antibody or hamster γ-globulins (control) were immobilized on 96 well plates with round-bottom wells. For that, the plates were incubated overnight at room temperature with 100 µl/well of either 5 µg/ml anti-CD3 antibody in PBS or 5 µg/ml hamster γ-globulin in PBS. Before using, the antibody solution was discarded and the plates were washed twice with PBS. Isolated lymph node T-cells were plated in the antibody-precoated plates at 5 x 10^5 cells/well, in 200 µl of RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0,05 mM 2-β mercaptoethanol, 10 mM HEPES (pH=7,2), 100 U/ml penicillin, 100 µg/ml streptomycin per well, and allowed to grow for 48 h before being collected and analysed for their HA-binding properties.

2.2.3.6 Stimulation of embryonic limb bud mesenchymal cells with soluble FGF8

Embryonic limb bud mesenchymal cells, cultured at high density for 2-7 passages were plated at 2,8 x 10^4 cells/well into the flat-bottom 96 well plate in DMEM containing 15% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin. This resulted in a 60-80% confluency next day. Cells were then washed in DMEM and serum-starved in DMEM medium for 12 h. Thereafter the medium was replaced with DMEM containing various amount of soluble FGF8, from 0 to 200 ng/ml, and cells were allowed to grow another 12 h, before pulsing them with [^{3}H]thymidine to measure the proliferation rate.

2.2.3.7 Transformation of primary mouse embryonic fibroblasts with *ras* and *myc* oncogenes using the retroviral gene transfer system

To generate the stable ectopic virus producing cell line, pZip *ras-myc* plasmid (a kind gift of H. Land), a murine retrovirus shuttle vector containing the v-Ha-*ras* and avian myelocytomatosis virus-derived *gag-myc* oncogens was co-transfected together with pcDNA3.1+ carring a "neo" selective marker, into a ωE packaging cell line using the calcium phosphate method. The weight ratio between pZip *ras-myc* and pcDNA3.1+ was 17:1, which

corresponds to the 10:1 molar ratio. The stably transfected clones were selected under G418 containing medium. Virus containing medium was produced by culturing separate clones of virus-producer cells in 6 cm culture dishes for 3 days with 3 ml (a half of the normal volume) of DMEM containing 10% FCS, 100 U/ml penicilin and 100 µg/ml streptomycin. The medium was then collected, filtered through a 0,45 µm sterile filter, and used immediately for infection or stored at -20^{0}C.

The virus titer of different virus producing clones was estimated by the ability to transform NIH3T3 cells in a plaque assay. For that, the day before infection a confluent NIH3T3 culture was split 1:20 into 6 cm culture dishes. Then the growth medium was discarded and cells were infected with 2 ml of fresh cell culture medium containing 8 µg/ml of polibrene and various amount (from 0,1 to 1 ml) of virus containing medium. After 2,5 h incubation at 37^{0}C in a standard conditions, 6 ml of NIH3T3 growth medium was added to the dishes and agitated slightly; the cells were further grown for 8 days under standard conditions, with regular replacement of growth medium by fresh one. Cells were then rinsed twice in PBS, fixed for 10 min in 100% methanol and dried overnight. The plaques were visualized by staining in a 1,5% crystal-violet solution for 10 min. The excess of dye was removed by washing the plates in water. The 2 clones producing the highest plaque number upon infection of NIH3T3 cells were chosen for further experiments.

The primary embryonic fibroblasts were infected with 2 ml of cell culture medium containing 8 µg/ml polibrene and 1 ml of the virus containing supernatant from one of the chosen virus producer clones. The infection was further carried out in the same way as for NIH3T3 cells. The infected cells were cultured for 6-8 weeks, and the transformed phenotype of the resulting cultures was confirmed by the ability of cells to grow and form colonies in a soft agar.

2.2.3.8 The colony formation in a soft agar

0,2 ml of FCS was added to 1,5 ml of DMEM and supplemented with 100 µl of cell suspension containing 4000, 2000 or 800 cells. 3,3 M sterile agarose in PBS was melted completly and allowed to cool for 5 min at room temperature. 0,2 ml of melted agarose was then added to the resulting cell suspension, mixed immediately, and the mixture was distributed into wells of 24 well culture plate, 500 µl per well. The plates were incubated for 5-10 min at $+4^0$C, and then placed in a normal cell incubator. The colonies were counted and photographed 8 days later.

2.2.3.9 Flow cytometry

To assess the expression of cell surface proteins, the adherent cells were detached from the plates with EDTA solutition. Adherent or suspension cells were washed twice in 5% FCS in PBS. 5-10 x 10^5 cells were resuspended in 50 µl of 5% FCS in PBS containing the primary antibody and left for 30 min on ice. When the primary antibodies were not directly labelled, the cells were then washed twice in 5% FCS in PBS and resuspended in 50 µl of 5% FCS in PBS containing fluorescently-labelled secondary antibody. After 30 min of incubation on ice, in the dark, cells were washed twice in 5% FCS in PBS, resuspended in PBS and the fluorescence was measured at the FACStar flow cytometer. The primary antibodies dilutions used were as follows: for directly labelled or unlabelled anti-CD44 antibody (clone IM7) -1 µg/ml; for anti-CD69 - 1:100 from the supplier solution; for anti-B220 - 5 µg/ml, the anti-CD4 and anti-CD8 antibodies were used in dilution 10 µg/ml, anti-Mac-1 antibody -1:100 from the supplier solution. To measure the cell surface-bound heparan sulfate, cells were detached as above, washed once in PBS and once with PBS containing 0,3% BSA. 0,5-1 x 10^6 cells were resuspended in 50 µl of 0,3% BSA in PBS with or without 4 u/ml Heparinase II (Sigma) and incubated for 3 h at 37^0C with occasional agitation. After that cells were washed 4 times in PBS containing 3% FCS and incubated for 30 min with 20 µg/ml of anti-HS antibody in 3% FCS in PBS on ice. After two subsequent washings cells were incubated with biotinylated anti-mouse IgM antibody in 1:500 dilution in a 3% FCS in PBS for 30 min on ice. Cells were washed twice and incubated in the dark for 30 min with streptavidin-FITC conjugate, diluted 1:500 in 3% FCS in PBS on ice. After two washing steps the fluorescence was measured at the FACStar flow cytometer (Becton Dickinson).

2.2.4.10 Binding of cells to the immobilized hyaluronic acid

The plastic caps were incubated with 1 ml of either 1 mg/ml HA in PBS or PBS alone overnight at room temperature. Directly before use, the solution was washed away by two washings with PBS, and the caps were further incubated for 30 min at room temperature with 2 ml of 10% FCS in PBS to block the uncovered plastic. The cells, when adherent, were detached from the culture plates with EDTA solution. 3,5 x 10^6 cells were resuspended in a methionine-free medium, supplemented with 1% lysine, 2 mM L-glutamine and 10% FCS dialyzed against 140 mM NaCl in 10 mM HEPES, pH=7,4. [^{35}S]-methionine/cysteine mix was added to the cell suspension up to a final concentration of 30 µCi/ml. The tubes were filled with CO_2, closed and incubated for 2 h at 37^0C, rotating. Labelled cells were pelleted by centrifugation , washed 3 times in PBS and resuspended in 3,5 ml of 10% FCS in PBS. 500 µl of the cell suspension was transferred into separate Eppendorf tube, lysed with 500 µl of 1% Nonidet P-40 in PBS and kept as a 100% control. The rest of the cell suspension was distributed into plastic caps coated with HA or with PBS alone, 500 µl per cap, and left there at room temperature for 30 min. Thereafter the unbound cells were carefully washed away by 3 changes of PBS, the liquid was removed and the bound cells were lysed by 500 µl of 1% Nonidet P-40 in PBS. The caps with the lysates were shaken for 10 min at room temperature, and 250 µl from each sample was taken to measure the radioactivity. The results were expressed as a percentage of the total cell lysate radioactivity from the 100% control samples. This value was taken as a measure for cell binding to the immobilized HA.

2.2.3.11 Cell binding of soluble hyaluronic acid

The cellular binding of soluble HA was assayed by the flow cytometry. Adherent cells were detached from the plates with EDTA solution. Adherent or suspension cells were washed twice in 5% FCS in PBS. 5-10 x 10^5 cells was resuspended in 50 µl of 5%FCS in PBS containing 5 units of hyaluronidase and incubated at 37^0C for 1 h. Hyaluronidase was washed away by 4-5 washings with 5% FCS in PBS. For blocking of the CD44-mediated HA binding, cells were resuspended in 50 µl of 3,5 µg/ml KM81 antibody in 5% FCS in PBS and left for 15 min in ice. Cells were then pelleted, resuspended in 50 µl of fluorescent or biotinylated HA solution, usually

diluted 1:100 in 5% FCS in PBS, and incubated 30 min on ice, in the dark. When biotinylated HA was used, cells were washed 3 times and incubated with 50 µl of fluorescent streptavidin conjugate, diluted 1:500 in 5% FCS in PBS. After 3 washing steps the fluorescence was measured at the FACStar flow cytometer.

Chapter 3. Results

The importance of the CD44 family of molecules has been shown in a number of *in vitro* and *in vivo* systems. They play a role in embryogenesis, hematopoiesis, immunological processes, tumor development and metastasis formation. However, disruption of the CD44 gene had no significant effect on the phenotypic characteristics of CD44-null mice (Schmits et al., 1997). The lack of CD44 expression must therefore be compensated for in the CD44-null mice, and the mechanism of compensation is the issue of the following investigation.

3.1 Analysis of the HA binding properties of cells derived from CD44-/- mice

HA binding is the best characterized feature of CD44. In order to see if CD44-mediated HA binding is taken over by one or several other HA binding molecules in CD44-deficient mice, different cell types from CD44-positive mice and their CD44-negative counterpartners were compared for their ability to bind hyaluronate. The aim was to identify CD44-/- cells which nevertheless bind to hyaluronate. These cells would then serve as a source of the HA binding molecule which compensates for CD44 in CD44-/- mice, enabling this compensatory molecule to be characterized.

3.1.1 HA binding in CD44-positive and CD44-negative mouse embryonic fibroblasts

Firstly, HA binding in primary and immortalised mouse embryonic fibroblasts was investigated. These cells were chosen since, when obtained from wild-type mice, they express CD44, can be easily grown in culture and can be immortalised, providing a useful source of experimental material.

3.1.1.1 Establishment of CD44-positive and CD44-negative fibroblasts in culture

In order to establish mouse fibroblasts in culture, day E14,5 embryos descended from mating between CD44 heterozygous mice were dissected and the tissues were enzymatically disaggregated with trypsin. Outgrowing cells passaged for longer than 10 days give the primary fibroblast cultures. They were spontaneously immortalised by passaging them and expanding the cells which overcame crisis. Embryonic fibroblast cultures from individual embryos were then screened by Southern blot to detect their genotype (Fig.1).

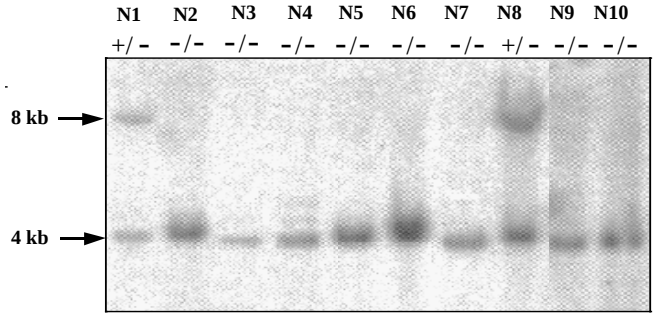


Figure 1. Screening of embryonic fibroblasts for the presense of wild-type and null CD44 allele. DNA isolated from embryonic fibroblasts was digested with Kpn I and analysed on Southern blot. A hybridization probe which was used recognizes both the wild-type and mutant CD44 allele, since it targets a flanking 500 bp fragment upstream of the mutation in a CD44-mutant allele. However, the CD44 null allele can be distinguished from the wild-type CD44 allele by the size of gene-targeted Kpn I fragment: it is 4 kb in the CD44-mutant allele, compared to 8 kb in wild-type CD44 allele. (T.W Mak, personal communication; Schmits et al, 1997). The revealed CD44 genotype of embryos from N1 to N10 is indicated at the top of each line.

3.1.1.2 Spontaneously immortalised fibroblasts do not bind to soluble or immobilized HA

Soluble HA binding of primary and spontaneously immortalised fibroblasts from embryo N1 (CD44+/-) and embryo N9 (CD44-/-) was measured by flow cytometry after

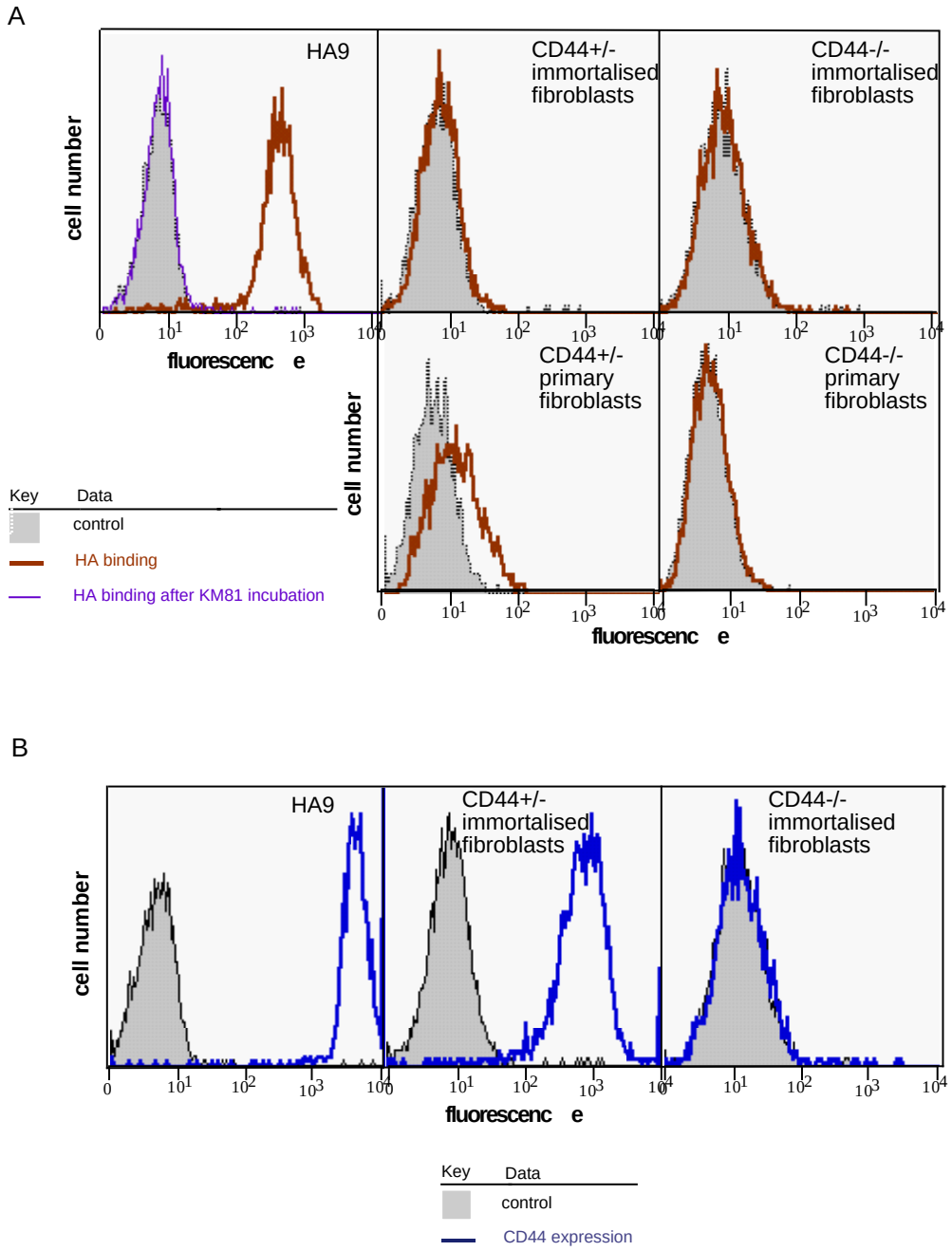


Figure 2. Binding of CD44+/- and CD44-/- mouse embryonic fibroblasts to soluble HA.

A. Primary and immortalised fibroblasts of indicated genotype were detached from plates with EDTA and stained with fluorescently labeled HA. Soluble HA binding was measured by flow cytometry (bold brown lines). HA9 cells, used as a control were stained and measured the same way (bold brown line) or after incubation of the cells with KM81 (fine violet line). B. CD44 expression of CD44+/- and CD44-/- immortalised fibroblasts was measured by flow cytometry after staining of the cells with fluorescent anti-CD44 ab (bold blue lines). H9 cells were used as a positive control. Gray shadowed curves on both A and B represent the autofluorescence of cells, when neither antibody or fluorescent HA was added.

staining of EDTA-detached cells with fluorescein-labeled soluble HA. CD44+/- cells are an appropriate control since they express high levels of CD44, as measured by flow cytometry (Fig. 2b). Both CD44-positive and CD44-negative immortalised fibroblasts, as well as CD44-negative primary fibroblasts did not show any HA binding. HA binding of CD44+/- primary fibroblasts was minor, compared to the HA binding of HA9 cell line, used as a positive control. (Fig. 2a). HA9 cells are known to bind HA in a CD44-dependent manner. In my experiment the HA binding of HA9 cells was completely abolished by treatment with KM81, an antibody blocking CD44-HA interaction, indicating that the FACS signal is due to the specific binding of fluorescently labeled HA.

Since the binding to immobilised HA is often a more sensitive method to detect cellular HA binding than binding of cell to soluble HA, spontaneously immortalised CD44+/- and CD44-/- fibroblasts were also assayed for their ability to bind to immobilised HA (Fig. 3). Radioactively labeled cells were allowed to attach to immobilized HA, and the HA binding was calculated as a percent of bound cells radiactivity to the radioactivity of all cells prior their attachment to HA. Neither CD44-positive nor CD44-negative fibroblasts bound to immobilised HA while ASpSV14 cells used as a positive control in this experiment did so. Cell attachment to mock coated plastic was measured for each cell type as a control for specificity of HA binding.

From these data I conclude that neither CD44+/- or CD44-/- fibroblasts constitutively bind to HA.

3.1.1.3 Mouse embryonic fibroblast HA-binding is not induced by several mitogens

It was puzzling that the CD44+/- fibroblasts did not bind HA, despite expressing CD44. However, CD44 binding of HA is known to be inducible in some cell systems (Lesley et al., 1993a). Therefore I set out to determine whether it was possible to stimulate HA binding mouse embryonic fibroblasts.

Phorbol esters are known to be a potential activators of CD44 function (Hirano et al, 1997; Liu and Sy, 1997). Therefore the influence of TPA stimulation on fibroblast attachment to immobilized HA was tested. However overnight incubation of both CD44+/- and CD44-/- immortalised fibroblasts with 10 ng/ml of TPA did not increase their binding to HA-coated surfaces (Fig. 3).

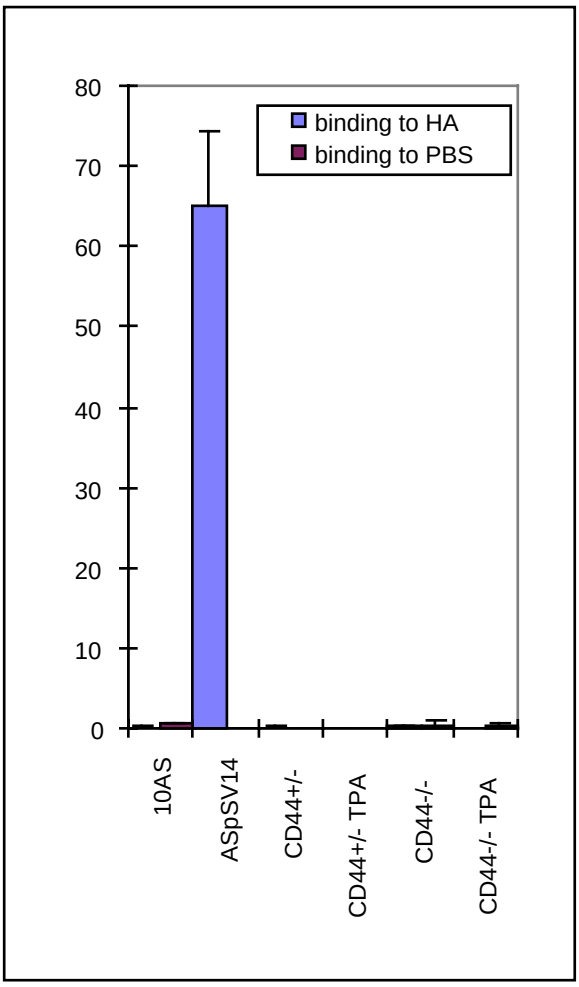
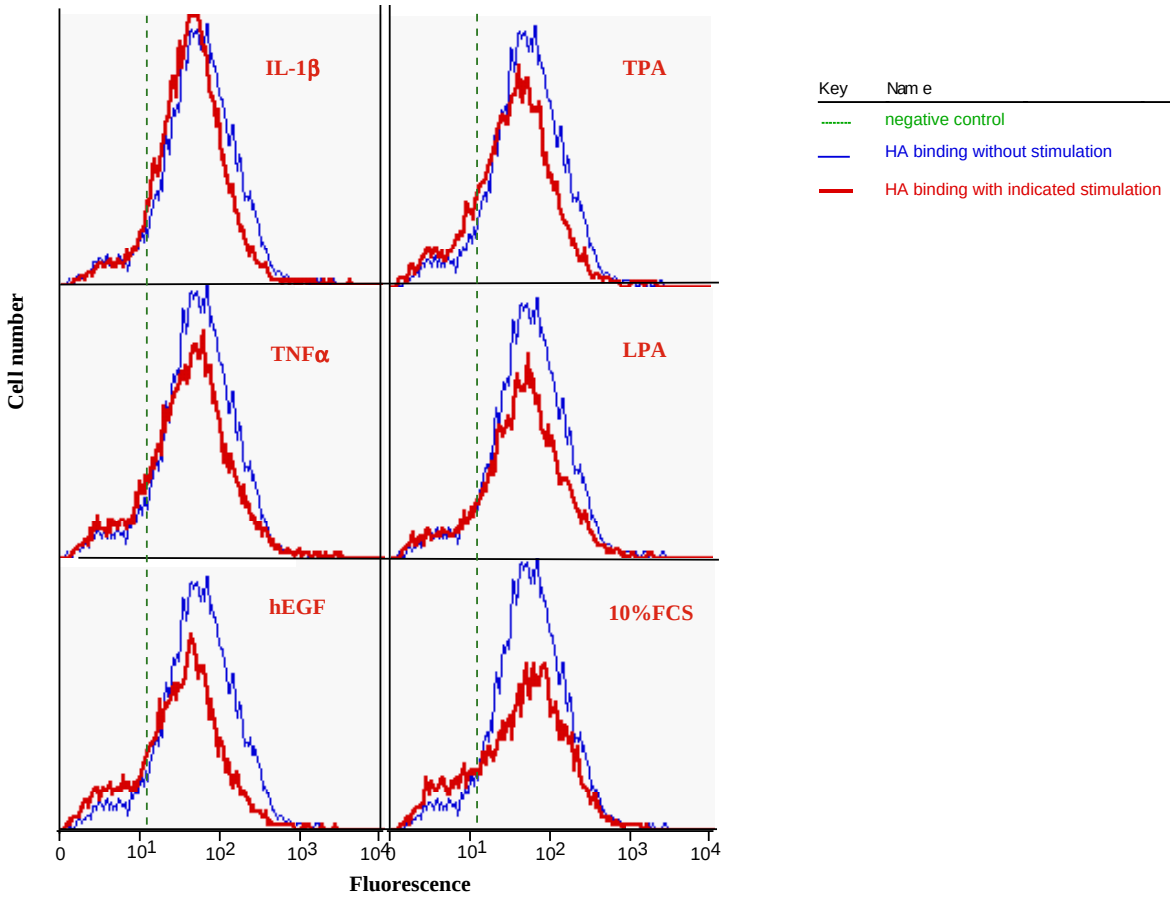


Figure 3. Binding of CD44-positive and CD44-negative embyonic fibroblasts to immobilised HA. Spontaneously immortalised CD44+/- and CD44-/- embryonic fibroblasts, with or without o/n TPA incubation, were labeled with [^{35}S] methionine/cysteine mixture and allowed to attach for 30 min to the bottom of plastic cups, coated with either HA or PBS. Unbound cells were washed away, bound cells were lysed, and radioactivity of lysates was measured. Bars represent the radioactivity of attached cells as a percent of total radioactivity incorporated. The HA-binding ASpSV14 cells and HA-non-binding 10AS cells were used as a positive and negative controls. All experiments were done in triplicate.

Since at least some binding to soluble HA was detected in a flow cytometry assay in the CD44-positive primary fibroblasts (Fig. 2a), this system was exploited in further experiments. Several mitogens and potential activators were tested for their ability to induce binding of soluble HA in wild-type primary embryonic fibroblasts. The rational behind this approach is that if a given mitogen induces CD44-mediated HA binding in CD44 -positive cells, the same mitogen may induce a protein which compensates for CD44 in CD44-/- cells.

Unfortunately, soluble HA binding of primary embryonic fibroblasts was not altered by IL-1β, TNFα, human EGF, TPA, lysophosphatidic acid (LPA) or 10% serum (Fig. 4a), although all these treatments induced intracellular signalling in the fibroblasts, as it was detected by activation of MAP kinases (Fig. 4b): all stimuli induced partial or complete phosphorylation of both Erk1 and Erk2, compared with the uninduced control (first line on Fig. 4b).



B.

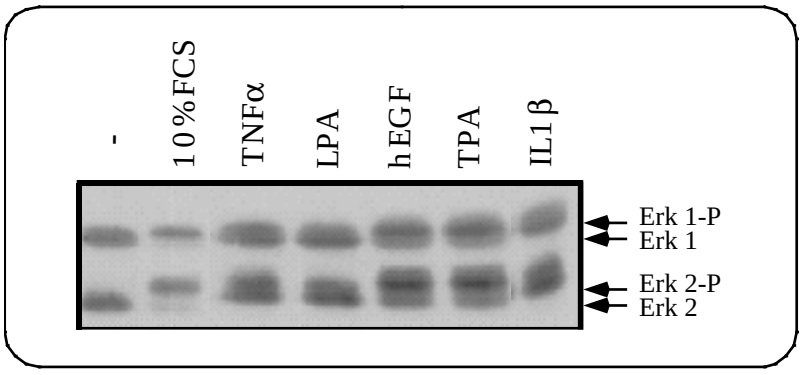


Figure 4. Stimulation of primary embryonic fibroblasts with Il-1β, TNFα, hEGF, TPA, LPA or 10% FCS does not change their HA binding.

A. HA binding of unstimulated (fine blue lines) and stimulated (bold red lines) primary embryonic fibroblasts. Serum starved wild-type embryonic fibroblasts were incubated overnight with IL-1β (10 ng/ml), TNFα (10 ng/ml), hEGF (10 ng/ml), TPA (100 ng/ml), LPA (25 µM) or 10% FCS, as indicated on each histogramm. Cells were

detached from the plates with EDTA, stained with fluorescently-labeled HA and analysed by flow cytometry. The green broken line shows the median autofluorescence, when no fluorescent HA was added.

B. MAP kinase phosphorylation, induced in embryonic fibroblasts by the described stimuli. Cells were incubated for 15 min with the same mitogen or cytokine concentrations as in Fig. 3a. They were lysed and protein extracts were subjected to Western blot analysis using Erk 1,2 specific antibodies. The shift from inactive nonphosphorylated forms (arrows Erk1 and Erk2) to the active, phosphorylated forms (arrows Erk1-P and Erk2-P) of the MAP kinases can be noticed by retardation of migration of the proteins.

3.1.1.4 HA binding of primary embryonic fibroblasts depends on cell density

Besides mitogenic stimuli, the expression and function of cellular proteins may be regulated by cell density and be associated with the proliferation rate. Since CD44 is often upregulated in highly proliferative tissues, such as epithelium, activated lymphocytes and tumour cells, it is imaginable that its expression and HA-binding function in cultured cells may depend on the state of the culture.

In order to check whether confluency can influence their HA-binding capacity, CD44+/+ primary embryonic fibroblasts were allowed to grow to different densities and their binding to soluble HA was investigated. HA binding of primary embryonic fibroblasts was found to inversely correlate with confluency (Fig. 5a). CD44+/+ fibroblasts cultured at low density are able to bind HA. Also CD44 expression appeared to be dependent on cell density, increasing with a decrease of confluency (Fig. 5b).

These experiments identify conditions in which HA binding by CD44-positive fibroblasts can be detected.

3.1.1.5 Primary embryonic fibroblasts from CD44 -/- mice do not bind HA

Since the wild-type primary fibroblasts collected at low density bind HA, the same may be true for the fibroblasts from CD44-deficient mice. In order to find this out, I compared the HA binding ability of CD44-positive and CD44-negative primary fibroblasts grown at low confluency. The primary fibroblasts cultures from the CD44+/+, CD44+/- and CD44-/- embryos generated in a new heterozygote mating were grown to the equivalent low confluency and analysed for HA-binding in flow cytometry. As demonstrated in Fig. 6, HA binding in

these cells correlates with their CD44 expression: CD44 -/- primary embryonic fibroblasts did not bind HA while CD44+/+ fibroblasts did, and HA binding of CD44+/- cells was intermediate. Both CD44+/+ and CD44+/- fibroblasts HA binding was mediated only by CD44, since a pretreatment of these cells with the anti-CD44 ab, KM81, which blocks CD44-mediated HA binding, completely abolished HA binding by these cells.

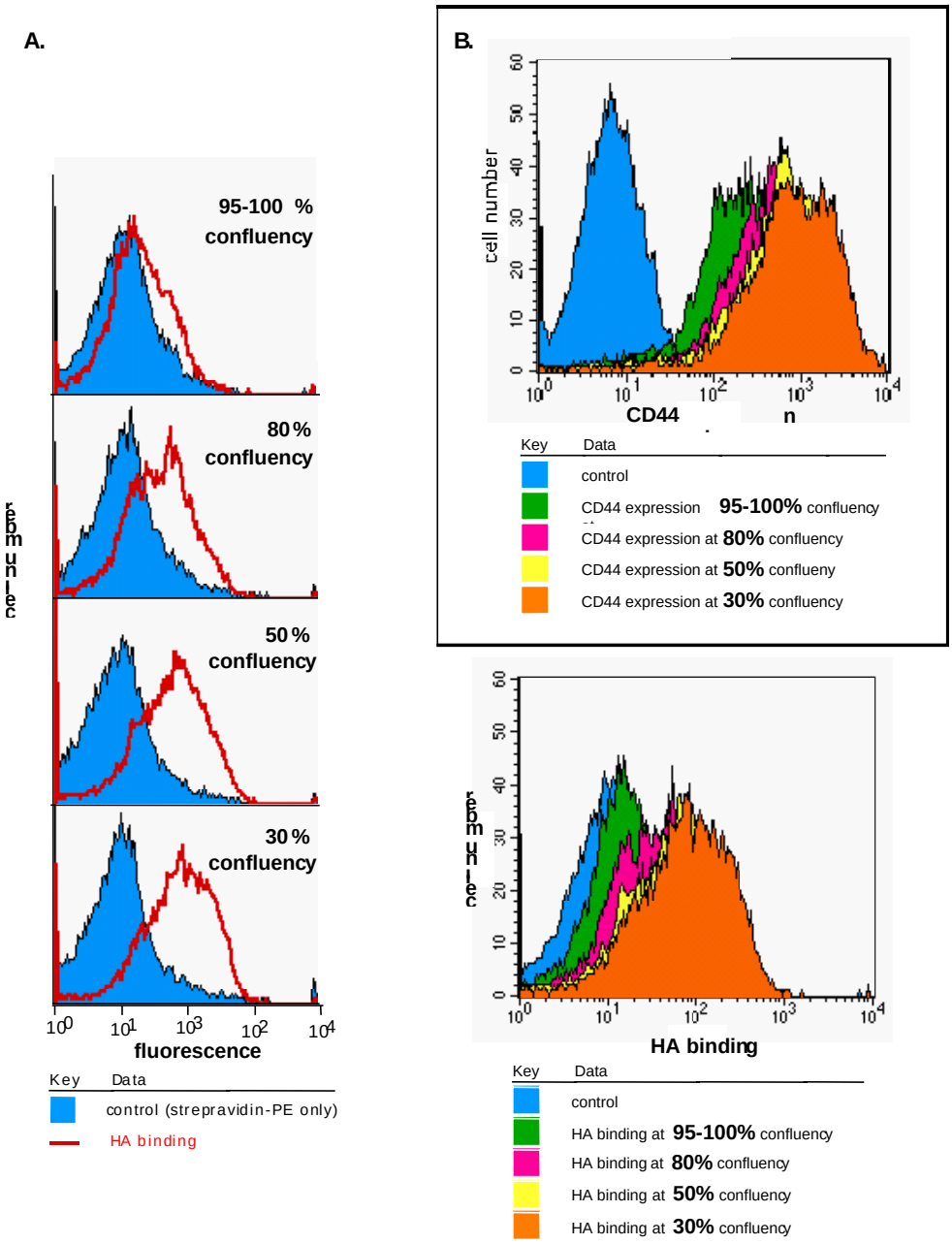


Figure 5. HA binding of primary embryonic fibroblasts is dependent on confluency.
A. HA binding at different confluencies. Cells were collected at indicated confluency and stained with biotinylated HA. HA binding was visualized by a flow cytometry after staining of cells with a fluorescent streptavidin conjugate. Left panel and right (bottom) histogram show the gradual increase of HA binding with the decline of confluency. B. CD44 expression of primary embryonic fibroblasts measured at decreasing confluencies.

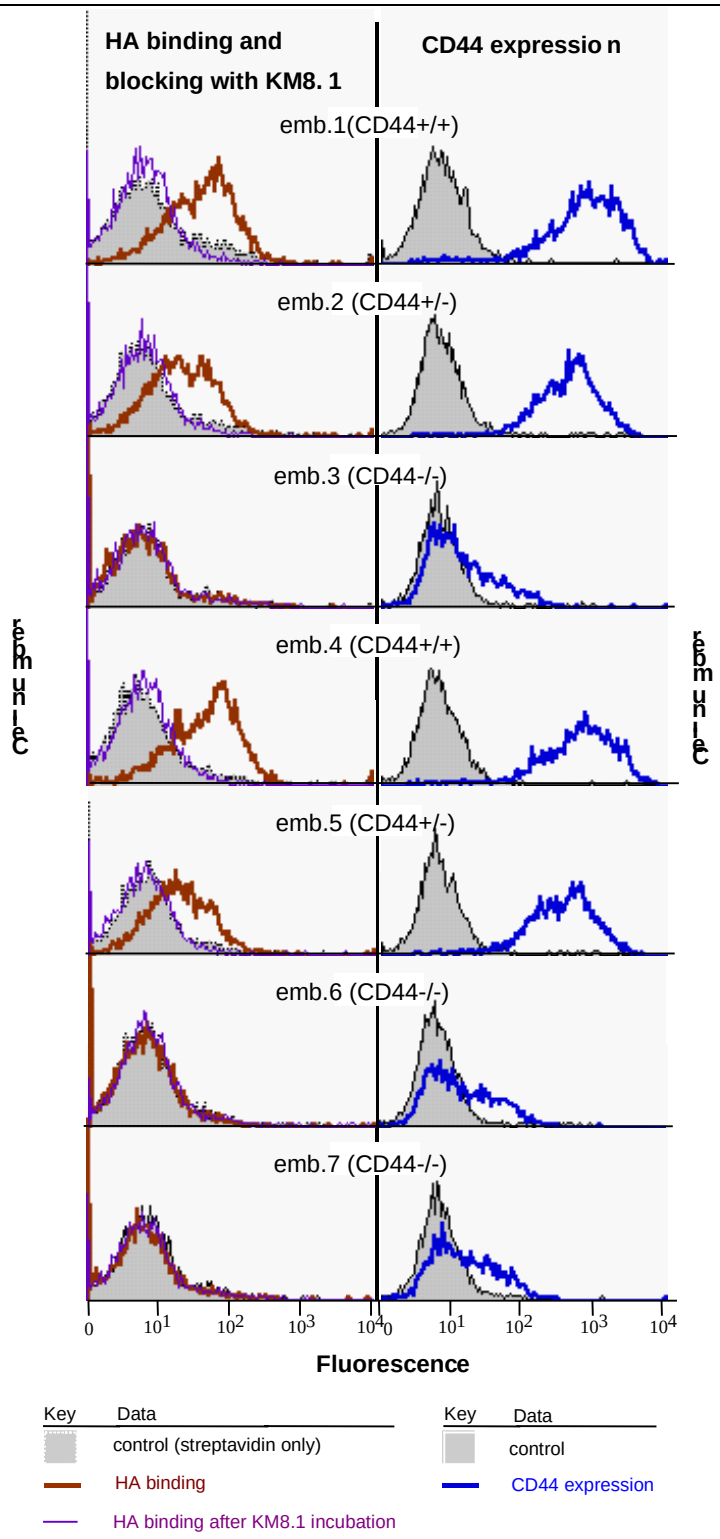


Figure 6. HA binding of primary fibroblasts from CD44+/+, CD44+/- and CD44-/- embryos correlates with the presence of wt CD44 allele.

Cultured primary embryonic fibroblasts of indicated CD44 genotype were collected at low density and stained with biotinylated HA, with (fine violet lines) or without (bold brown lines) KM81 preincubation. HA binding was visualized by flow cytometry after staining with fluorescent streptavidin conjugate (left panel). Controls show the fluorescence of cells stained with fluorescent streptavidin conjugate only. CD44 expression, measured in parallel, is shown on the right panel (bold blue lines).

Therefore in CD44-deficient primary embryonic fibroblasts the HA binding property of CD44 is not substituted.

3.1.1.6 Transformation of CD44 -/- embryonic fibroblasts does not stimulate their HA binding

Another way to activate CD44-dependent HA binding in CD44-positive fibroblasts and, perhaps, the binding of HA in CD44-negative fibroblasts would be a cell transformation.

The *Ras* -induced transformation of cultured fibroblasts was shown to increase the CD44 expression (Hofmann et al., 1993; Kogerman et al., 1996) and confer CD44-dependent HA binding to the cells (Kogerman et al., 1996). Furthermore, the most tumour-derived cells and tumorogenic cell lines express a high level of CD44, and CD44-mediated HA binding was shown to promote tumour growth and metastasizing in some cases (Bartolazzi et al, 1994, 1995). It is therefore possible that both enchanced CD44 expression and increase in HA-binding are the important features of the transformed cellular phenotype. For this reason I set out to transform CD44-/- fibroblasts, in the hope of inducing a HA binding protein which might compensate for he lack of CD44 in the transformed CD44-deficient cells.

Since transformation of primary fibroblasts requires activation of at least 2 oncogenes (Land et al., 1983), it was chosen to introduce both *ras* and *myc* oncogenes in mouse embryonic fibroblasts in order to achieve their full transformation.

p*Zip ras/myc* plamid carrying v-*Ha-ras* - and v-*gag-myc* - containing provirus (the kind gift of H. Land) was introduced into the ωE packaging cell line. Two stable virus producing clones with the highest transformation activity, as it was determined in screening for transformation of 3T3 cells, were selected for further experiments. Embryonic fibroblasts of different CD44 genotypes were transformed with virus-containing supernatants from these clones. The transformed phenotype was confirmedby the ability of the fibroblasts infected with *ras-myc* containing virus to form colonies in the soft agar (Fig. 7 a-c; Table I), in contrast to the primary fibroblasts, used as a negative control.

Table I. The efficiency of colony formation in soft agar by the *ras-myc* transformed mouse embryonic fibroblasts.

cell type	*ras-myc* CD44+/+	*ras-myc* CD44+/-	*ras-myc* CD44-/-	*ras*-NIH3T3 *)	primary CD44+/-
percent of colonies	65,5 ± 1,5 %	66,4 ± 8,1 %	12,9 ± 1,9 %	34,8 ± 5,0 %	0 ± 0 %

*) NIH3T3 cell line transformed with *ras* oncogene was used as a positive control.

The HA binding of resulting *ras-myc* transformed fibroblasts was measured as described previously. Soluble HA binding of transformed CD44-positive (CD44+/+ and CD44+/-) cells showed some clonal variation, and still no HA binding was detected in *ras-myc* transformed CD44-/- embryonic fibroblasts (Fig. 8a). CD44 expression did not change significantly after *ras-myc* transformation in any of the fibroblast cultures (Fig. 8b).

Thus, embryonic fibroblasts from CD44-/- mice did not bind to the immobilised or soluble HA. Both culturing the cells at the low density and oncogenic transformation led to the activation of CD44-dependent HA-binding in wild-type mouse embryonic fibroblasts but did not induce HA binding in fibroblasts originated from CD44-null mice. Therefore it was decided to look for compensatory HA binding in other cell types from CD44-/- mice.

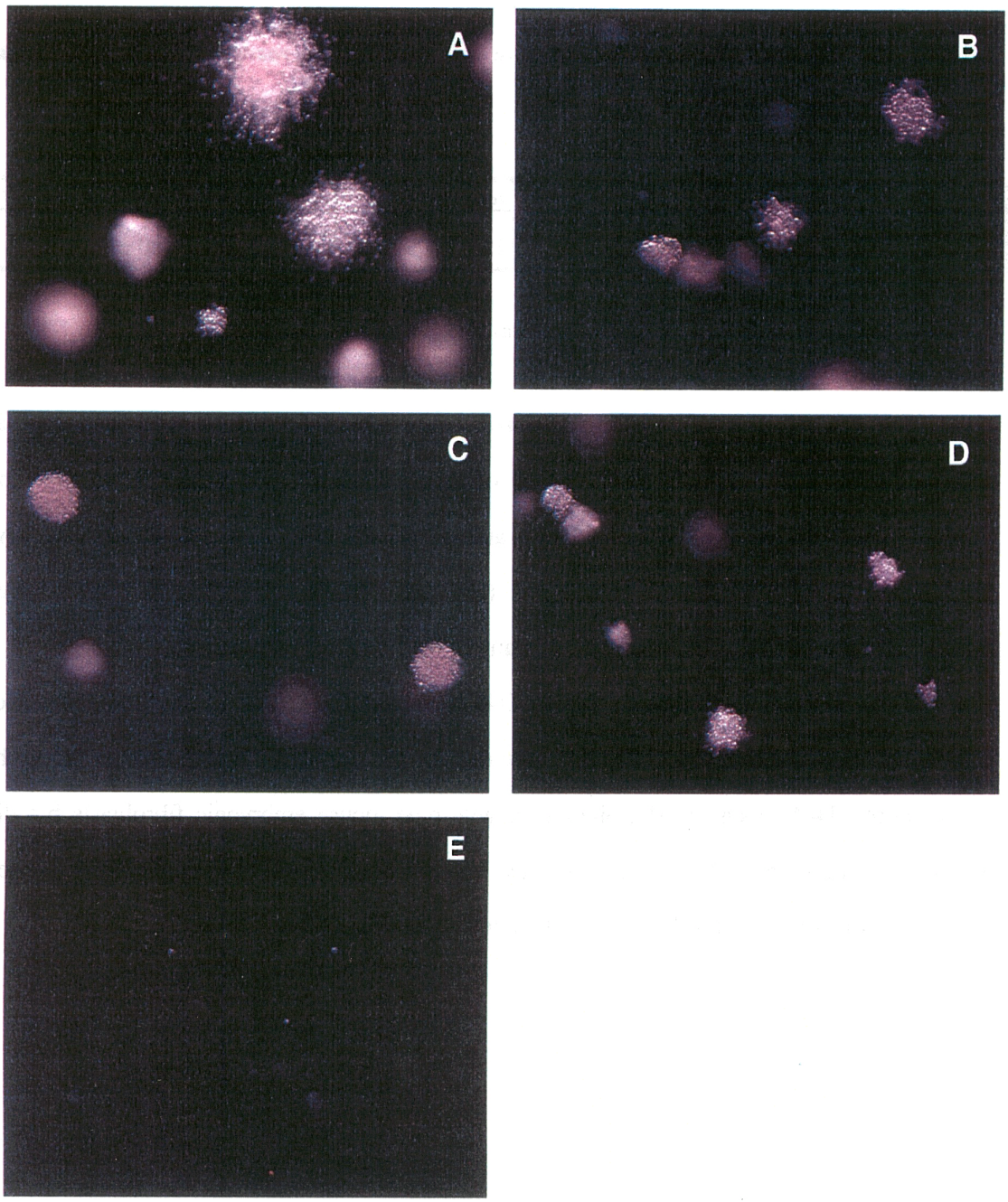


Figure 7. Growth property of *ras-myc* transformed mouse embryonic fibroblasts in a soft agar. CD44+/+ (A), CD44+/- (B) and CD44-/- (C) mouse embryonic fibroblasts, infected with *ras-myc* containing retrovirus, were plated in soft agar at density 500 cells per 500 µl. Colonies were photografed 8 days later. *Ras*-transformed NIH3T3 (D) and primary CD44+/- mouse embryonic fibroblasts (E) were grown in the same way and used as a positive and a negative control, respectively.

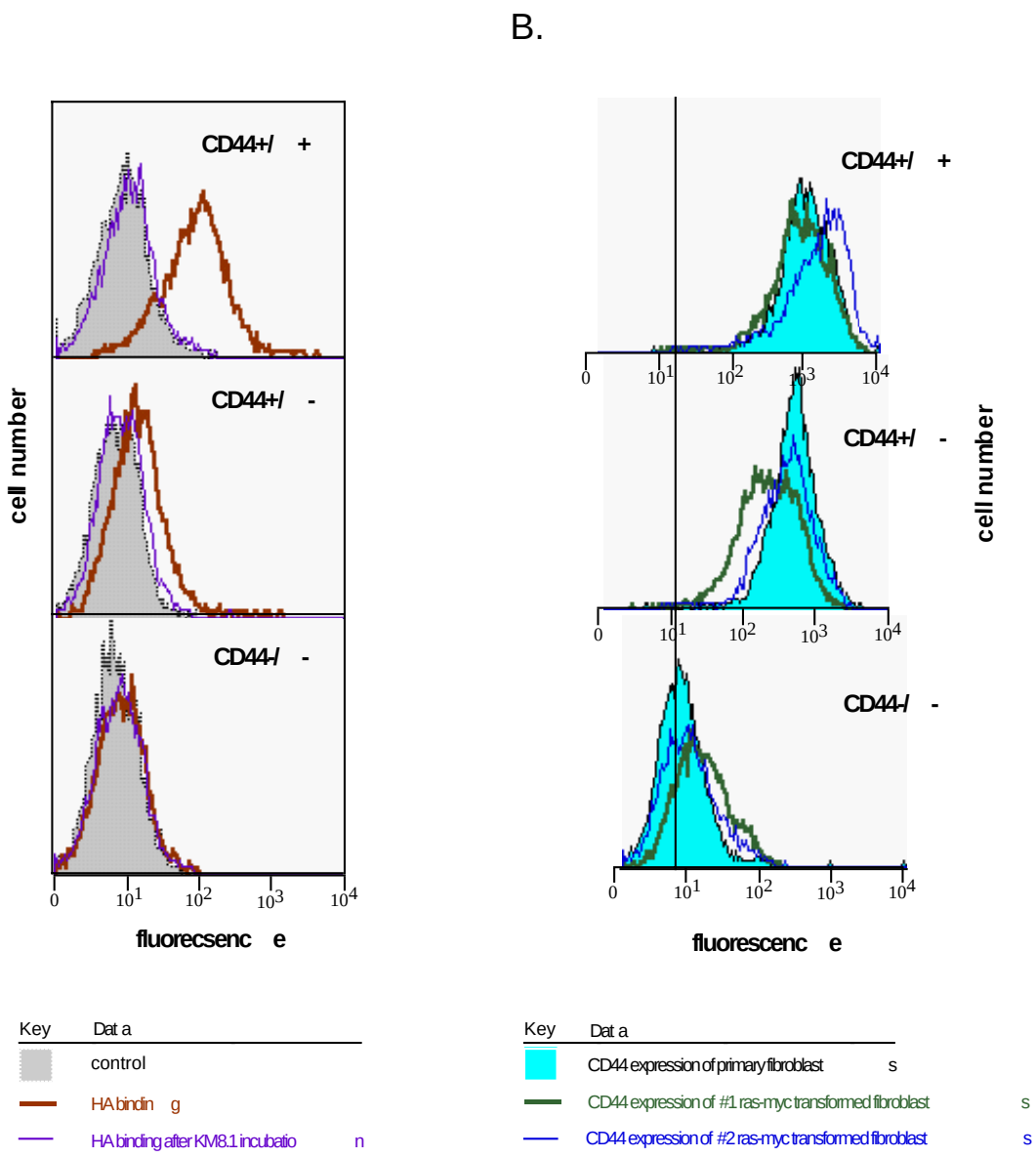


Figure 8. HA binding and CD44 expression of *ras-myc* transformed mouse embryonic fibroblasts of different CD44 genotype. Primary mouse fibroblasts from CD44 +/+, CD44+/- and CD44 -/- embryos (embryos N4, N5 and N6 in Fig. 5, respectively) were infected with *Zip ras/myc* virus. Two months after infection the resulting cultures were analysed for HA binding and CD44 expression.

A. HA binding. Cells of indicated genotype were collected at low confluency , stained with fluorescent HA with (fine violet lines) or without (bold brown lines) KM81 preincubation and analysed on flow cytometry. Controls show autoflurescence of cells without any staining. B. CD44 expression. Primary fibroblasts (shadowed curve) and cells infected with *Zip ras/myc* virus (fine blue lines and bold green lines demonstrate the results of two independent infections) were collected at low density and analysed for CD44 expression by a flow cytometry. CD44 genotype is indicated. Black bar shows a median of cell autofluorescence without an addition of abs.

3.1.2 HA binding of CD44-positive and CD44-negative cells of immune system

CD44 is described to play a role in many immunological processes. Nevertheless, CD44-null mice have no immune disfunctions (Schmits R., 1997). Receptor-mediated HA-binding has been detected in such immune cells as macrophages and B- and T-lymphocytes. Therefore these cells represent a potential source of HA binding proteins which compensate for the HA binding function of CD44 in CD44-/- animals. For this reason the HA binding properties of macrophages and T- and B- lymphocytes were investigated.

3.1.2.1 Thioglycollate-elicited and resident peritoneal macrophages bind HA in a CD44-independent manner

Macrophages are known to bind HA in a receptor-mediated fasion (Green et.al., 1988). Indirect evidence for a role of CD44 in macrophage HA binding comes from a number of studies, where HA-induced activation of macrophages could be prevented, at least in part, by anti-CD44 abs. (Noble et al., 1993; McKee et al., 1996; Hodge-Dufour et al., 1997). This makes macrophages a promising cell model to look for CD44 substitution in CD44-/- mice.

Peritoneal thioglycollate-elicited murine macrophages have been reported to respond to HA by cytokine production, and this effect was blocked by the anti-CD44 mAb KM81 (Hodge-Dufour., 1997). Therefore in a preliminary experiment I aimed to find out if CD44 is directly involved in the HA binding in these cells in CD44-positive mice. I used wild-type C57Bl6/J mice in order to be able to directly compare the HA binding of macrophages in further experiments from CD44-positive and CD44-negative mice, which are of C57Bl6/J background.

Peritoneal cells from healthy C57Bl6/J mice or mice previousely injected i.p. with Brewer thioglycollate medium were stained with the macrophage-specific marker Mac-1 and fluorescent HA and analysed by flow cytometry. Thioglycollate induces the inflammatory response in the injected mice, which results in an increase in the number of macrophages (Mac-1-positive cells) and in changes in their physical characteristics such as size and granularity (Fig. 9a). In order to define the macrophage populations, gates were set up around Mac-1 positive cells (coloured in red) on dot plots. The gated populations, consisting on 93 and 95 per cent of macrophages in

control and inflammatory case, respectively , were analysed for their binding to soluble HA (Fig. 9b).

Both thioglycollate-elicited (inflammatory) and control (resident) peritoneal macrophages bound HA (Fig. 9b). However, preincubation of the cells with KM81 did not have any effect on HA binding, suggesting that HA binding of the macrophages is not mediated by CD44. The MS5 cell line known to bind HA in a CD44-dependent manner was used as a control. HA staining in MS5 cells was completely blocked by KM81.

Since CD44 is not involved in HA binding of both resident and inflammatory peritoneal macrophages in C57Bl6/J mice, it does not need to be substituted in these cells in CD44-/-mice, and therefore the peritoneal macrophages are not a suitable cell model for the identification of HA binding molecule which compensate for CD44 in CD44-deficient mice.

3.1.2.2 B-lymphocytes from C57Bl6/J mice do not develop an HA-binding subpopulation in response to an *in vitro* IL-5 stimulation

IL-5 has been reported to induce a B-cell subpopulation that binds hyaluronate in a CD44-dependent manner (Murakami et.al, 1990). In CD44 -/- mice this subpopulation may therefore express a protein which compensates for CD44. I set out to investigate this possibility.

IL-5 is a product of T helper cells, promoting growth and differentiation of mature B-cells into antibody-producing plasma cells (Takatsu, 1997). The stage of B-cell differentiation can be traced by the expression of a number of cell-surface markers, including MHC class II (Ia atigens), B220, CD43, CD44 and so on. In the experiments described by Murakami et al. the IL-5-induced B-cells or splenocytes developed into two distinct subpopulations: one was CD44 dull/ Ia bright/ B220 bright and the other was CD44 bright/ Ia dull/ B220 dull. The last subpopulation was shown to bind HA in a CD44-dependent manner.

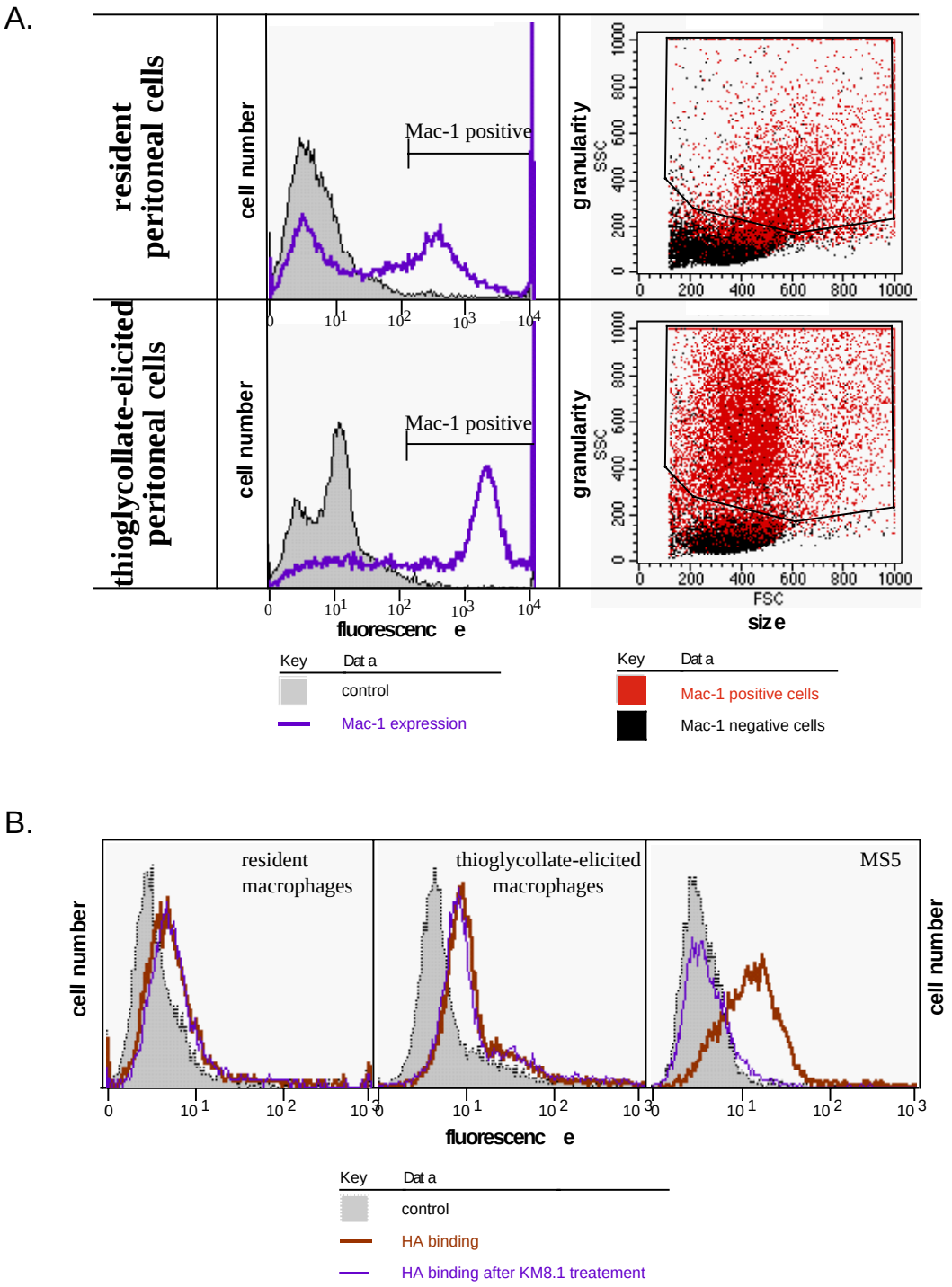


Figure 9. HA binding of resident and thioglycollate-elicited peritoneal macrophages.

Wild-type C57Bl/6J mice were intraperitoneally injected with Brewer thioglycollate medium, and inflammation was allowed to develop for 7 days. Peritoneal cells from injected and control healthy mice were collected and stained with PE-labeled Mac-1 abs and fluorescent HA with or without KM81 preincubation. A. Thioglycollate induces the inflammatory response in injected mice . Resident cells from control mice (upper part) and thioglycollate-elicited

cells (lower part) were analysed by flow cytometry for Mac-1 expression (bold violet lines on histograms). Control (shadowed) curves on histograms show autofluorescence of cells without addition of abs. FSC/SSC dot plots show size and granularity of the peritoneal cells. Red dots denote the Mac-1 positive cells, marked on the histograms. B. HA binding of peritoneal macrophages. Resident or thioglycollate-elicited peritoneal cells, gated on MAC-1 positive cells on dot plots (gates are shown on Fig.9a), were analysed for HA binding , with (fine violet lines) or without (bold brown lines) KM81 preincubation. Controls represent the autofluorescence of the cells. Control MS5 cell line was treated with fluorescent HA and KM81 and analysed in the same way as macrophages.

I attempted to induce the CD44 bright/B220 dull HA-binding subpopulation in B-cells or total splenocytes derived from C57Bl6/J mice. The HA binding of this subpopulation would be then compared with the HA binding of an analogous subpopulation developed by B-cells from CD44-deficient C57Bl6/J mice.

B-cells were isolated from splenocytes by T-cell depletion. B-cells or the total splenocyte population were incubated with LPS, with various amount of IL-5 or without any stimulus for 3 days. The B220-CD44 profile and HA binding of the resulting cells was investigated using three-colour flow cytometry.

Stimulation with IL-5 did not lead to separation of the desired CD44-bright/B220 dull subpopulation in C57Bl6/J -derived B-cells (Fig.10). HA binding of the total B-cell population was not altered by IL-5 incubation (not shown). An increase of the IL-5 concentration (Fig.10 shows the two examples with 1 and 100 ng/ml of IL-5) or of the incubation time did not improve the results: still there was no formation of a CD44-bright/B220 dull subpopulation. LPS stimulation resulted in an increase both in CD44 and B220, but not in HA-binding, consistent with the published data. This served as a positive control, indicating that the cells were viable and inducible. IL-5 was able to induce a dose-dependent proliferation of C57Bl6/J splenocytes, as it was tested by thymidine incorporation assays, indicating that splenocytes were stimulated by the concentration of IL-5 used.

In contrast to my results obtained with C57BL6/J splenocytes, when I used splenocytes derived from the mouse strain DBA/2 originally used by Murakami et.al, stimulation with 100 ng/ml IL-5 gave a rise to a CD44 high/B220 dull subpopulation (Fig.10). This population was described to bind HA in a CD44-dependent manner. This suggests that a strain background could be a crucial factor for B-cell development in response to IL-5-stimulation: in DBA/2 mice

it leads to the separation of CD44 high/B220 HA-binding subpopulation, while in C57Bl6/J mice this subpopulation is not detectable. Since it was impossible to induce HA-binding in wild-type C57Bl6/J B-cells, this cell model can not be used to look for a substitution of HA binding function of CD44 in CD44-deficient mice, which are in a C57Bl6/J background.

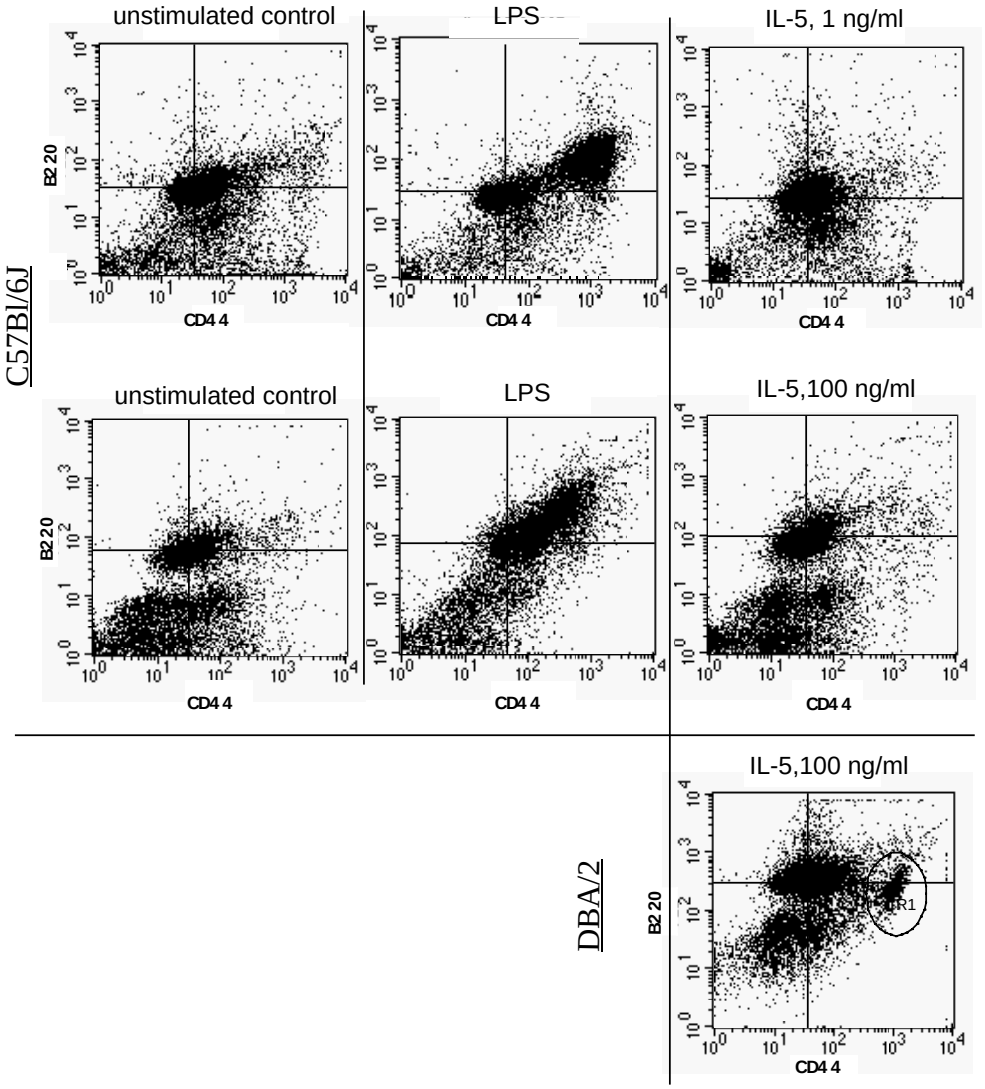


Figure 10. IL-5 stimulation of C57Bl6/J B-cells does not lead to the formation of the CD44 bright/B220 dull subpopulation. B-lymphocytes were isolated from C57Bl6/J splenocytes by T-cell depletion on a magnetic column after incubation of splenocytes with anti-Thy 1.2-coupled magnetic beads. B-cells (upper part) or splenocytes (middle part) were incubated for 3 days with 15 µg/ml LPS, the indicated amount of IL-5 or without any stimulus. They were then stained with PE-labeled B220 and Cy-Chrome -labeled anti-CD44 abs and analysed by flow cytometry. Splenocytes from DBA/2 mice, treated and analysed in the same way as cells in a middle part, were used as a control. R1 gate marks the CD44 bright/B220 dull subpopulation, which is described to bind to HA. These are the data from 3 different experiments.

3.1.2.3 The T-Cell Receptor cross-linking by anti-CD3 antibody induces CD44-dependent HA binding in C57Bl6/J murine T-lymphocytes

The treatment of T-cells with different TCR-stimulating agents, including anti-CD3 abs, induces a CD44 high subpopulation which binds HA in a CD44-dependent manner (DeGrendele at al, 1997b). Since CD44-null mice are of C57Bl6/J background, it was necessary to check if this phenomenon could be reproduced in T-cells derived from C57Bl6/J mice. If so, anti-CD3-treated T cells from CD44-/- mice may also express a compenatory molecule for CD44.

Lymph node cells from Balb/c (the strain used in the publication) and from C57Bl6/J mice were incubated with immobilized anti-CD3 abs or hamster γ-globulin as a control. After 48 hours, cells were collected and analysed for CD44 expression and HA-binding.

Stimulation with anti-CD3 abs, but not with hamster γ-globulin control, induced HA binding in both Balb/c and C57Bl6/J derived T cells (Fig.11). HA binding of these cells was measured with and without KM81 preincubation. This allowed a subpopulation of T-cells which binds HA in a CD44-dependent manner to be defined. This subpopulation was slightly bigger in Balb/c derived T-cells (12-15%) than in C57Bl6/J-derived T-cells (8-11% of total cell population). As a control in a CD44-dependent manner, KM81 preincubation totally blocked HA-binding of HA9 cells, known to bind HA (the histogram in Fig.11).

Thus, T cells derived from CD44-positive C57Bl6/J mice can be induced to bind HA in a CD44-dependent manner. This provides a suitable cell system to investigate whether a potential CD44 substitutive molecule could be found in CD44-/- mice.

3.1.2.4 Stimulation of T-lymphocytes from CD44-/- mice with anti-CD3 antibody does not induce their HA binding

If anti-CD3-stimulated T cells from CD44-deficient mice would develop HA binding subpopulation, analogous to that found in CD44-positive C57Bl6/J T cells, they could be a source of the HA-binding molecule which compensates for CD44 in CD44-/- mice.

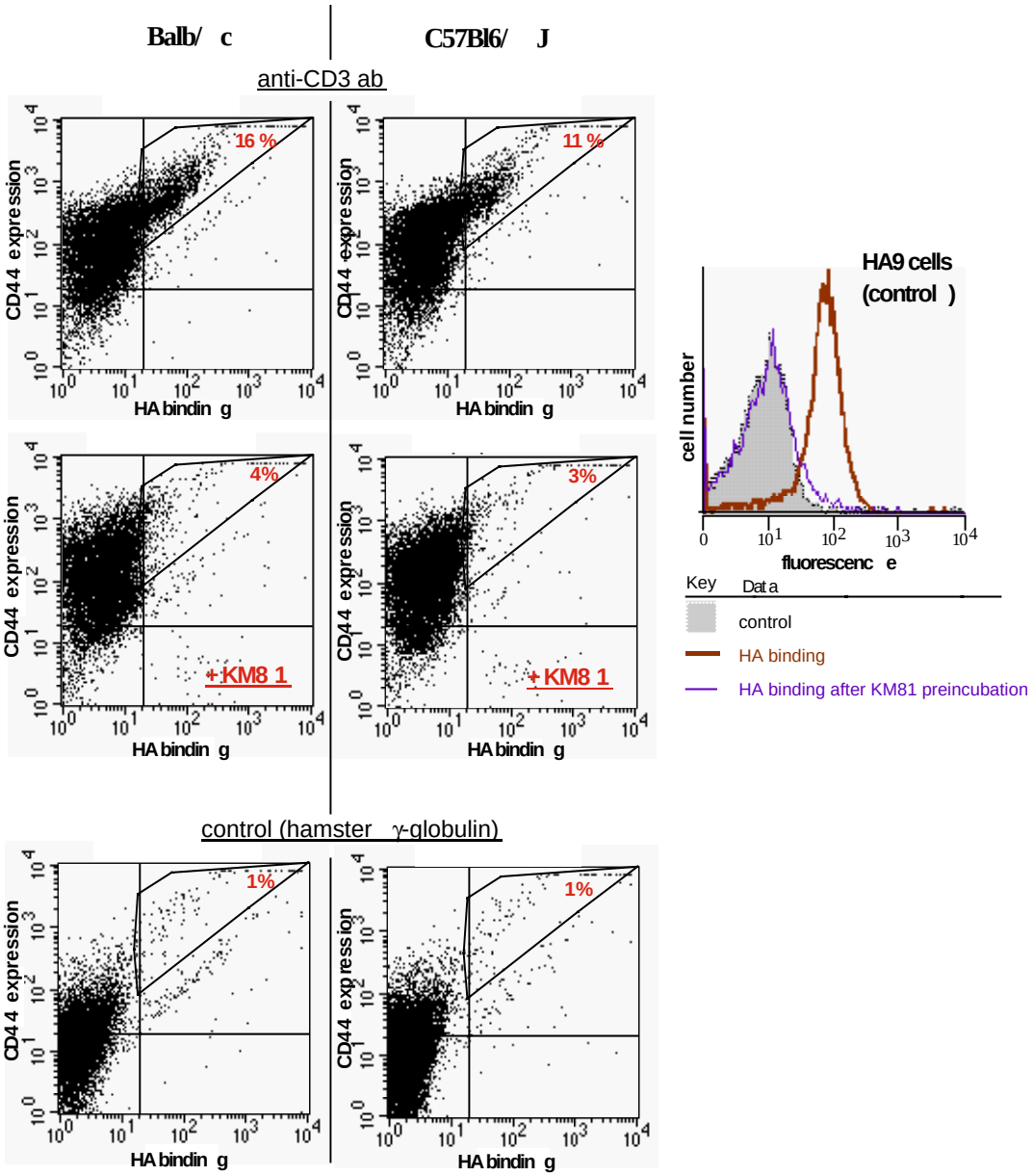


Figure 11. CD44-dependent HA binding is induced in both Balb/c and C57Bl6/J T-lymphocytes by the CD3 crosslinking. Lymph node cells from Balb/c andC57Bl6/J mice were plated in 96 well plates with immobilized anti-CD3 ab or hamster γ-globulin for 48 h and then stained with anti-CD44 abs and biotinylated HA with (when indicated) or without KM81 preincubation, followed by staining with fluorescent streptavidin conjugate. CD44 expression and HA binding of the cells were analysed by the two colour flow cytometry. The gate was set around the CD44-dependent HA-binding subpopulation, and the percent of gated cells was noted in each case (red numbers within the gates). The histogram shows HA binding with (fine violet line) or without (bold brown line) KM81 preincubation of HA9 cells line, used as a control for KM81 blocking capacity. Control represents the cell fluorescence after fluorescent streptavidin conjugate staining only.

Therefore I compared the HA-binding capacity of anti-CD3 ab stimulated T-cells from CD44-positive and CD44-negative mice.

Lymph node cells from wild-type and CD44-deficient C57Bl6/J mice were treated as above and analysed for CD44 and CD69 expression, as well as for HA-binding. CD69 is a T-cell differentiation marker, which is useful for double-stainings when CD44 is not available, as in cells from CD44-null animals, allowing the HA-binding cells to be resolved in a two-dimentional picture.

As shown in a Fig.12, after stimulation with anti-CD3 abs, CD44-positive T-cells upregulated both CD44 and CD69 compared to the unstimulated control, and developed a subpopuplation which binds HA in a CD44-dependent manner. This subpopulation could be detected in both types of dot plot, CD44 versus HA-staining and CD69 versus HA-staining. Since in CD69 versus HA plots it was not possible to gate precisely on the subpopulation binding HA in a CD44-dependent manner, this subpopulation can be seen in this case by the drop in percentage of total HA-binding cells (cells on the right part of the plot) after KM81 preincubation. The size of this subpopulation in T-cells from CD44-positive C57Bl6/J mice was 10-11%.

However, there was no increase in HA binding of CD3-stimulated T-cells from CD44-/- mice compared to the unstimulated control (Fig. 12). That means that CD3-crosslinking does not induce the formation of additional HA binding subpopulation in CD44-/- T-cells. Clearly, KM81 preincubation did not have any effect on CD44-/- T-lymphocytes. The level of CD44-independent HA binding stayed the same in both CD44+/+ and CD44-/- cells, and was not altered by CD3-stimulation.

These results show that T-cells from CD44-null mice do not develop an HA binding subpopulation, analogous to the one formed by CD44-positive T-cells. It suggests that CD44-dependent HA binding is not substituted in T-cells from CD44-deficient mice.

Apparently, a CD44-dependent HA binding is not substituted by any other HA binding molecule in CD44 -/- mice, at least in the *in vitro* situation.

Since it was not possible to trace the substitution of CD44 in CD44-deficient mice by the hyaluronic acid binding, other CD44 features such as growth factor presentation were exploited in a search for compensation for lack of CD44 in CD44-null mice.

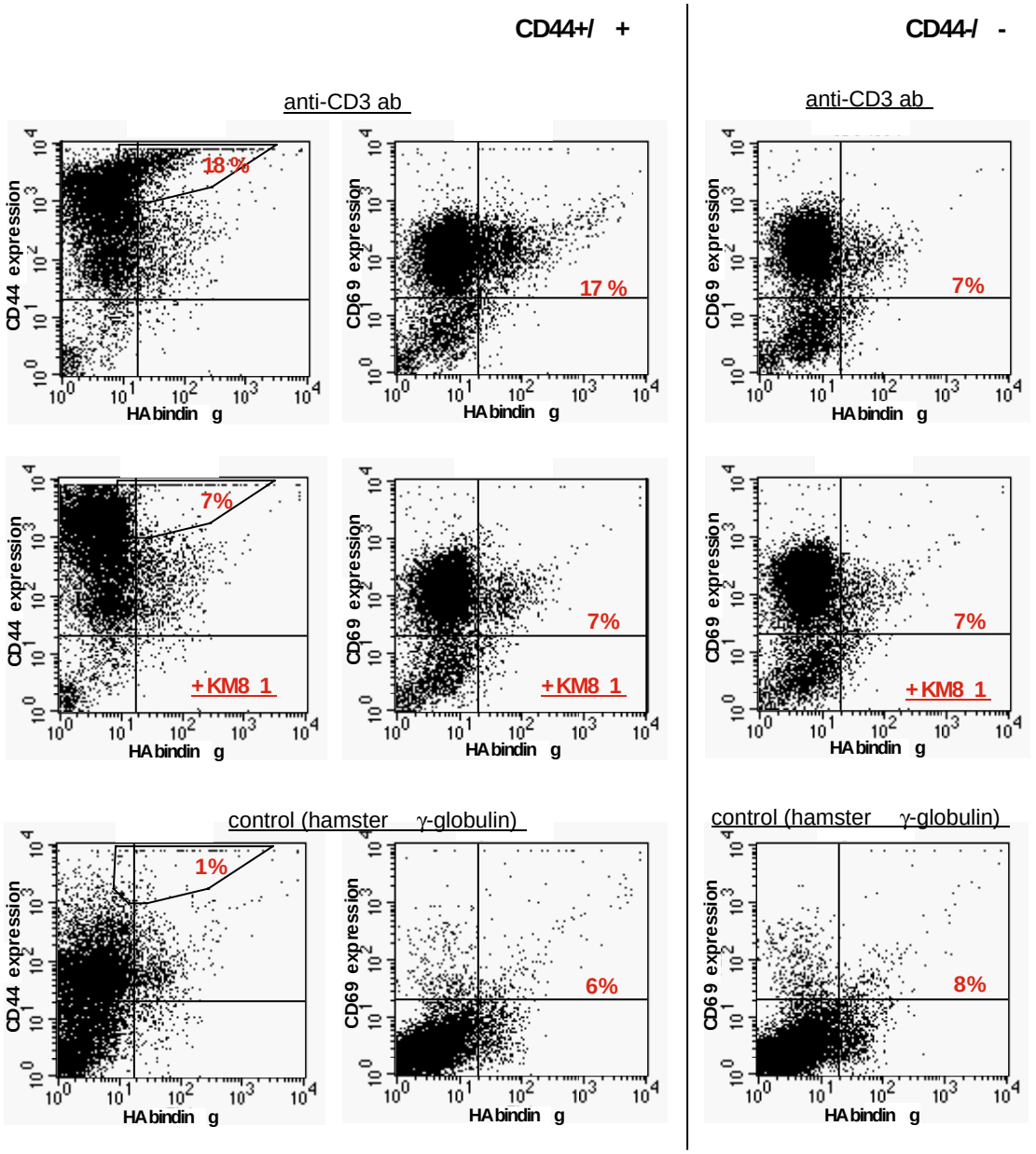


Figure 12. The stimulation with anti-CD3 abs induces the formation of HA-binding subpopulation in T-lymphocytes derived from wt but not CD44-null mice. Lymph node cells from CD44+/+ and CD44-/- C57Bl6/J mice were plated in 96 well plates with immobilized anti-CD3 abs or hamster γ-globulin for 48 h and then stained with anti-CD44 abs, anti-CD69 abs and biotinylated HA with (when indicated) or without KM81 preincubation, followed by staining with fluorescent streptavidin conjugate. CD44 or CD69 expression and HA binding of the cells were analysed by two colour flow cytometry. The gates on CD44 versus fluorescent HA plots were set around the CD44-dependent HA-binding subpopulation. The numbers indicate percent of gated cells or, when there is no gate, percent of cells in the right half of the plots.

3.2 Analysis of heparan sulfated proteoglycans in cells derived from CD44-/- mice

The outgrowth of the developing limb bud is controlled by the signals from the apical ectodermal ridge (AER) which promote the proliferation of underlying mesenchymal cells, keeping them in the undifferentiated stage (Kosher et al.,1979). The effect of the AER on mesenchymal cells is mediated by different members of FGF family produced by AER cells, including FGF2, FGF4 and FGF8 (Crossley at al, 1996; Fallon at al., 1994; Niswander at al., 1993; Vogel at al, 1996). In order to exert its growth-promoting potential on responsive cells, FGF must be first associated with its low affinity receptor - a heparan sulfate proteoglycan (HSPG). This enables FGF to induce signalling via the specific high affinity tyrosine kinase receptor, FGFR. Thus, FGFs have to be presented to their specific receptors by HSPGs.

CD44 variants containing exon v3 have a covalently attached heparan sulfate chain, which joins these CD44 isoforms to the HSPG family. CD44v3-10 and CD44v3, 8-10 are able to bind growth factors and present them to their high affinity receptors (Jackson et al., 1995; Sherman et al, 1998).

In the context of the developing limb buds in mouse and in rat, CD44v3-10 plays the role of a major HSPG presenting AER-produced FGF8 and FGF4 to underlying mesenchymal cells (Sherman et al, 1998). Expression of CD44v3-10 in the developing limb buds is restricted to AER, and anti-CD44 abs applied on AER can block limb development to a similar extent as the complete removal of AER. Because of such an important role for CD44 in developing limb buds, and the fact that CD44 deficient mice have normal limbs, it is conceivable that CD44-deficient mice develop a substitutive mechanism for the absense of CD44. One can expect that other than CD44v3-10 heparan sulfated proteoglycan/proteoglycans take over the FGF presentation function of CD44 in CD44-/- limb buds.

From the previous work of D.Wainwright (Waiwright, 1998) it appears that the compensation for the lack of CD44 occurs both in AER and in limb bud mesenchyme in CD44-null mice. AER from CD44-null mice is able to induce proliferation of the wild-type mesenchymal cell, in contrast to anti-CD44 ab-pretreated AER from wild-type animals (Wainwright, 1998) In its turn, CD44-deficient mesenchyme aquires the capacity to respond to soluble FGF8 by proliferation, while the wild-type limb bud mesenchymal cells are not responsive to soluble FGF8. It is likely that mesenchymal cells from CD44-deficient mice

themself express a HSPG, which plays a role of low affinity receptor for FGF8, thus compensating for the absense of CD44.

In order to find this compensatory molecule, the limb bud mesenchymal cells from CD44-positive and CD44-negative mice were analysed for their expression of surface heparan sulfated proteins. HSPGs selectively upregulated in CD44-/- mesenchymal cells compared to the wild-type cells would be the proteins of interest.

3.2.1 Establishment of CD44-positive and CD44-negative limb bud mesenchymal cells in culture

The high density cultures of CD44+/+ and CD44-/- mesenchymal cells were established from the pooled mesenchymal limb bud tissues of the embryos of corresponding genotype, with at least 50 limbs in each pool. After several passages in culture, the cells were tested for their responsiveness to soluble FGF8: serum-starved CD44-positive and CD44-negative limb bud mesenchymal cells were exposed for 12 h to the increasing concentrations of soluble FGF8 in serum-free medium. The cell proliferation was measured by the [^{3}H]-thymidine incorporation. The results (Fig. 13) were consistent with the previous findings, that CD44-/- limb bud mesenchymal cells are able to respond to soluble FGF8 by proliferation, while CD44+/+ cells are not. This confirms that the isolated cell cultures have the earlier described properties and can be used as a model for investigation of CD44-substitutive molecule.

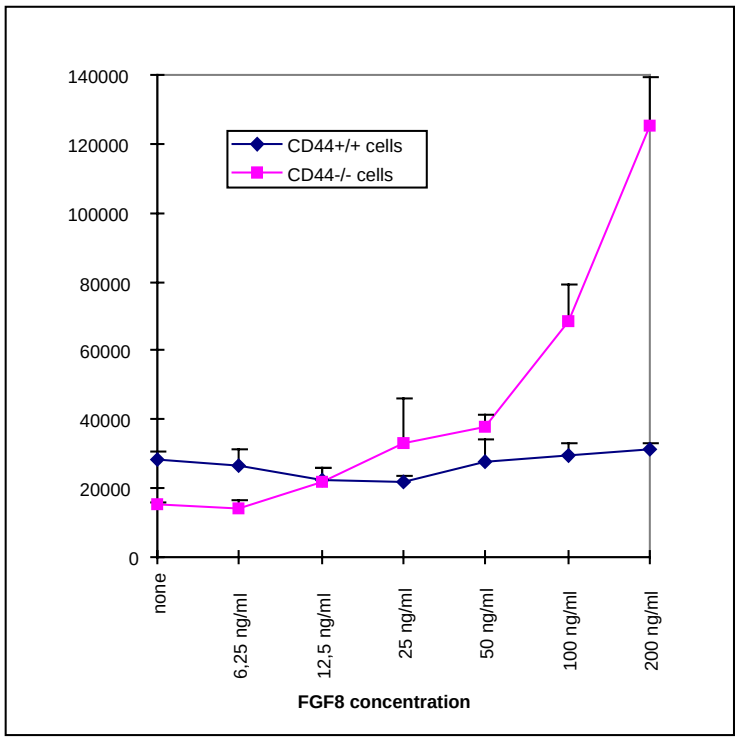


Figure 13. Responsiveness of wild-type and CD44-negative limb bud mesenchymal cells to soluble FGF8. The cultured limb bud mesenchymal cells from CD44+/+ (blue line) and CD44-/- (red line) mice embryos were serum starved for 12 hours, followed by incubation with different concentrations of soluble FGF8 in serum-free medium for next 12 hours. Cells were pulsed with [^{3}H]-thymidine and the proliferation was measured by thymidine incorporation in 5-6 h. Each point represents the mean of triplicate samples.

3.2.2 CD44+/+ and CD44-/- limb bud mesenchymal cells have an equivalent amount of heparan sulfate on their surfaces

In order to find out if CD44-negative limb bud mesenchymal cells carry more of heparan sulfate on their surfaces then the CD44-positive ones, the limb bud mesenchymal cells were analysed by flow cytometry with an anti-heparan sulfate (anti-HS) antibodies recognizing the intact heperan sulfate chain on any of HSPGs (Fig. 14). The mesenchymal cells from both CD44+/+ and CD44-/- limb buds had a significant amount of surface heparan sulfate. Pretreatment of the cells with heparinase abolished the anti-HS ab staining (histograms in a left part of Fig. 14), showing that the antibody staining was specific for heparan sulfate. As it is seen on the right histogram in a Fig. 12, CD44-positive and CD44-negative cells had the same level of surface heparan sulfate.

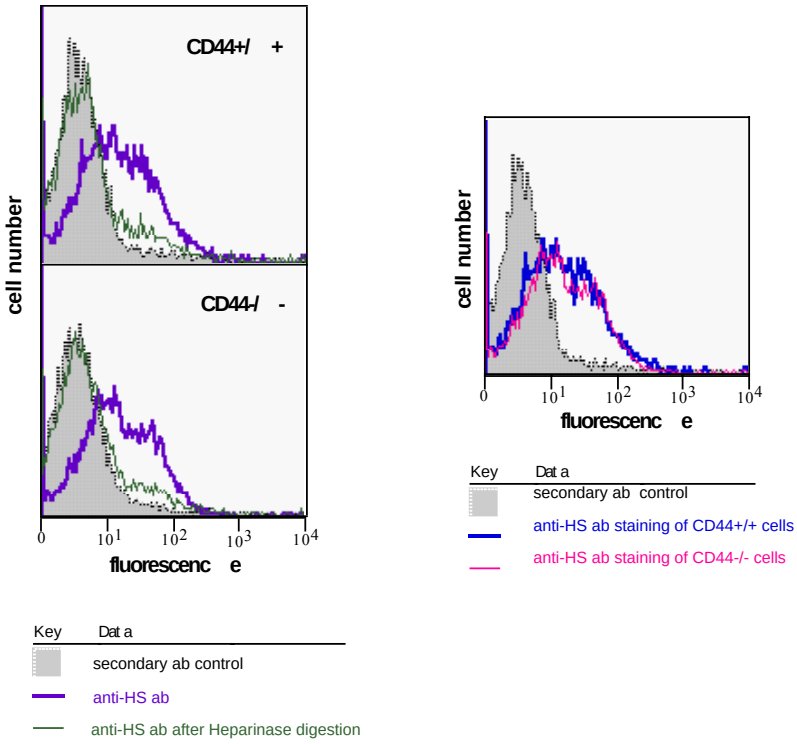


Figure 14. An amount of surface heparan sulfate in wild-type and CD44-negative mouse embryonic limb bud mesenchymal cells. Cultured CD44+/+ and CD44-/- mouse limb bud mesenchymal cells were detached from the plates with EDTA solution and stained with anti-HS abs with (fine green lines on left histograms) or without (bold violet lines on left histograms) 3 hours pretreatment with heparinase. The amount of heparan sulfate on the cell surfaces was analysed by flow cytometry, after incubation of cells with biotinylated secondary antibodies followed by fluorescent streptavidin conjugate. Gray shadowed curves represent the fluorescence of the cells without the addition of primary (anti-HS) abs. The right histogram shows the comparison of heparan sulfate level in CD44+/+ (bold blue line) and CD44-/- (fine red line) limb bud mesenchymal cells.

Thus, surprisingly, there was no difference in the total amount of heparan sulfate on the cell surface in CD44+/+ and CD44-/- limb bud mesenchymal cells.

3.2.3 The limb bud mesenchymal cells from CD44-deficient mice have an increased level of the low molecular weight heparan sulfated proteoglycans

Despite the fact that the total amount of heparan sulfate on CD44-positive and CD44-negative mesenchymal cells appeared to be equal in a flow cytometry analysis, it does not exclude the possibility that there is a switch in expression from one HSPG to another in CD44-deficient mesenchyme. In order to see which proteins are contributing in the total level of heparan sulfate in both CD44-positive and CD44-negative cells, limb bud mesenchymal HSPGs were analysed on a Western blot.

The anti-HS antibodies which have been used in flow cytometry assay do not work in Western blots. For this reason, another available antibodies, anti-Δ HS, which are suitable for Western blots analysis of HSPGs were used for investigation of HSPGs in limb bud mesenchymal cells. They specifically recognize the newly created epitope in HSPGs resulting from the digestion of the proteoglycan with heparinase.

The limb bud mesenchymal cells from CD44+/+ and CD44-/- embryos were treated with heparinase in order to create the epitope for an anti-Δ HS ab. Both heparinase digested and undigested cells were subjected to Western blot analysis with the anti-Δ HS abs (Fig.15). Heparan sulfated proteoglycans can be detected as bands specific for heparinase digested samples (indicated with the arrows). There is a clear upregulation of at least 3 bands in a range of 55-65 kDa (black arrows), in CD44-deficient cells compared to the wild-type cells (lines 4 and 2, correpondingly). The HSPG whose heparinase digestion product runs as a 120 kDa band is expressed at the same level in both cell types, and the high molecular weight HSPG (more than 210 kDa after heparinase digestion) is downregulated in CD44-/- samples.

Probing with anti-β-tubulin antibodies showed that the lanes with heparinase digested samples were equally loaded.

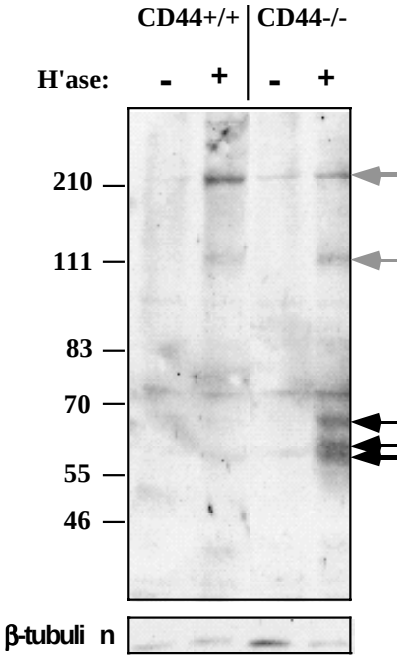


Figure 15. Expression of heparan sulfated proteoglycans by limb bud mesenchymal cells from wild-type and CD44-negative mice embryos. CD44+/+ and CD44-/- cultured limb bud mesenchymal cells were treated for 3 hours with 4 units/ml heparinase (H'ase) or PBS along, as indicated. Cells were then lysed and lysates were subjected to Western blot analysis with anti-Δ HS antibodies. The heparan sulfated proteoglycans are marked with arrows. The probing of the same blot with an anti-β-tubulin abs serves as a loading control.

These results mean that CD44-deficient limb bud mesenchymal cells have a different expression of heparan sulfated proteoglycans compared to the CD44-positive limb bud mesemchymal cells. The low molecular weight HSPGs overexpressed in CD44-null cells are the likely candidates for the FGF-presenting molecules responsible for the increased sensitivity of these cells to the soluble FGF8. Therefore the lack of CD44 in the CD44-deficient animals is possibly compensated for by the overexpression of these low molecular weight HSPGs.

Chapter 4. Discussion

Compensatory mechanisms in CD44 deficient mice

The involvement of CD44 in many physiological and pathological processes suggested that animals lacking this molecule would have a severe phenotype with multiple abnormalities in different systems. However, the gene targeting disruption of CD44 in mice (Schmits et al., 1997) resulted in minor changes and did not affect embryonic development or immune system functions (with one exception noted below). It was concluded therefore that CD44-deficient mice utilise some compensatory mechanisms which allow them to overcome the need for CD44.

This work aimed to investigate changes in CD44-mutant mice which compensate for the lack of CD44. I found no substitution of CD44-dependent HA binding in either embryonic fibroblasts or activated T-lymphocytes. However, CD44-deficient limb bud mesenchymal cells showed an increased responsiveness to soluble growth factor. In these cells low molecular weight heparan sulfated proteoglycans were upregulated compared to CD44-positive mesenchymal cells. These proteins might be involved in the compensation for the lack of the heparan sulfated proteoglycan form of CD44 during limb morphogenesis.

4.1 The substitution of CD44-dependent HA binding in CD44-deficient cells

To study compensatory mechanisms in CD44-deficient mice, a simple approach was first chosen: since the hyaluronic acid (HA) is a major ligand for CD44, it was assumed that the compensation for the lack of CD44 would upregulate some substitutive molecule/molecules which bind hyaluronic acid instead of CD44 and thus perform the CD44 functions. To isolate this substitutive HA binding protein, several different cell types derived from CD44-deficient and wild-type mice were tested for their ability to bind HA, in order to find a suitable source for the biochemical purification of the protein of interest. The sought tissue or cell type had to satisfy the following criteria: i) in the wild-type mice this cell type should bind HA in a CD44-dependent manner and ii) this type of cell should bind HA in CD44-deficient mice. It was desirable that in addition to the criteria mentioned above, the tissue/cell type expressing molecule(s) which

compensate for lack of CD44 would be easy to expand *in vitro* so that it would be possible to obtain a sufficient amount of material for the purification procedure.

4.1.1 HA binding in mouse embryonic fibroblasts from CD44-positive and CD44-negative mice

The first cell type studied was embryonic fibroblasts. It turned out however, that despite high CD44 expression, both primary and immortalised fibroblasts obtained from CD44 heterozygous mutant mice did not exhibit any significant HA binding. The same was true for the fibroblasts obtained from CD44-null mice. Attempts to enhance HA binding by stimulation with different mitogens failed. However, in a search for conditions which could induce the CD44-dependent HA binding, I found that in wild-type primary embryonic fibroblasts the CD44-dependent HA binding is inversely correlated with cell density.

These results are in agreement with the previous investigations (Lesley et al., 1993a, 1993b) showing that HA binding of CD44 in some cell systems is inducible and not constitutive. My data indicate that also in primary embryonic fibroblasts CD44-dependent HA binding is inducible under some circumstances, such as low density conditions.

Most of the studies concerning CD44-mediated HA binding in fibroblasts were made on established fibroblasts cell lines, which are often transformed. It is difficult therefore to compare the HA binding of these cells with that of the primary fibroblast cultures. Nevertheless, there is some indication that CD44 dependent HA binding in fibroblasts can be induced, for example, by maintaining them in medium containing 5% serum (Kogerman et al., 1996). Furthermore, CD44 expression and CD44-dependent HA binding was upregulated by FGF in a FGF-receptor transfected mouse fibroblast cell line (Zhang et al., 1997). Moreover, the enhanced CD44-dependent HA binding in NIH3T3 fibroblasts after transfection with $p185^{neu}$, one of the EGF-receptor family members (Zhu and Bourguignon, 1996), suggests that EGF may stimulate CD44-dependent HA binding in primary mouse fibroblasts. This was not the case, however, in my experiments, as the overnight incubation of primary mouse fibroblasts with EGF failed to increase their HA binding. The failure could not be ascribed to the absence of a functional EGF-

receptor in these cells, since the same concentration of EGF induced intracellular signalling, as was detected by increased MAP-kinase phosphorylation.

In contrast, a decrease in confluency of cultured primary mouse embryonic fibroblasts led to an increase in both CD44 expression and in CD44-dependent HA binding. The influence of cell density on the expression level of different proteins has been described before. The expression and activity of ouabian-sensitive Na/K ATPase pumps in epithelial cells declined with increased cell confluency and did not depend on the proliferation rate (Burke et al., 1991). In contrast, the expression of T-cadherin, a new lipoprotein-binding glycoprotein in aortic smooth muscle cells increased with higher cell density and achieved maximum level at confluency (Kuzmenko et al, 1998). Low density conditions in mammary epithelial cell lines led to a decrease of erbB-2 expression due to protein degradation, without affecting the erbB-2 mRNA level (Kornilova et al., 1992). Interestingly, several protein kinase C (PKC) subspecies were differently regulated by the density of Swiss 3T6 fibroblasts. While the level of PKC-α and -δ increased with the increase in the cell density, PKC-ε was upregulated at low confluency and gradually declined as the cells became more dense. The level of PKC-ζ did not depend on confluency (Littlebury et al., 1997). So far there is no published data about the regulation of CD44 by cell density.

HA binding in different cell systems appeared to be dependent on the amount of CD44 on the cell surface (He et al., 1992; Lesley et al., 1993a). It was observed, for example, that in LB-melanoma cells induced by PMA the aquisition of HA binding requires that the level of CD44 reaches a certain threshold (Sionov and Naor, 1998). Furthemore, in a cell-free enviroment, a CD44-immunoglobulin (CD44/Ig) fusion protein immobilized on beads also showed HA binding only above a certain threshold of amount of the fusion protein, increasing further with the increase of CD44/Ig density. The threshold level was dependent on the cell type in which a CD44/Ig fusion protein was produced and on the glycosylation pattern of the protein (English et al., 1998). It is possible therefore that HA binding in primary mouse fibroblasts cultured at low density is partially a consequence of the increased amount of surface CD44. However, since the increase in CD44 expression was rather moderate compared to the increase in HA binding, it is not clear if the change in HA binding ability is a result of only a "permissive" accumulation of CD44 on the surface, or also of some alterations in CD44 structure or/and signalling.

In accordance with my aim to find the substitutive molecule for CD44 expression in CD44-deficient mice, I compared the HA binding of CD44-positive and CD44-negative mouse embryonic fibroblasts at low confluency. I could show that there is no substitution for CD44-dependent HA binding in CD44-deficient cells: the HA binding in fibroblasts obtained from heterozygous CD44-mutant was decreased compared to the wild-type littermate controls, and was absent in cells derived from CD44-null embryos. All detectable HA binding in CD44+/+ and CD44+/- fibroblasts was CD44-dependent, since it was completely abolished by KM81, anti-CD44 antibody interfering with HA binding. Apparently, the only protein which is able to bind HA in primary mouse embryonic fibroblasts is CD44, and in the absence of CD44 expression its HA binding function is not taken over by any other protein.

My next attempt to induce HA binding in CD44-deficient fibroblasts via transformation was inspired by the fact that many tumour cells and transformed cell lines bind HA constitutively. Since the HA binding capacity of transformed cells was shown to be beneficial for their tumorigenic and metastatic behaviour (Bartolazzi et al, 1994, 1995), it was possible to think that *ras* transformed fibroblasts might upregulate their HA binding independently of the presence of CD44. However, the transformation of primary embryonic fibroblasts with a retroviral construct containing both *ras* and *myc* oncogenes did not lead to an increase in HA binding in any cell tested, independently of their CD44 genotype. There was still no HA binding in CD44-deficient fibroblasts after their transformation.

Surprisingly, the CD44 expression and CD44-dependent HA binding in CD44-positive cells was also not upregulated by transformation. This observation is in contradiction to the multiple previous reports about the influence of *ras* transformation on CD44 expression and CD44-mediated HA binding of fibroblast cell lines. For example, Balb/c 3T3 fibroblasts showed an increase in CD44 expression and CD44-dependent HA binding upon transformation with the *ras* oncogene, independently of cell density and serum concentration (Kogerman et al., 1996). *Ras* -transformation of rat intestinal epithelial cells dramatically increased their CD44 expression and resulted in HA-dependent cell aggregation (Jamal et al., 1994). However, I observed no increase in CD44-expression and CD44-dependent HA binding of wild-type *ras-myc* transfected fibroblasts compared with the nontrasfected cells. This could be due to the fact that both of these parameters were measured at low density conditions, favouring high CD44-expression and HA

binding in nontransfected cells. One can speculate that under these conditions CD44 expression in mouse embryonic fibroblasts perhaps reaches a maximum, which can not be further augmented by transformation or mitogen stimulation. Transformation may confer an HA binding ability on cells independently of density conditions, but not greater than that observed in nontransformed cells at low density. This model could also explain the failure of mitogens to induce HA binding by primary fibroblasts. The mitogen induction experiment was done in a way that cells were plated at low density a day before stimulation. Perhaps maximum HA binding was already reached under these conditions, and no additional upregulation of HA binding could be induced by the mitogens applied.

Thus, the CD44-deficient fibroblasts, transformed or not, did not showed any detectable HA binding. Since HA binding is not substituted in CD44-deficient fibroblasts, this suggests that either HA binding properties are not necessary in this cell type or the compensation for the lack of CD44-dependent HA binding involves some other receptor-ligand interaction partners.

4.1.2 HA binding in immune cells derived from CD44-positive and CD44-negative mice

CD44 is implicated in the inflammatory response *in vivo*, since anti-CD44 antibody administration inhibited disease manifestation in models such as 2,4 dinitro-1-fluorobenzene (DNFB) - induced Delayed-Type Hypersensitivity (DTH) (Camp et al., 1993),
Bacillus Calmette Guerin (BCG)-induced granuloma formation (Rösel et al., 1997), murine proteoglycan (PG)-induced arthritis (Mikecz et al., 1995) and migration of inflammatory T-lymphocytes into the site of SEB-induced inflammation (DeGrendele et al., 1997a). It is puzzling, however, that the DNFB-induced DTH in CD44-/- mice was not significantly altered compared with the heterozygous littermate controls, as measured by the increase in ear swelling after challenging of sensitised animals (Schmits et al., 1997). Furthermore, the number and size of granulomas formed in response to the injection of heat-killed *Corynebacterium parvum* was even 1.7 fold higher in CD44-mutant mice compared with wild type controls (Schmits et al., 1997). The obvious difference between the outcome of antibody-blocking and gene-targeting CD44-elimination in the DTH response might be due to the compensatory changes taking place in

CD44-deficient mice. During the induction and progression of inflammation, CD44 may play a role at several different steps such as the rolling interaction between T-cells and inflammatory endothelium, T-lymphocyte activation, T- and NK cell-mediated cytotoxicity and macrophage activation. The possible compensation processes would have to take over CD44 functions exactly at these points in CD44-deficient mice. I therefore investigated whether the HA binding function of CD44 is compensated for in cells of the immune system in CD44-/- mice.

None of the analysed cells of the immune system I analysed were suitable for the isolation of a CD44-substitutive HA binding molecule from CD44-deficient mice. Both IL-5-induced B-cells and inflamed and resident peritoneal macrophages did not show any CD44-dependent HA binding when obtained from wild-type C57Bl6/J mice. The anti-CD3 antibody-stimulated T-lymphocytes from C57Bl6/J mice formed a CD44-dependent HA binding subpopulation, in agreement with the previous data (DeGrendele et al., 1997b), but there was no upregulation of HA binding following the anti-CD3 antibody treatment in T-cells obtained from CD44-deficient mice, indicating that HA binding function of CD44 is not substituted in these mice.

4.1.2.1 CD44 and HA binding in T-cells

CD44 and its interaction with HA was suggested to be involved in the T-lymphocyte extravasation through the microvascular endothelium at the inflammatory site. Therefore it is conceivable that in CD44-deficient mice the compensation for the lack of CD44 would engage another HA binding molecule/molecules on activated T-cells. However, the results presented here indicate that in contrast to the wild-type, the activation of T-cells obtained from CD44-deficient animals does not lead to the upregulation of their HA-binding. T-cell stimulation *in vitro* with the anti-CD3 antibody used in these experiments is an artificial but appropriate model, since a) the crosslinking of CD3 mimics to some extend the crosslinking of TCR complex by the natural ligand (an antigenic peptide bound to the MHC of APC) and leads to the differentiation and proliferation of T-cells (Weiss and Imboden, 1987) and b) the same stimulation leads to the induction of HA-binding in CD44-positive cells. Assuming that T-lymphocytes from CD44-deficient mice are also not able to bind HA during the immune response *in vivo*, the migration of

T-lymphocytes into sites of inflammation must be mediated by the other ligand-receptor interaction partners. What could they be? Since the primary rolling adhesion of lymphocytes to the inflammatory endothelium is supposed to be a site of CD44 function (Siegelman et al., 1999), the other proteins involved in primary adhesion such as members of the selectin superfamily (Staite et al., 1999, Bullard et al., 1996, Tedder et al., 1995) could be responsible for this process in CD44-deficient mice during skin contact hypersensitivity. It is interesting to speculate that these molecules may compensate for the lack of CD44.

Recent studies on CD44-null mice provide evidence that at least some T-lymphocyte and NK-cell functions are altered in these mice. The cytotoxicity of IL-2-activated LAK (lymphokine-activated killer) cells against different tumour cell lines was significantly lower in cultures derived from CD44-deficient animals compared to the wild-type controls (Matsumoto et al., 1998a; Rafi-Janajreh et al., 1999). Furthermore, the IL-12 induced vascular leak syndrome (VLS), characterised by the endothelial cell injury and likely resulting from the detrimental action of own activated cytolytic lymphocyte on the endothelial cells, was diminished in CD44-deficient mice. My results demonstrate that HA binding of activated T-cells is not compensated for in CD44-deficient mice. Exactly this defect may be responsible for the impaired interaction between activated cytotoxic T-cells and HA on endothelial cells or tumour cell lines, resulting in a decreased cytotoxicity in CD44-deficient mice, which becomes apparent during the development of IL-2-induced VLS. This possibility deserves to be investigated further.

4.1.2.2 CD44 and HA binding in macrophages

Macrophages play an important role in the progression of inflammation. Low-molecular weight fragments of HA can trigger, in part via CD44, the production by macrophages of tumour necrosis factor α (TNFα), interleukin-1 β (IL-1β), and insulin-like growth factor-1 (IGF-1) (Noble et al., 1993), MIP-1α, MIP-1β and RANTES (McKee et al., 1996) as well as interleukin -12 (IL-12) (Hodge-Dufour et al., 1997). The normal inflammatory response of CD44-deficient mice (Schmits et al., 1997) can be explained in two ways. It could firstly mean that the stimulation of macrophages by HA fragments *in vivo* is not significant compared with the other

stimuli which activate macrophages during the immune response. The other possibility is that another molecule on CD44-mutant macrophages is responsible for their activation by HA.

In this work I attempted to use the binding of fluorescently labelled HA as a feature by which the existence of a potential substitutive molecule could be examined in CD44-deficient macrophages. Surprisingly, in preliminary experiments both resident and thioglycollate-elicited peritoneal macrophages from wild-type mice bound HA in a CD44-independent manner. This is in contradiction with the previous findings that at least some macrophage subtypes bind HA in CD44-dependent manner (Green et al., 1988; Culty et al., 1994). It is possible, however, that macrophages may bind HA via CD44 dependent and CD44 independent mechanisms, as it was suggested by McKee et al., 1996.

The reasons why I was not able to detect CD44-dependent HA binding in wild-type macrophages in my experiments are unclear. Besides CD44, macrophages have been shown to express several other hyaluronic acid binding proteins (HABP). One of them, a 60 kDa protein was found on J774 macrophage cell line (Gustafson and Forsberg, 1991). Another group has demonstrated the presence of a 34-kDA HABP on J774.G8 macrophages, which was identical to C1q complement protein. This molecule was also expressed on peritoneal hamster macrophages and was involved in the interaction between macrophages and the intracellular parasite *Leishmania donovani* (Rao et al., 1999).

4.1.2.3 The influence of genetic background

My experiments suggest that CD44-dependent HA binding in immune cells is not critical for CD44-deficient mice. One possible reason for this could be the strain of CD44-deficient mice used. In my experiments I had some evidence that the HA-binding function of CD44 differs between mice strains. The size of the HA-binding subpopulation formed by lymph node T-cells in response to CD3-crosslinking was bigger in the BALB/c derived T-lymphocyte population then in C57Bl6/J derived one. Even more striking, B-cells from C57Bl6/J mice did not develop a $CD44^{high}$ HA binding subpopulation when stimulated with IL-5, in contrast to the DBA/2-derived

B-cells, where this phenomenon was originally described (Murakami et al., 1990). This speaks for the importance of the genetic background for CD44 function in immune cells.

The notion that strain influences CD44 function is supported by several other observation. For example, in the experiments showing almost complete inhibition of early phase of delayed-type hypersensitivity by anti-CD44 antibodies, (BALB/c x DBA/2)F1 mice were used (Camp et al. 1993). The administration of anti-pan CD44 antibody at the effector phase of DNFB-induced DTH in the C57Bl6/J mice caused only a partial reduction of the ear swelling (Weiss et al., 1997). CD44-deficient mice were generated on a 129 x C57Bl6/J background. The C57Bl6/J strain differs from BALB/c and DBA/2 mice strains in the allotype of CD44: BALB/c as well as DBA/2 strains carry the CD44.1 allele while C57Bl6/J mice express the CD44.2 allele. Beside the difference in CD44 structure which is detectable by using different monoclonal antibodies (Lesley and Trowbridge, 1982), CD44.1 mice have a higher percentage of CD44 positive T-cell and thymocytes compared to the CD44.2 mice (Lesley et al., 1988; Lynch et al., 1989). It is likely that the difference in expression is reflected by the difference in the realisation of CD44 functions. Perhaps the degree of involvement of CD44 in immune processes in general varies between mice strains: its role may be more significant in mice expressing the CD44.1 allele than in mice expressing CD44.2 allele. Currently, several groups are trying to establish CD44 deficient mice on BALB/c or DBA/1 background (Schmits et al., 1997; M.Hegen, personal communication).

4.1.3 Is HA binding functionally redundant ?

One of the most surprising finding of my work is that the HA binding properties of many cells are not required for normal life. In two cell systems the CD44-dependent HA binding was detected in a wild-type mice. This HA binding was not substituted by any other HA binding molecule in cells derived from CD44-deficient animals.

The interaction of different cell types with the ECM component hyaluronate is thought to regulate tissue modeling. As an example, the ability of mesenchymal cells to form HA-dependent pericellular matrix regulates the conversion of the loose embryonic limb bud mesoderm to its condensation state prior to chondrocyte differentiation (Toole et al., 1989). HA binding has also

been implicated in cell migration as well as other aspects of cell behavior such as tissue-specific differentiation and activation. In CD44-/- animals it may be that other components of the extracellular matrix (ECM) and their receptors are able to compensate for the absense of functional contact with HA. Fibronectin, collagen and laminin and their receptors are possible alternatives. These members of the ECM are recognised by cells via a number of receptors, many of which are integrins. For example, members of the integrin family bind to collagens, such as $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha10\beta1$ (Camper et al., 1998). At least eight integrins have been shown to bind fibronectin, and at least 7 are capable of binding to laminin (Fässler et al., 1996). In this regard, it would be interesting to investigate integrin expression in CD44-/- mice to determine whether these receptors and their ligands may take a part in compensating for lack of CD44.

HA binding is also involved in interactions between different cell types, for example between endothelial cells and migrating leukocytes. However, this process involves many other ligand-receptor interaction partners, such as immunoglobulin family members recognised by various integrins, or selectins binding their carbohydrate ligands. Therefore the HA-binding during leukocyte migration may be redundant. As noted above, selectins are likely candidates to substitute the HA-binding function of absent CD44, since they participate in the same primary adhesion step and mediate a similar rolling type of attachment.

The proposed role of CD44-mediated HA binding in leukocyte activation may be overtaken by other known co-stimulatory molecules. The signal exerted by the B7-CD28/CTLA-4 interaction is known to be important for the normal immune response, as it was demonstrated in CD28-deficient mice (Kondo et al., 1996). Other molecules, such as LFA-1 triggered by ICAMs, CD2 binding LFA-3, CD40 engaged by its ligands CD40L and OX40L, VLA-4 interacting with VCAM-1 as well as a heat-stable antigen have been described as co-stimulatory for activation of different lymphocyte subsets (reviewed by Sharpe, 1995). Hence it is possible that HA may participate but is not necessarily required for the induction of lymphocyte proliferation during an immune response.

Together, these observations could explain why the HA binding function of CD44 is not critical for the normal life in C57Bl6/J mice.

4.2 Compensation for the growth factor presenting function of CD44 during limb development

It has been demonstrated that CD44 is able to bind to and to present both FGF4 and FGF8 to the limb bud mesenchymal cells, resulting in the proliferative response of mesenchyme to these growth factors. Furthermore, the pretreatment of AER with anti-CD44 antibody blocked limb outgrowth, indicating that CD44 is necessary for the transduction of morphogenic signals from the AER toward limb mesenchyme (Wainwright , 1998; Sherman et al., 1998).

Since CD44-null mice exhibit no limb abnormalities, the developing limb bud was proposed to have some compensation for the lack of CD44. Indeed, mesenchymal cells from CD44-deficient mice showed a dose-dependent proliferative response to soluble FGF8, while analogous cells from wild-type mice were not inducible by soluble FGF. Further experiments revealed that chlorate treatment of the mesenchymal cells, which prevents sulfation and therefore inhibits the heparan sulfate synthesis, abolished the increased sensitivity of CD44-deficient mesenchyme to soluble growth factor. This suggests that the compensatory changes in these cells may involve the overexpression of some heparan-sulfated molecules, possibly taking over the FGF8-presenting function in embryonic limb bud in the absence of CD44 (Wainwright D., 1998).

On this basis in a search for the CD44 substitutive molecule I compared the total amount of surface heparan sulfate and the pattern of HSPGs expression in the CD44-positive and CD44-negative mesenchymal cells. Although the total amount of heparan sulfate measured by flow cytometry appeared to be very similar on these two cell types, a clear difference in the pattern of HSPG expression was found using the western blot technique. The CD44-deficient mesenchyme, which showed a dose-dependent proliferation in response to soluble FGF8, had elevated levels of at least 3 low molecular weight HSPGs, in comparison to the wild-type limb bud mesenchyme. These low molecular weight heparan sulfated proteoglycans are the likely candidates to play an FGF8 presentation function in CD44-mutant limb bud mesenchymal cells and to be a reason for the gained FGF-responsiveness of these cells.

What are these low molecular weight proteoglycans? The 3 major groups of HSPGs are known as syndecans, glypicans and perlecan. The involvement of perlecan is unlikely because of its huge molecular weight: the core protein of mouse perlecan was described to be 396 kDa

(Noonan et al., 1991). According to their molecular weight, the HSPGs upregulated in CD44-deficient limb bud mesenchyme match to the glypican family or to some of the known syndecans.

Glypicans are a family of closely related HSPGs, all attached to the plasma membrane via unique glycosylphosphatidylinositol (GPI) anchor. The family includes glypicans 1, 3, 4, 5 and cerebroglycan. All of them have a similarly sized core protein, which varies between 55 and 65 kDa. The ability of glypicans to modulate the response to growth factors has been described in different cell systems. Glypican-1 promoted the binding of FGF2 and HB-EGF by several pancreatic tumour cell lines and thus induced their growth-factor dependent proliferation. The removal of glypican by cleavage with GPI-specific phospholipase C abolished the proliferation of these cells (Kleeff et al., 1998). Also, glypican-1 was shown to enhance keratinocyte response to FGF1 but inhibit their response to FGF7 (keratinocyte growth factor), as a result of positive and negative regulation of binding of these growth factors to the FGF-receptor 2 (Bonneh-Barkey et al., 1997). In chicken, a strong glypican expression was observed in the AER and proximal region of developing limbs, suggesting a possible role for glypicans in limb formation (Niu et al., 1996).

Syndecans are a family of transmembrane HSPGs. They have a broader molecular weight range compared to the glypicans. For example, syndecan-3 (N-syndecan) cloned from rat schwann cells has a core protein size of 120 kDa (Carey et al., 1992), while for chicken syndecan-4 a 19,6 kDa core protein is calculated. The mouse syndecan-2 has a "promising" pattern of expression: it is expressed mostly in mesodermal cells at the sites of mesenchymal-epithelial interaction during morphogenesis (David et al, 1993). Syndecan-3 is expressed in the mesenchyme underlying AER in the developing limbs in chicken embryo (Dealy et al., 1997). Syndecan-3 was shown to play a critical role in chicken limb bud development. It regulats the FGF2-stimulated limb outgrowth, as an antibody against syndecan-3 could block the formation of limbs in embryonic explants where limb development was maintained by FGF2 (Delay et al., 1997). Thus, there are many candidates for the low molecular weight HSPGs in CD44-deficient mesenchyme. However, these HSPGs may also turn out to be unknown proteins.

4.3 Compensatory mechanisms in mice with targeted gene disruption

The gene targeting technique has opened a new door for investigators in many fields of biology. The generation of mice lacking a particular molecule allows more precise and critical examination of the roles of the genes than it was ever possible before. Another lesson one can learn from "knock-out" animal models is the way in which the organism deals with the absence of an important gene product.

Apparent compensation for the lack of a certain protein may be due to functional redundancy of the protein. As an example, in a moesin-deficient mice the absence of this member of ERM family did not affect any of the phenomena previously described as being moesin-dependent, such as platelet aggregation, formation of stress fibres in fibroblasts or microvillar formation in mast cells. The expression of other family members ezrin and radixin was not elevated in any tissue or in any *in vitro* cell culture established from the moesin-mutant mice. The authors concluded that the function of moesin as a member of ERM family is redundant (Doi et al., 1999).

In knock out animals, compensation for a gene which has been destroyed by genetic manipulation may occur through upregulation of another gene, which is able to perform the same function. Often this involves the upregulation of the gene/genes belonging to the same family as the mutated one, as in the case of mice with a targeted disruption of the RIIβ subunit of the cAMP-dependent protein kinase A (PKA). Normally only the RIIβ isoform is expressed in brown and white adipose tissues. In mutant mice these tissues expressed the RIa isoform of the PKA subunit, which together with the normal C subunits formed a functionally active cAMP-dependent protein kinase A. Notably, the resulting type I-PKA had an elevated avidity for cAMP compared to the usual type II-PKA in these tissues. This PKA "overfunctioning" caused the lean phenotype of RIIβ subunit-deficient mice (Cummings et al., 1996).

A switch of function from the disrupted gene to the another one without an increase in the expression of the compensatory molecule can be observed, for example, in $p27^{Kip-1}$-deficient mice. The mitogen starvation-induced cell cycle arrest, normally mediated by $p27^{Kip-1}$ which inactivates the cyclin-E/CDK-2 complex, is driven by p130, an Rb-related protein, in $p27^{Kip-1}$-deficient mouse embryonic fibroblasts. Serum starvation upregulates p130 in both wild-type and

$p27^{Kip-1}$-deficient cells to a similar level, but only in $p27^{-/-}$ fibroblasts was p130 found to co-immunoprecipitate together with cyclin-E. The compensatory role of p130 was confirmed by the fact that in $p27^{-/-}/p130^{-/-}$ double knock-out mice the mild phenotype of $p27^{Kip-1}$ single knock-out was dramatically enhanced. The mechanism of this functional substitution is unclear, but it may involve the availability of substrate in the absence of the natural CDK inhibitor (Coats et al., 1999).

Compensation occuring at the posttranscriptional level is illustrated by the myosin light chain 2 (mlc-2v) heterozygous mutant mice. Mlc-2v plays a role in embryonic murine heart muscle contraction, and for this function the stoichiometric ratio between mlc-2 and other sarcomeric proteins is important. Although the mRNA level of mlc-2 in the heterozygous mutants was decreased to 50% of wild-type, the mlc-2v protein level was unaltered compared to the wild-type controls (Minamisawa et al., 1999). This uncovers a posttranscriptional regulatory mechanism which perhaps normally serves to keep the correct stoichiometry in the sarcomer assembly but is compensatory in a case when one mlc-2v allele does not function. Similar posttranscriptional compensation was shown to enhance α-tropomyosin protein expression to the wild-type level in α-tropomyosin heterozygous mutant mice (Blanchard et al., 1997; Rethinasamy et al., 1998).

The compensatory alteration in the "knock-out" mice may include not only molecular, but also systemic changes in the tissue or organ affected by the introduced mutation. An example of such a compensation is the multiple changes in myoglobin-deficient mice. In heart muscle cells from normal mice, myoglobin is supposed to facilitate the oxygen diffusion from sacrolemma to the mitochondria. The increase in coronary flow, coronary reserve and in the density of the capillaries in the hearts of myoglobin "knock-out" mice together steepen the O_2 gradient from the capillary bed to the mitochondria in cardiac myocytes, which promotes O_2 diffusion to the mitochondria in the absence of myoglobin (Gödecke et al., 1999).

Sometimes it is difficult to distinguish between redundancy, when the unaltered functions of "compensatory" proteins are unmasked by the absence of the targeted gene, and real compensation, when other proteins take over an unusual function. An example of this situation is observed in NK (natural killer) cells derived from Syk-deficient mice. In normal NK cells the protein tyrosine kinase Syk was shown to be important for IL-15 responses, cytotoxicity and

cytokine production. However, almost none of these functions were affected by the lack of Syk in Syk-deficient NK cells. It was further shown that Zap-70, a T-cell specific protein tyrosine kinase, appeared to play a crucial role in the signalling in these cells. However it is not clear if this is the result of a compensatory activation of Zap-70 (perhaps due to cell selection during lymphopoiesis) or it is because in the normal NK cells both Syk and Zap-70 PTKs have an overlapping redundant function (Colucci et al, 1999).

An additional complication comes from the fact that in mice compensation may develop in a strain-dependent manner and cover some but not other alterations caused by targeted gene disruption. This may be mirrored by the different phenotypes of the same mutation observed in the mice with different genetic backgrounds. A classical example of this phenomena is the strain-dependency in defects caused by mutation of the EGF receptor. Mutants generated on the 129/Sv genetic background died at mid-gestation from a placenta defect, while in 129/Sv x C57Bl6 or 129/Sv x C57Bl6 x MF1 backgrounds the death of the EGFR-null animals was delayed until birth or even longer. The main reason of death in these cases was multiple epithelial insufficiency (Sibilia and Wagner, 1995). Generation of EGFR-deficient mice on a CF-1 background resulted in an embryonic lethality due to degeneration of inner cell mass, while CD-1 mice lacking the EGF receptor survived up to 3 weeks and showed multiple abnormalities (Threadgill et al., 1995).

Thus, the search for compensatory changes in gene-targeted mice is a complicated task, since the alterations occur at different levels and include a variety of systems. The CD44-deficient mice are probably an example of multiple cell type/tissue-specific compensation for the disruption of CD44 gene, due to the multifunctional nature of this protein. The CD44 function is likely to be redundant in lymphocytes migrating to inflammatory tissues. The absence of CD44 and its HA-binding properties in lymphokine-activated killer cells is not compensated for and results in impaired IL-2-induced cytotoxicity. In the developing limb bud the lack of FGF-presenting function of CD44 is possibly overtaken by the upregulation of some other heparan sulfated proteoglycans. Moreover, the compensatory alterations in CD44-deficient mice is determined by the genetic background.

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