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Date Dec 13/1993
ABSTRACT:

This thesis set out to determine how regulatory factors, such as possible cytoskeletal associations or cytoplasmic proteins, could affect how CD44 expressing cells bind hyaluronan. Cell lines expressing CD44 were used as a tool to determine if associations between the cytoskeleton and CD44 could account for the variable hyaluronan binding in cell lines with different binding abilities towards hyaluronan. Analysis of CD44-cytoskeletal associations in lymphoid and fibroblast cell lines suggested no direct link between CD44 and actin in lymphoid cells nor between CD44 and actin, vimentin or keratin in NIH 3T3 fibroblast cells that could regulate hyaluronan binding.

It was shown that in one cell line, the T-cell lymphoma WH(4).1 in which hyaluronan binding was induced with the phorbol ester, Phorbol 12-Myristate 13-Acetate (PMA), that the increase of hyaluronan binding was prevented by an inhibitor of protein synthesis and this inhibition was shown to be reversible.

L-cells expressing CD44 but lacking the glycosaminoglycans (GAG's) heparan sulphate or chondroitin sulphate showed no differences in their ability to bind hyaluronan or in their distribution of CD44 before or after Triton X-100 solubilization. However, L-cells lacking chondroitin sulphate were unable to be activated to bind hyaluronan with a CD44 monoclonal antibody that normally induces CD44 dependent hyaluronan binding. The distribution of CD44 following Triton X-100 solubilization of NIH 3T3 cells displayed some similarities to that of Thy-1, a glycoprophosphoinositol lipid linked protein. In addition, both these proteins were insoluble in Triton X-100 in these cells but were solubilized by a different non-ionic detergent octyl glucoside.
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I would like to express thanks to Conan Young and the other graduate students and technicians within the department for their support and discussions. I would also like to thank Pauline Johnson for allowing me to carry out this work in her lab.
DEDICATION:

This Thesis is dedicated to the memory of my mother, Beth Bader.
INTRODUCTION:

CD44 is a structurally and functionally diverse molecule present on many cell types. It is involved in cellular adhesion (Miyake et al. 1990b), lymphocyte homing (Berg et al. 1989) and lymphocyte migration (Trowbridge et al. 1982). It was first cloned in 1989 (Idzerda et al. 1989, Nottenburg et al. 1989, Stamenkovic et al. 1989, Zhou et al. 1989) and identified as a unique receptor distinct from the selectin, integrin or immunoglobulin families of adhesion receptors. It contains 342 amino acids divided into three domains; the extracellular domain (250aa's), transmembrane domain (20aa's) and cytoplasmic domain (72aa's) (Idzerda et al. 1989). The extracellular domain can be further divided into two functional domains, the membrane proximal domain and the N terminal domain. The membrane proximal domain is differentially glycosylated with N- and O- linked sugars (Planagan et al. 1989), and contains a splice site for alternatively expressed exons. The N terminal domain displays homology to cartilage link protein (Goldstein et al. 1989, Stamenkovic et al. 1989) which binds hyaluronan (HA). CD44 was subsequently shown to bind HA (Aruffo et al. 1990, Lesley et al. 1990, Miyake et al. 1990a) as well as other ligands such as mucosal addressins (Picker et al. 1989), MHC Class II invariant chain (Naujokas et al. 1993), fibronectin (Jalkanen and Jalkanen 1992) collagen Type I (Carter and Wayner 1988, Faassen et al. 1993, Faassen et al. 1992), MIB -1β (Tanaka et al. 1993) and chondroitin sulphate (Underhill 1989). Possible associations with the cytoskeleton have also been described (Camp et al. 1991, Kalomiris and Bourguignon 1989, Lacy and Underhill 1987, Neame and Isacke 1992, Neame and Isacke 1993). CD44 exists as many distinct forms that exhibit differential glycosylation patterns (Brown et al. 1991, Faassen et al. 1992) or differential exon expression (Gunthert et al. 1991, Jackson et al. 1992, Stamenkovic et al. 1989). These modifications that occur mainly in the membrane proximal domain could lead to the diverse ligand binding of CD44.
Characterization of CD44 as a Hyaluronan Receptor

The binding of CD44 to HA has been extensively studied. An HA receptor was described in SV 3T3 cells and BHK cells as early as 1979 (Toole 1990) and this receptor was later shown to be CD44 (Aruffo et al. 1990, Culty et al. 1990). Since then many groups have characterized the HA binding of CD44 expressed in different cellular environments. Lesley et al. characterized the HA induced aggregation of different hematopoietic cell lines that expressed CD44 and showed that the HA induced aggregation could be blocked by antibodies to CD44 (Lesley et al. 1990). One interesting observation was that some cell lines that expressed CD44 did not aggregate in response to HA. Some of these cells could be induced to aggregate following a 16 hour incubation with PMA. Miyake showed that a B-cell line that bound to a stromal cell line was dependent on a CD44/HA interaction. This group also described a monoclonal antibody to CD44 that could directly compete with HA to bind CD44 in murine cells (Miyake et al. 1990a). Aruffo et al. created a soluble CD44-Immunoglobulin fusion protein and showed that this protein could bind tissue sections of lymph node high endothelial venules (HEV) in an HA dependent manner (Aruffo et al. 1990). An epithelial form of human CD44 has been described that will not bind to HA when expressed in a CD44 negative B-cell line (Stamenkovic et al. 1991). This isoform contains a 132 amino acid insert in the membrane proximal region of the extracellular domain. Conversely, Dougherty et al. have also described a human CD44 isoform containing a 132 amino acid insert that will bind HA (Dougherty et al. 1991). Murakami et al. showed that peripheral murine B cells did not bind HA. These cells could, however, bind HA following a 3 day exposure to IL 5 (Murakami et al. 1990). These results, along with the results of Lesley (Lesley et al. 1990) who described cell lines whose binding could increase following activation with PMA suggest that CD44/HA binding correlates with lymphoid cell activation. However, Lesley et al. found that T and B cells isolated from murine spleens did not bind HI-HA following cellular activation with anti-
CD3 monoclonal antibody and LPS (Lesley et al. 1990). Thus activation seems to play a role in some instances but it does not necessarily determine if a CD44 expressing cell will bind HA.

Hyman and associates determined that the expression of CD44 does not correlate with HA binding (Hyman et al. 1991). This group characterized the CD44 expression and HA binding of three derivatives of one T-cell line. The parental cell line, SAKRLT512.1, expressed low levels of CD44 and did not bind HA. A clone was selected that bound HA and had the same level of CD44 expression as the parent. A different clone was then selected that expressed high levels of CD44 but did not bind HA. When these cell lines were treated with PMA for 16 hours, all three clones showed increased CD44 expression and HA binding. The increased CD44 expression did not directly correlate to increased HA binding, i.e., a two fold increase in expression did not equal a two fold increase in HA binding (Hyman et al. 1991).

CD44 has been isolated from human placenta and two forms were identified. One 85Kd HA binding form and a 200Kd form that did not bind HA (St Jacques et al. 1993). The role of CD44 in this tissue is to maintain the structural integrity of the placenta by acting as an adhesion molecule to HA. Glioblastoma cells produce HA that is bound by CD44 expressed on these cells (Asher and Bignami 1992). These cells will bind exogenous HA only following treatment with hyaluronidase, an enzyme that degrades HA. Some CD44 positive glioblastoma cells did not bind HA suggesting that CD44 on these cells may bind to a different ligand. Another possibility is that these cells must be activated, as in the case for some of the lymphoid cells, to bind HA. CD44 has also been shown to be involved in the uptake and degradation of HA in SV 3T3 cells (Culty et al. 1992).

Functional Domains of CD44

The domains of CD44 responsible for HA binding have been examined.
Mutagenesis of the extracellular domain has shown that the membrane proximal region of CD44 is not crucial for HA binding (He et al. 1992, Peach et al. 1993). Variants of CD44 containing insertions in the membrane proximal domain did not show differences in CD44 binding to soluble or immobilized HA. Partial deletion of the membrane proximal domain (Val 161 - Arg 244) reduced CD44 binding to immobilized but not soluble HA (He et al. 1992). Site-directed mutagenesis of basic amino acids within the extracellular domain reduced HA binding by soluble CD44-Immunoglobulin fusion proteins (Peach et al. 1993). One particular amino acid deletion in the N-terminal domain, Arg 41, completely abolished HA binding by the fusion protein. Thus, basic residues seem to be important for CD44 to bind HA. An interesting anomaly here is the epithelial variant described by Stamenkovic that could not bind HA (Stamenkovic et al. 1991). This variant was later shown to have a three amino acid deletion upstream of the variant insertion site resulting in the loss of a basic amino acid (Peach et al. 1993). This loss may account for the decreased HA binding seen by this isoform. Lesley and co-workers transfected a CD44 negative T-cell line with a CD44 allele lacking most of the cytoplasmic domain. This cell line displayed a decreased ability to bind immobilized HA and an even lower ability to bind soluble HA compared to a transfectant expressing full length CD44 (Lesley et al. 1992). This suggests a role for the cytoplasmic domain of CD44 in regulating HA binding.

An antibody, IRAWB14.4, directed to CD44 was shown to increase HA binding by CD44 positive cells (He et al. 1992, Lesley et al. 1992, Lesley et al. 1993). This antibody could induce binding in fixed cells at 0°C suggesting that a change in CD44 conformation is important for increasing HA binding. Alternatively the antibody could be mimicking the binding of another CD44 ligand that could increase CD44/HA binding. Fab fragments of the antibody are ineffective at inducing HA binding (Lesley et al. 1993). This suggests that altered distribution of CD44 is important to increase HA binding. This altered distribution may not be due to CD44 mobility but to the concentration of cross linked extracellular domains that allow CD44 to bind HA.
Other HA Binding Proteins

HA receptors other than CD44 have been described (Toole 1990). Aggrecan and Versican were the first HA binding proteins studied to also show homology to cartilage link protein (Turley 1991). These proteins make up the proteoglycan core of cartilage. Recently another protein that contains a region with sequence similarity to cartilage link protein was identified (Lee et al. 1992). This tumour necrosis factor-inducible protein (TSG-6) was isolated from fibroblasts stimulated with tumour necrosis factor. The HA binding domain of this protein shows more sequence similarity to the CD44 N-terminal domain than it does to cartilage link protein. It binds specifically to HA and not to the related glycosaminoglycans chondroitin sulphate or heparan sulphate. It is thought to be involved in adhesion events during inflammation and tumorigenesis where TNF is produced. A distinct HA receptor has been described on motile fibroblast cells. This HA receptor, RHAMM, is associated with a protein kinase (Turley 1989, Turley et al. 1991). One similarity this protein has to CD44 is the necessity of basic amino acids in the HA binding domain to allow HA binding (Yang et al. 1993).

Cytoskeletal Associations and Phosphorylation of CD44

The association of CD44 with the cytoskeleton has been extensively studied. CD44 has been described to have associations with actin in SV 3T3 fibroblast cells (Jacobsen et al. 1984, Lacy and Underhill 1987), with vimentin but not actin in human lung fibroblast cells (Carter and Wayner 1988) and ankyrin when CD44 was isolated from lymphoid cells (Kalomiris and Bourguignon 1989). Associations with actin and vimentin were evaluated by assessing the Triton X-100 solubility of CD44. Triton X-100 solubility has been widely used to assess cytoskeletal associations, although other interactions can also lead to Triton X-100 insolubility such as the interactions of GPI-linked proteins (Luna and Hitt 1992). These reports thus do not provide direct evidence for a link between CD44 with the cytoskeleton but they do provide some indication that a possible association may exist. Kalomiris has shown that CD44 can associate with
ankyrin in vitro following phosphorylation by protein kinase C (Kalomiris and Bourguignon 1989). This group also showed that CD44 can bind GTP in vitro and that this interaction enhances CD44 association with ankyrin (Lokeshwar and Bourguignon 1992).

Further work assessing CD44’s Triton X-100 solubility showed a role for phosphorylation in affecting its Triton X-100 solubility (Camp et al. 1991, Neame and Isacke 1992, Neame and Isacke 1993). They extrapolated the Triton X-100 solubility of CD44 to indicate a cytoskeletal association, which is suggested but clearly not proven. Two populations of CD44 are expressed by resting macrophages. One population is soluble in the detergent NP-40 and is phosphorylated. Another population is insoluble in NP-40 and is not phosphorylated (Camp et al. 1991). Upon macrophage activation CD44 becomes NP-40 soluble and phosphorylated. Thus Camp et al. suggest that the phosphorylation of CD44 causes it to dissociate from the cytoskeleton to become NP-40 soluble. Neame et al. transfected mutated CD44 constructs lacking conserved phosphorylated serines in the cytoplasmic domain into polarized epithelial cells (Neame and Isacke 1992, Neame and Isacke 1993). The resulting proteins localized to the basolateral membrane of the polarized epithelial cells and were Triton X-100 soluble. This result was reproduced with non-mutated CD44 transfectants containing conserved phosphorylated serines. This result contrasts the results of Camp who found that phosphorylation did affect detergent solubility. Similar experiments using a construct lacking 69 amino acids of the cytoplasmic domain showed that the truncated CD44 molecule was also soluble in Triton X-100 but did not localize to the basolateral membrane of the polarized epithelial cells as the normal CD44 protein did. This result supports the role of the cytoplasmic domain in localizing CD44 in this cell. NIH 3T3 fibroblast cells were transfected with the CD44 mutants lacking the phosphorylated serines or the cytoplasmic domain. These mutants, along with the wild type CD44, were insoluble in Triton X-100. This is in contrast to the results with the epithelial cells where CD44 was soluble in Triton X-100. Interestingly, the cytoplasmic domain deletion
transfectant was also insoluble in Triton X-100. Thus the mechanism of CD44 insolubility in Triton X-100 does not involve phosphorylation or the cytoplasmic domain since the absence of either does not result in CD44 becoming solubilized.

In conclusion, cytoskeletal associations of CD44 and CD44 phosphorylation have been studied but these events have not been correlated to the ability of the cells to bind HA. Thus it is currently unknown if these events regulate CD44/HA binding in CD44 expressing cells. The objectives of this thesis were to further evaluate possible cytoskeletal or membrane associations that could regulate CD44/HA binding. Cell lines expressing CD44 but varying in their ability to bind HA were used as a tool to elucidate if cytoskeletal or membrane associations were involved in regulating CD44/HA binding. The work described in this thesis further analyzed the HA binding ability of CD44 in lymphoid and fibroblast cells and explored CD44's potential association with other proteins, particularly cytoskeletal proteins, in order to determine how CD44 was regulated with respect to HA binding.
MATERIALS AND METHODS:

Cell Cultures and Antibodies

The murine T cell lymphomas used include; AKR1.G.1ova^
1.26 - CD44 negative cell line, TFX591.2 - AKR1.G.1ova^
1.26 transfected with the CD44.1 allele, WT(3).6 - AKR1.G.1ova^
1.26 transfected with the CD44.1 allele lacking all but the first six amino
 acids of the cytoplasmic domain, WH4.1 - SAKRRTLS12.1 derived cell line selected for
 high CD44 expression of the CD44.2 allele, with low HA binding, and WB3.6 - SAKRRTLS12.1 derived cell line selected for high HA binding. These were maintained in DMEM supplemented with 10% horse serum. The B lymphoma RAW253.ITB.1.OVA^
1 - pre B cell line with high levels of CD44 expression and no ability to bind to HA was also maintained in DMEM supplemented with 10% horse serum. These lymphoid cell lines were obtained from Robert Hyman of the Salk Institute. WEHI231, a mature B cell line, NIH 3T3 and L-fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum. The L-cells were obtained from Dr. Frank Tufaro including the heparan sulphate deficient gro2c, chondroitin sulphate deficient gro29, and the heparan sulphate, chondroitin sulphate deficient L-cell line sog9. All cell lines were incubated at 37°C in an atmosphere containing 5% CO2. Normal purified lymph node T-cells were harvested from 8-12 week old C57BL/6 or BALB/cJ mice. The B-cells were removed following incubation with Dynabead M-450 conjugated to sheep anti-mouse IgG using 4X10^7 beads per 1X10^7 cells. Purity of the T-cells was assessed by labeling with goat anti-mouse FITC IgG (Southern Biotechnology) which binds to B-cells but not to T-cells and it was shown that no negative peak was present in the purified T-cell samples. The CD44 specific monoclonal antibodies, IRAWB14.4 and KM201, were obtained from Dr. Robert Hyman of the Salk Institute and used as a 1/10 dilution of tissue culture supernatant for FACS analysis and microscopy. All dilutions were made in phosphate buffered saline (PBS - 138mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.2mM KH2PO4 pH 7.2) plus 2% horse serum (PBS/HS). The secondary antibody was used at a 1/150
dilution of Goat anti mouse FITC from Silenus. Rabbit anti-mouse Thy-1 was obtained from Dr. Wilfred Jefferies and used at a 1/1000 dilution of rabbit serum. MHC Class I monoclonal antibody, 34.1.2s anti K\(^d\)D\(^d\) (mouse anti-mouse), was also obtained from Dr. Wilfred Jefferies and used as straight tissue culture supernatant for immunofluorescent microscopy. The secondary antibody used against the Thy-1 antibody was goat anti rabbit FITC from Southern Biotechnology used at a 1/100 dilution in PBS/HS. The secondary antibody used against MHC Class I antibody was goat anti mouse FITC from Southern Biotechnology used at a 1/100 dilution in PBS/HS. Monoclonal antibodies to vimentin and keratin were obtained from Dr. Brett Finlay and used as a straight preparation.

**Preparation of FL-HA:**

Bovine trachea hyaluronic acid was obtained as a sodium salt from Sigma and fluorescenated by the method of de Belder et al (1975). Briefly, 5 mgs of HA was dissolved in 4 mls of distilled water to which was added 2 mls of DMSO. To this was added 150\(\mu\)l of acetaldehyde (Aldrich), 2.5\(\mu\)l cyclohexylisocyanide (Aldrich), and 2.5\(\mu\)gs fluoresceinamine Isomer I (Aldrich). The pH was maintained between 5 and 7 and the reaction was allowed to incubate in the dark with constant stirring for 5 h at room temperature. The mixture was then cleared of unconjugated fluoresceinamine by precipitating the hyaluronate with 80mls NaCl saturated ethanol. The precipitate was redissolved in distilled water. Following two cycles of this procedure the Fl-HA was resuspended in PBS and dialyzed against PBS to remove any remaining free fluoresceinamine. The presence of soluble fluoresceinamine was assessed by thin layer chromatography with chloroform-methanol (3:1) whereby spots of various Fl-HA concentrations were compared to a spot of free fluoresceinamine on a TLC plate. No free fluoresceinamine was present in the Fl-HA lanes that corresponded to the fluoresceinamine only lane as assessed by inspection under u.v. light. Assuming total recovery of fluoresceinated hyaluronate, the concentration of Fl-HA was determined to
be 1.2mg/ml. Final concentrations of Fl-HA used in all experiments was 3μg/ml in PBS/HS.

Treatment of Cells with PMA

PMA was obtained from Calbiochem. Incubations with PMA were carried out for 16 h at 37°C in an atmosphere containing 5% CO2. The concentration of PMA ranged from 10ng/ml to 50ng/ml. PMA was dissolved in 100% ethanol to make a 1mg/ml stock solution and stored aliquoted at -70°C.

Flow Cytometry

1-3 X10^5 suspension cells were incubated for 20 min on ice in microtitre wells with 100μl IRAWB14 monoclonal antibody tissue culture supernatant diluted 1/10 in PBS/HS. This antibody was used in all FACS analysis to determine the level of CD44 expression. The cells were then washed two times with 200μl PBS/HS and incubated for another 20 min on ice with 100μl of 1/150 diluted goat anti-rat FITC (Silenus). Cells to be labeled with Fl-HA were incubated for 20 min on ice with 100μl of 3μg/ml Fl-HA prepared as described. Cells activated to bind Fl-HA with IRAWB14.4 antibody or prevented from binding Fl-HA with KM201 monoclonal antibody were treated for 5 min, on ice, with 50μl straight tissue culture supernatant prior to addition of 50μl 6μg/ml Fl-HA. Following 2 washes with 200μl PBS/HS the cells were transferred from the microtitre plates to Falcon tubes to be assayed on a Becton Dickenson FACSCAN. NIH 3T3 or L-cells were removed from 100mm² tissue culture dishes following a 10 minute incubation with 2mls of 1mM EDTA in PBS. 1-3X10^5 cells were aliquoted into separate wells for staining and stained as described for the suspension cells. L cells treated with xyloside were incubated overnight with 0.1μM xyloside in DMEM with no FBS at 37°C. Cells were then removed from the dish and incubated with the activating antibody and Fl-HA as previously described.
Western Blot Analysis

8X10^5 cells were lysed in 15µl of lysis buffer for 10 min on ice (20mM Tris, pH7.5, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.2mM phenylmethylsulfonyl fluoride, 1mg/ml leupeptin and aprotinin). The lysates were then centrifuged at 10,000 rpm for 10 min at 4°C. The soluble fraction was removed to a separate tube and equal volumes of 2X - non-reducing sample buffer (2X - NRSB - 250mM Tris pH6.8, 4% SDS, 20% glycerol, 2mg bromophenol blue) was added just prior to boiling. The volume of these samples did not exceed 30µl. The remaining pellets were washed with 1 ml lysis buffer and again centrifuged at 10,000 rpm for 10 min at 4°C. The wash was removed and 20µl of 1X - NRSB (1:1 dilution of 2X - NRSB with dH20) was added to the pellets. The samples were boiled for 10 min at 100°C. Each sample was loaded into a 40µl well of a 7.5% SDS-polyacrylamide gel. The proteins were then transferred to an Immobilon PVDF membrane (Millipore) with a Biorad transfer apparatus. Biorad broad range pre-stained standards were used to estimate molecular weights. Following transfer the membranes were blocked for 2 h with TBS buffer (20mM Tris pH7.5, 150mM NaCl, 5% BSA, 0.2mM phenylmethylsulfonyl fluoride, 1.0 mg/ml leupeptin and aprotinin). Following one wash with 0.1% Tween 20 in TBS the blots were incubated for 90 min with 1/20 diluted KM201 tissue culture supernatant in 0.1% Tween 20 in TBS with 0.2mM phenylmethylsulfonyl fluoride, 1.0 mg/ml leupeptin and apotinin. The blots were washed 5 times over 30 min with 0.1% Tween 20 in TBS. The secondary incubation contained 1/10,000 goat anti-rat HRP (Horse Radish Peroxidase - Southern Biotechnology) in 0.1% Tween 20 in TBS with 0.2mM phenylmethylsulfonyl fluoride, 1mg/ml leupeptin and apotinin and was carried out for 30-45 min. The blots were again washed 5 times over 30 min with 0.1% Tween 20 in TBS. Labeled proteins were then visualized using Amersham ECL Western blotting detection system.

Treatment of Suspension Cells with Cytochalasin D

1-3 X 10^5 cells were incubated with 20µM cytochalasin D in DMEM
supplemented with 10% horse serum at 37°C in an atmosphere containing 5% CO2 for 20 - 30 min. Cytochalasin D was obtained from Sigma and dissolved in DMSO as a 2mM stock solution.

Treatment of Cells with the Protein Synthesis Inhibitors Puromycin, Emitine or Cycloheximide and the Transcription Inhibitor Actinomycin D

WH(4).1 was incubated with 10μg/ml cycloheximide (Sigma), 1μg/ml puromycin (Sigma), 10μg/ml actinomycin D (Calbiochem) or 0.1μM emitine (Sigma) in the presence or absence of PMA used at a concentration of 30ng/ml for 16 h and assayed for Fl-HA binding by FACS analysis as described previously. Cells treated with cycloheximide were further grown to dilute the cycloheximide and were again induced with PMA for 16 h and assessed for Fl-HA binding.

Preparation of Adherent Cells for Fluorescent Microscopy

NIH 3T3 or L-cells from an 80% confluent, 100mm² dish were removed with 1mM EDTA in PBS. The cells were washed once and resuspended in DMEM supplemented with 10% FBS. 1 X10⁴ cells were placed on a sterile microscope cover slip and incubated overnight. Cells to be treated with cytochalasin D were washed once with DMEM and incubated with 20μM cytochalasin D for 20 min at 37°C in an atmosphere containing 5% CO2. Cytochalasin D was obtained from Sigma and dissolved in DMSO and stored at -20°C, percentage of DMSO in the final incubations was 0.01%. Cells were then washed with cold PBS prior to staining. 0.5% Triton X-100 or 60mM β-octyl glucoside (Calbiochem) in cold PBS was used to solubilize the cells prior to fixation and staining. Cells that were not solubilized were fixed and blocked prior to staining. Cells were stained with 100μl of 1/10 diluted IRAWB14.4 monoclonal antibody, 1/1000 diluted Thy-1 antibody or undiluted 34.1.2s anti K^{dD}d antibody for 20 min, washed twice with 1 ml PBS/HS, followed by a 20 minute incubation with 100μl 1/150 diluted goat anti-rat FITC (Silenus), 100μl of 1/100 goat anti rabbit FITC
(Southern Biotechnology) or 100μl of 1/100 goat anti mouse FITC (Southern Biotechnology) respectively. The cells were further washed and mounted on a slide containing a drop of Fluoromount (Molecular Probes) with 2% 2,4, diazobicyclo (2,2,2) octane (Sigma) to prevent photobleaching. 2,4, diazobicyclo (2,2,2) octane was prepared by dissolving the solid in 10mM Tris pH 8.6 to make a 10% solution. A 1/5 dilution in Fluoromount was prepared fresh for each experiment.

Actin, vimentin or keratin were stained by first fixing the cells with 3% paraformaldehyde, blocking with 10mM glycine, followed by permeabilization with 0.5% Triton X-100. Actin was labeled with 100μl of 0.1mg/ml Phalloidin-TRITC (SIGMA, dissolved in EtOH). A 30 minute incubation with 100μl of monoclonal antibody to vimentin or keratin, followed by incubation with 100μl of a 1/100 diluted goat anti mouse FITC was used to visualize vimentin and keratin. Cells were observed using a Zeiss Axiophot Fluorescent microscope. Photos were taken on TMAX 400 Black and White film.
RESULTS:

1.0 Characterization of CD44 Binding to HA in Lymphoid Cells

1.0.1 CD44 Expression and HA Binding by Lymphoid Cell Lines

The lymphoid cell lines used in these experiments and described in the table below were obtained from Robert Hyman and Jayne Lesley of the Salk Institute. Initial experiments were designed to confirm CD44 expression and fluoresceinated hyaluronan (Fl-HA) binding properties of the different cell lines.

Table I: Summary of Lymphoid Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>CD44 expression</th>
<th>Ability to bind Fl-HA</th>
<th>Inhibition of Fl-HA binding with KM201†</th>
<th>Increase in Fl-HA binding with IRAWB14.4†</th>
<th>Increase in Fl-HA binding with PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1.G.1.22</td>
<td>no</td>
<td>no</td>
<td>no effect</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>TFX591.2</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no effect</td>
</tr>
<tr>
<td>WT(3).1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no effect</td>
</tr>
<tr>
<td>WH(4).1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>WB(3).6</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>RAW253</td>
<td>yes</td>
<td>no</td>
<td>no effect</td>
<td>yes</td>
<td>no effect</td>
</tr>
<tr>
<td>WEHI231</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

† antibody directed to CD44

These cell lines express CD44 and have different binding properties for HA. The different cell lines represent different stages of activation of normal lymphoid cells (Lesley et al. 1992), i.e.; RAW253 represents an inactive B cell stage, WH(4).1 represents an inactive T-cell stage, and WB(3).6 and WH(4).1+PMA represent an activated stage. Cell lines able to bind HA are considered to be at a more activated stage than cell lines unable to bind HA. Such is the case for WEHI231 a mature B-cell line that can bind HA, compared to RAW253, a pre-B-cell line that cannot bind HA. The T cell line, WH(4).1, can be shown to bind HA when activated with PMA.

All CD44 positive cell lines expressed high levels of CD44 (Figure 1.1) and
displayed different binding properties for Fl-HA (Figure 1.2) as assessed by FACS analysis. AKR1.G.1 is a CD44 negative cell line that does not bind Fl-HA (Figure 1.2A). TFX591.2 is a wild type CD44 transfectant of AKR1.G.1 that binds high levels of Fl-HA (Figure 1.2B). WT(3).1 is a CD44 cytoplasmic deletion transfectant of AKR1.G.1 that expresses the same CD44 isoform as TFX591.2 except it lacks 64 amino acids of the cytoplasmic domain (Figure 1.2C). Gly278 to the C terminus is deleted leaving only six amino acids of the cytoplasmic domain. This cell line will not bind high levels of soluble Fl-HA (Figure 1.2C), but will bind to immobilized HA although not as well as the full length CD44 transfectant (Lesley et al. 1992). Two other cell lines, WH(4).1 (Figure 1.2D) and WB(3).6 (Figure 1.2E), derived from the parental cell line SAKRTLS12.1 (CD44.2) both express a CD44 allele different from the one transfected into AKR1.G.1 (CD44.1). The identity of the allele was determined by the murine strain that the CD44 expressing cell line was derived from (Nottenburg et al. 1989). Cell lines derived from AKR/J or C57BL/6 mice express the CD44.2 allele. Cell lines derived from BALB/cJ express the CD44.1 allele. WH(4).1 expresses high levels of CD44 and binds low levels of Fl-HA. WB(3).6 expresses lower levels of CD44 compared to WH(4).1 and binds high levels of Fl-HA. CD44 expressed by the B-cell line RAW253 does not bind Fl-HA (Figure 1.2F). WEHI231, a mature B-cell line, binds high levels of Fl-HA (Figure 1.2G). The level of CD44 expression cannot be accurately determined in this cell line since a secondary FITC labeled antibody was used that could bind to the cells via the Fc receptor expressed by WEHI231 thus giving higher than normal background levels. A CD44 monoclonal antibody directly coupled with FITC would provide a better estimation of CD44 expression.

1.0.2 CD44 is the Hyaluronan Receptor

To confirm that CD44 was the lone HA receptor on these cells a CD44 monoclonal antibody, KM201, was shown to inhibit Fl-HA binding (Figure 1.2A - G). This antibody has been previously shown to inhibit CD44 binding to HA in splenic B
cells (Miyake et al. 1990a). This provides evidence that CD44 is responsible for all HA binding observed on these cells.

1.0.3 Induction of HA Binding with a CD44 Specific Monoclonal Antibody
IRAWB14.4

Fl-HA binding increased following incubation with IRAWB14.4, another CD44 monoclonal antibody (Figure 1.2A - G). IRAWB14.4 slightly increased HA binding in cell lines able to bind HA (TFX591.2, WB(3).6, WEHI231), and remarkably increased HA binding in cell lines normally unable to bind HA (WT(3).l, WH(4).1, RAW253). This suggests that not all of the CD44 molecules on the cell lines are binding HA and the antibody is inducing more molecules to bind. The ability of the antibody to allow CD44 to bind HA could be due to conformational changes induced by the antibody, or a redistribution of CD44 to increase HA binding by the cell lines (Lesley et al. 1993).

1.0.4 Verification that HA Binding is Dependent on the Receptor CD44

Fl-HA binding by these cell lines is titratable and this experiment (Figure 1.3) approaches saturation. This suggests that there is a limited number of HA binding receptors on these cell lines. Data from one cell line, WB(3).6, is representative of all the HA binding cell lines. CD44 binding to Fl-HA increased following IRAWB14.4 incubation. This suggests that HA binding by the cells was not at its optimal level and that not all of the CD44 molecules bind Fl-HA even though they are capable of doing so.
Figure 1.1 CD44 expression on lymphoid cell lines. A. CD44 negative cell line AKR1.G.1ova r1.26, B. TFX591.2 - AKR1.G.1ova r1.26 transfected with the CD44.1 allele, C. WT(3).1 - AKR1.G.1ova r1.26 transfected with the CD44.1 allele lacking all but the first six amino acids of the cytoplasmic domain, D. WH(4).1 - SAKRTLS12.1 derived cell line selected for high CD44 expression and low HA binding, E. WB(3).6 - SAKRTLS12.1 derived cell line selected for high HA binding, F. RAW253.ITB.1.ova r1.1 - pre B cell line with high levels of CD44 expression and no ability to bind to HA. G. WEHI231 - Mature B cell line.
Figure 1.2 Hyaluronan binding by cell lines and the effect of anti-CD44 monoclonal antibodies. A. CD44 negative cell line AKR1.G.1ova1.26, B. TFX591.2 - AKR1.G.1ova1.26 transfected with the CD44.1 allele, C. WT(3).1 - AKR1.G.1ova1.26 transfected with the CD44.1 allele lacking all but the first six amino acids of the cytoplasmic domain, D. WH(4).1 - SAKRTLS12.1 derived cell line selected for high CD44 expression and low HA binding, E. WB(3).6 - SAKRTLS12.1 derived cell line selected for high CD44 expression and high HA binding, F. RAW253.ITB.1.ova1.1 - pre B cell line with high levels of CD44 expression and no ability to bind to HA. G. WEHI231 - B cell line with high CD44 expression and HA binding.
Figure 1.3 Titration of Fl-HA binding by a T-cell line, WB(3).6, constitutively able to bind HA. Values for median fluorescent intensity correspond to the fluorescent intensity of the sample as assessed by FACS analysis.
1.0.5 Molecular Weight Determination of CD44 in Different Cell Lines

Different isoforms of CD44 have been shown to have different binding abilities for HA (St Jacques et al. 1993, Stamenkovic et al. 1991). The molecular mass of CD44 in the different cell lines was determined to assess whether or not differences in isoforms were responsible for the differences in HA binding. Different isoforms result from differential glycosylation or from alternative exon expression and this usually results in a CD44 molecule expressed which corresponds to a higher molecular mass variant than the standard 95 Kd isoform. The molecular mass of CD44 was assessed in the different cell lines by western blot analysis of whole cell lysates (Figure 1.4). The molecular mass of CD44 was 85Kd, in the WT(3).1 cell line. This value appears slightly higher than it should due to skewing of the gel during electrophoresis. The molecular mass of full length CD44 was 95Kd on TFX591.2, WB(3).6 and one of the species expressed by WH(4).1. The higher molecular mass proteins in the TFX591.2 lane are not thought to be CD44 specific proteins. The B-cell lymphoma, RAW253, expressed a molecule of slightly higher molecular mass (100Kd). The identity of the lower molecular mass bands seen in the RAW253 lane are presently unknown. Interestingly, WH(4).1, which normally binds low levels of Fl-HA, expressed three additional CD44 variants (130, 145, 160 Kd). Activation of this cell line with PMA increased the expression of all variants. The exact nature of increased HA binding by WH(4).1 cannot be attributed to the CD44 isoform expressed as assessed by this method since all the variants increased expression. It may be that the 95 Kd form has the highest affinity for HA since the cell lines that expressed only this form could bind high levels of HA (TFX591 and WB(3).6). Alteration of CD44 in RAW253 could result in the inability of this cell line to bind HA.
Figure 1.4 Western blot analysis of whole cell lysates of different CD44 positive cell lines following Triton X-100 solubilization. Cell lysates were prepared as described in materials and methods. Triton X-100 soluble CD44 is described as 'soluble', CD44 remaining in the Triton X-100 insoluble fraction is described as 'pellet'.
1.0.6 Determination of CD44 Expression and Fl-HA Binding on Murine T-cells

T-cells from two different murine strains were isolated as described in Materials and Methods. The normal murine T-cells allowed for the study of CD44 expression and Fl-HA binding in normal unactivated lymph node T-cells. Comparison of the normal T-cells to cell lines that represent unactivated lymphoid cells supports the notion that unactivated cells do not bind HA and that the cell lines can represent cells in vivo that exhibit different HA binding capabilities. Lymph node T-cells from the 2 murine species, BALB/cJ and C57BL/6, expressed high levels of CD44 and neither bound Fl-HA (Figure 1.5A - B). CD44 expressed by the lymph node T-cells were induced by IRAWB14.4 to bind Fl-HA. Thus CD44 expressed by the T-cells are capable of binding Fl-HA, but for some reason do not. T-cells isolated from BALB/cJ (CD44.1) mice expressed high levels of CD44 as assessed by FACS analysis. When the activating antibody induced Fl-HA binding in this cell line one high Fl-HA binding population was present. T-cells isolated from C57BL/6 (CD44.2) mice displayed populations with low, medium and high levels of CD44 expression. When these cells were induced with the activating antibody, low, medium and high Fl-HA binding populations were present. Thus, there is variability in CD44 expression but not Fl-HA binding ability in normal inactive murine T-cells isolated from different murine strains. This result supports the necessity of activation of T-cells in regulating CD44/HA binding since unactivated T-cells do not bind HA, but binding could increase during later stages of activation. The inability of the unactivated T-cells to bind HA parallels the HA binding ability of RAW253 and unactivated WH(4).1, cell lines that represent the inactive state of normal lymphoid cells that do not bind HA.
Figure 1.5 CD44 expression and Fl-HA binding by normal lymphoid T-cells isolated from A. BALB/cj and B. C57BL/6 showing the effects with the CD44 monoclonal antibodies IRAWB14.4 and KM201.
1.0.7 Effects of Disrupting the Lymphoid Cytoskeleton with Cytochalasin D

CD44 has been suggested to be associated with the cytoskeleton in SV transformed 3T3 fibroblast cells by extrapolating from Triton X-100 solubilization experiments. The Triton X-100 insolubility of CD44 increased following treatment with cytochalasin B suggesting that CD44 is associating with cytoskeletal elements (Lacy and Underhill 1987). Therefore, cytochalasin B and a more specific disrupter of the cytoskeleton, cytochalasin D (CD) was used to disrupt filamentous actin in normal lymphoid cells in order to assess whether disruption of filamentous actin induced CD44 to bind hyaluronan (Figure 1.6). Only data showing the effects with CD is shown; data with cytochalasin B was comparable. CD did not induce CD44 to bind hyaluronan on normal T-cells isolated from C57BL/6 mice. Since CD44 does not bind HA in these normal lymph node T-cells it was necessary to assess the effects of CD on cell lines able to bind HA. Thus, the ability of CD to abolish HA binding in the cell lines able to bind HA was determined. Fl-HA binding was also unaffected by CD in the HA binding cell lines (data not shown). The exact role of filamentous actin was not conclusively determined by these experiments with the cell lines since it was difficult to control for the possibility that CD was not working in the cell lines. CD may not be disrupting actin in the cell lines since actin may already be disrupted in this transformed cellular environment. Alternatively, CD may not enter these cells to disrupt filamentous actin; however, it should be noted that prolonged incubations of the cells with CD reduced cell viability, thus it does seem that CD enters the cells to cause the reduced viability. Another explanation was that filamentous actin does not regulate CD44/HA binding in these cell lines. To try and assess possible cytoskeletal associations, another method which determines the Triton X-100 solubility of CD44 in the cell lines was employed to evaluate whether there was any correlation between Triton X-100 solubility and the ability of CD44 to bind HA. This would give an indication as to whether a correlation between HA binding and the potential association of CD44 with the cytoskeleton exists.
Figure 1.6 Effects of cytochalasin D on CD44 expression and HA binding on C57BL/6 normal murine T-cells.
Effects of Triton X-100 Solubilization on CD44 in the Lymphoid Cell Lines

The solubility of CD44 in the non-ionic detergent Triton X-100 was determined in the different cell lines to see if a correlation between Triton X-100 solubility and the differences in HA binding occurred. If a correlation between HA binding and Triton X-100 solubility occurred it would suggest that cytoskeletal associations may be involved in regulating HA binding. Further experiments would have to be employed to ensure that the Triton X-100 insolubility is indeed due to cytoskeletal associations. CD44 was found to be predominantly Triton X-100 soluble in all cell lines regardless of their ability to bind HA (Figure 1.4). Two exceptions include the non-HA binding B-cell line, RAW253, which expresses a small portion of CD44 that appears to be Triton X-100 insoluble, and the WH(4).1 cell line that has a greater Triton X-100 insoluble population following PMA activation where HA binding increases. WH(4).1 also expresses a small amount of Triton X-100 insoluble CD44 prior to PMA activation. Thus it seems that Triton X-100 solubility does not correlate with Fl-HA binding ability in these cell lines, which is in agreement with previous data and suggests that cytoskeletal associations are not involved in regulating CD44/HA binding.

The Effects of Protein Synthesis Inhibitors on WH(4).1 Binding to Fl-HA Following PMA Incubation

The increase in HA binding by WH(4).1 following PMA incubation was further examined by addressing the possibility that a newly translated protein was responsible for the increased HA binding in this cell line. Since PMA, a diacyl glycerol analog, stimulates PKC, the effect of PMA could be due to PKC dependent phosphorylation resulting in activation of CD44. No induction of HA binding was seen during the time when PKC activation was maximal (30 min to two hours) but an effect was seen following longer incubations, after 16 hours. To address the question of increased protein synthesis resulting from a prolonged PMA incubation, four different inhibitors
of macromolecular synthesis were used. Actinomycin D, which inhibits DNA synthesis, and puromycin, emitine and cycloheximide, which inhibit protein synthesis, all prevented the increase in Fl-HA binding by this cell line (Figure 1.7A - D). Moreover, cells treated with cycloheximide grown for an additional 3 days in the absence of cycloheximide were once again induced to bind Fl-HA following a 16 hour PMA incubation (Figure 1.7E). These results suggest that PMA induced the synthesis of a protein that increased CD44/Fl-HA binding. This protein could either be an additional isoform of CD44 expressed on the surface or another regulatory molecule that increases the ability of CD44 to bind HA. However, earlier data suggests that no differences in isoform expression occurred, suggesting that the induction of HA binding is due to the expression of a regulatory protein.

Actinomycin D inhibits DNA transcription by intercalating between purine-pyrimidine (G-C) base pairs. Puromycin acts by reacting with the growing carboxyl end of elongating peptide chains by mimicking peptidyl tRNA to inhibit peptide chain elongation. Emitine also inhibits peptide chain elongation by an unknown mechanism. These inhibitors have irreversible effects. Cycloheximide inhibits protein synthesis by binding to 80s ribosomes. This reaction is reversible as was shown for WH(4).1 (Figure 1.7E). The reversibility of cycloheximide supports the role of translation and not a detrimental effect caused by side effects of this inhibitor to cause the inhibition of increased HA binding by CD44. It should be noted that all four inhibitors have potentially lethal side effects that could affect HA binding in this cell line. However, the fact that all four resulted in the same effect and one of the common functions of all four drugs is to prevent protein synthesis, provides evidence that the effect seen here is due to the inhibition of synthesis of a protein that increases HA binding in this cell line.
Figure 1.7 Effects of protein synthesis inhibitors on CD44 expression and HA binding on WH(4).1 cell line. A. Actinomycin D, B. Puromycin, C. Cycloheximide, D. Emitine, E. Reversal of Cycloheximide (CHX) inhibition.
1.0.10 Results Obtained with Lymphoid Cell Lines

The study of CD44/HA binding in these cell lines allowed for the elucidation of how CD44 is regulated with respect to HA binding. It was necessary to characterize cytoskeletal associations or the involvement of protein synthesis and how these actions effect CD44 in the different cell lines to gain more understanding about CD44 regulation.

1. CD44 is the principle HA receptor on lymphoid cells.
2. CD44 expressing cells do not necessarily bind HA.
3. CD44 expressed by the lymphoid cell lines is of similar molecular mass and there is no correlation between the molecular mass isoform of CD44 expressed and its ability to bind HA.
4. The Triton X-100 solubility experiments and disruption of filamentous actin by cytochalasin B and D provided no evidence that CD44 was directly associated with the actin cytoskeleton or that disruption of the cytoskeleton affected CD44's ability to bind HA.
5. A newly translated protein increases HA binding in some CD44 positive cell lines - possible candidates include new CD44 molecules or a regulatory protein. Data accumulated to date suggests that the newly synthesized protein is likely to be a regulatory protein.

The study of CD44 in fibroblast cells will provide further insight into cytoskeletal associations of CD44 since these cells have a more defined cytoskeleton than the lymphoid cells.
2.0 Evaluation of CD44 Expression and HA Binding in Fibroblast Cell Lines

2.0.1 CD44 Expression and Fl-HA Binding by NIH 3T3 and L-cells

CD44 expression and Fl-HA binding were examined in the mouse fibroblast cell lines, NIH 3T3 and L-cells. The use of the fibroblast cell lines allowed for further evaluation of cytoskeletal associations of CD44 since these cells have a well-defined cytoskeleton. Fibroblast cells express high levels of CD44 and have been shown to bind soluble[^3H]-HA (Culty et al. 1992, Lacy and Underhill 1987). Both cell lines tested expressed high levels of CD44, but neither bound high levels of Fl-HA unless induced by IRAWB14.4 (Figure 2.1A - B). This shows that CD44 was able to bind Fl-HA in these cell lines and binding was somehow prevented. NIH 3T3 cells were shown to bind higher levels of Fl-HA than the L-cells. Factors such as the glycosaminoglycans - heparan sulphate or chondroitin sulphate or filamentous actin, that might affect CD44's distribution, which in turn may affect its ability to bind HA.

2.0.2 The Effect of Glycosaminoglycans on Fl-HA Binding by CD44 Expressed by L-cells

The role of the glycosaminoglycans (GAGs) heparan sulphate and chondroitin sulphate in regulating CD44/Fl-HA binding was assessed in the L-cells by FACS analysis. Since GAG's are negatively charged, the presence of these molecules on the cell surface may affect how the negatively charged HA can bind CD44. The absence of GAG's may increase HA binding by L-cells and this was examined by testing the GAG deficient L-cells. In addition to normal L-cells, variants (Gruenheid et al. 1993) were tested that lacked the glycosaminoglycans heparan sulphate (Figure 2.2B), chondroitin sulphate (Figure 2.2C) or both (Figure 2.2D). None of the cell lines bound Fl-HA. Normal L-cells were induced by IRAWB14.4 to bind Fl-HA, as were the heparan
Figure 2.1 Characterization of CD44 expression and HA binding by the mouse fibroblast cell lines, A. NIH 3T3 cells and B. L-cells.
sulphate deficient cell lines - although to a lesser extent. The cell lines deficient in chondroitin sulphate could not be induced by IRAWB14.4 to bind Fl-HA. Furthermore, when the normal L-cells were treated with xyloside the IRAWB14.4 induced Fl-HA binding decreased (Figure 2.2E). xyloside effectively decreases the amount of glycosaminoglycans on the cell surface by preventing galactose attachment to core proteins (Hook 1984). These results suggest that glycosaminoglycans are important for IRAWB14.4 induced activation of CD44 binding to Fl-HA in L-cells.

2.0.3 Direct Assessment of Filamentous Actin Regulating CD44’s Potential to Bind Fl-HA in NIH 3T3 Cells

Results with the lymphoid cells suggest that CD44 does not associate with actin, and its ability to bind HA is not affected by disruption of filamentous actin. This is surprising in view of the data with fibroblast cells that suggest that CD44 is directly associated with actin (Jacobsen et al. 1984, Lacy and Underhill 1987). To clarify this apparent discrepancy, the association of CD44 with the actin cytoskeleton and the effect of an intact cytoskeleton on its ability to bind HA was evaluated. NIH 3T3 cells do not normally bind high levels of Fl-HA. NIH 3T3 cells were treated with Cytochalasin D and Fl-HA binding was assessed by FACS analysis (Figure 2.3) and immunofluorescent microscopy (data not shown). CD disrupts filamentous actin in NIH 3T3 cells (Figure 3.2C). Fl-HA binding by NIH 3T3 cells was unaffected following treatment with CD; thus, as with the lymphoid cells, filamentous actin does not regulate CD44/Fl-HA binding in this cell line.
Figure 2.2 CD44 expression and HA binding by A. normal L cells, B. heparan sulphate deficient L-cells, C. chondroitin sulphate deficient L-cells, D. heparan sulphate and chondroitin sulphate deficient L-cells, E. L-cells treated with xyloside (dotted line) or untreated (solid line) and labeled for IRAWB14.4 induced Fl-HA binding.
Figure 2.3 Effects of cytochalasin D on CD44 expression and HA binding on NIH 3T3 fibroblast cells
2.0.4 Summary of HA Binding by Fibroblast Cells

1. Fibroblast cells express CD44 but do not always bind FI-HA.
2. The GAG's chondroitin sulphate or heparan sulphate do not regulate CD44/HA binding.
3. Chondroitin sulphate is involved in the antibody-induced increase in CD44/HA binding.
4. Filamentous actin does not seem to regulate CD44/HA binding.

By evaluating possible cytoskeletal or other associations CD44 may have in the fibroblast cell lines it was hoped that this would lead to a better understanding of how CD44 may be regulated in the fibroblast cell lines.
3.0 Elucidation of CD44 Associations in Fibroblast Cell Lines

3.0.1 Assessment Of CD44 Associations with the Cytoskeletal Proteins Actin, Vimentin and Keratin in NIH 3T3 cells:

As previously mentioned CD44 has been described to have cytoskeletal associations. Since a role for the cytoskeleton was not apparent in the lymphoid cells, fibroblast cells were used which have a more defined cytoskeleton to examine if possible associations existed. The association of CD44 with actin, vimentin and keratin was assessed in NIH 3T3 cells. The distribution of CD44 both before and after Triton X-100 solubilization was compared with the distribution of actin, vimentin or keratin. The distribution of CD44 (Figure 3.1A and B) did not correlate with the distribution of actin (Figure 3.1C and D), vimentin (Figure 3.1E and F) or keratin (Figure 3.1G and H) in NIH 3T3 cells. This suggests that CD44 does not have a direct link with actin or the intermediate filaments vimentin or keratin in NIH 3T3 cells. Figure 3.1.2A - C are negative controls where NIH 3T3 cells were labeled with anti-rat FITC either before (Figure 3.1.2A) or after (Figure 3.1.2B) Triton X-100 solubilization or labeled with anti rabbit FITC following Triton X-100 solubilization (Figure 3.1.2C).

3.0.2 Direct Assessment of Filamentous Actin Regulating CD44 Distribution or Triton X-100 Insolubility

Triton X-100 insolubility is not a conclusive method to determine cytoskeletal associations. CD44 expressed by NIH 3T3 cells was shown to be insoluble in Triton X-100 both by immunofluorescent microscopy (Figure 3.1) and by immunoprecipitation where a population of CD44 was solubilized by Triton X-100 and a population remained insoluble (data not shown). To directly examine if actin was involved in retaining CD44 in the Triton X-100 insoluble fraction, NIH 3T3 cells were treated with CD. CD dissociates filamentous actin and should alter the distribution of CD44 in the cellular membrane and the Triton X-100 solubility if a strong CD44/actin association
Figure 3.1 Distribution of CD44 (A and B) and actin (C and D) before (A and C) or after (B and D) Triton X-100 solubilization.
Figure 3.1 Distribution of vimentin (E and F) and keratin (G and H) before (E and G) or after (F and H) Triton X-100 solubilization.
Figure 3.1.2 Labeling of NIH 3T3 cells with anti-rat FITC to show background fluorescent staining before (A) or after (B) Triton X-100 solubilization or anti-rabbit FITC (C) following Triton X-100 solubilization.
occurs. CD did not alter the distribution of CD44 following Triton X-100 solubilization in NIH 3T3 cells as assessed by immunofluorescent microscopy (Figure 3.2). A direct link between CD44 and filamentous actin is not clearly present in NIH 3T3 cells.

Since CD44 is not associating with the cytoskeleton in NIH 3T3 cells, the GAG's heparan sulphate and chondroitin sulphate were tested to see if they played a role in CD44 solubility or distribution. NIH 3T3 cells were treated with chondroitinase ABC lyase or heparitinase to degrade cell associated GAG's (data not shown). No effect was seen on CD44 solubility or distribution. Since these enzymes may not be totally efficient at degrading cell surface GAG's the use of L-cells lacking GAG's were examined with respect to CD44 distribution and solubility.

3.0.3 Assessing the Role of the Glycosaminoglycans Heparan Sulphate or Chondroitin Sulphate in the Triton X-100 Insolubility and Distribution of CD44 in L-cells

CD44 distribution following Triton X-100 solubilization was determined in normal L-cells (Figure 3.3A and B), L-cells lacking heparan sulphate (Figure 3.3C and D), and L-cells lacking chondroitin sulphate (Figure 3.3E and F). CD44 remained insoluble and retained a similar distribution compared to normal L-cells following Triton X-100 solubilization. Thus, these glycosaminoglycans are not responsible for the insolubility or distribution of CD44 following Triton X-100 solubilization. Figure 3.3G and H are negative controls to assess background fluorescent labeling where L-cells were labeled with anti-rat FITC either before (Figure 3.3G) or after (Figure 3.3H) Triton X-100 solubilization.
Figure 3.2 Effects of CD treatment on CD44 expression (A and B) or filamentous actin labeling (C and D) of unsolubilized (A and C) or Triton X-100 solubilized (B and D) NIH 3T3 cells.
Figure 3.3 CD44 expression on normal L-cells (A and B) and L-cells lacking heparan sulphate (C and D) before (A and C) or after (B and D) Triton X-100 solubilization.
Figure 3.3 CD44 expression on L-cells lacking chondroitin sulphate (E and F) or negatively controlled L-cells labeled with only anti-rat FITC (G and H) before (E and G) or after (F and H) Triton X-100 solubilization.
3.0.4 Comparison Of CD44 with a GPI Linked Protein (Thy-1) or a Protein with no Known Cytoskeletal Associations (MHC Class I) in NIH 3T3 Cells

In fibroblasts, the distribution of CD44 did not correlate with cytoskeletal components nor was it affected by the absence of GAG's since no change in distribution occurred when the cytoskeleton was disrupted or the GAG's were absent. The next step was to compare the distribution of CD44 with other membrane bound proteins with known associations. NIH 3T3 cells were solubilized with Triton X-100 and the distribution of CD44 (Figure 3.4.1A and B) was compared to the distribution of two other proteins with known cellular associations. The distribution of CD44 following solubilization was not mimicked by Thy-1 (Figure 3.4.1C and D), a GPI linked protein, or MHC Class I (Figure 3.4.1E and F), a protein that is not thought to be associated with the cytoskeleton. CD44 was solubilized by β-octyl glucoside (Figure 3.4.2A), a detergent that can solubilize GPI linked proteins such as Thy-1 (Figure 3.4.2B) and placental alkaline phosphatase (Brown and Rose 1992), but not cytoskeletal proteins such as actin (Figure 3.4.2C). These results suggest that CD44 is associating with something in the cell to prevent it from being solubilized by Triton X-100 but which still allows it to be solubilized by β-octyl glucoside. This association could be similar but not identical to associations Thy-1 may have since Thy-1 is also not solubilized by Triton X-100 but is solubilized by β-octyl glucoside. Since Thy-1's distribution following Triton X-100 solubilization is more localized and does not assume the same lacy pattern as CD44 would suggest that these two molecules have different molecular associations.
Figure 3.4.1 Distribution of CD44 (A and B), Thy-1 (C and D) and MHC Class I (E and F) before (A, C and E) or after (B, D and F) Triton X-100 solubilization.
Figure 3.4.2 Distribution of CD44 (A) Thy-1 (B) and actin (C) following solubilization with β-octyl glucoside.
3.0.5 Results Obtained with Fibroblast Cells;

These results have shown that unlike lymphoid cells, CD44 is Triton X-100 insoluble in NIH 3T3 and L-fibroblast cells. This is consistent with reports from other researchers who also found CD44 to be insoluble in other fibroblast cells (Lacy and Underhill 1987, Neame and Isacke 1993).

1. The distribution of CD44 does not correlate with the distribution of filamentous actin, vimentin or keratin in NIH 3T3 cells.
2. The GAG's chondroitin sulphate or heparan sulphate do not affect CD44's distribution in L-fibroblast cells.
3. The distribution of CD44 is not comparable to the distribution of Thy-1 or MHC Class I suggesting CD44 does not have similar associations as these molecules.
4. The solubility of CD44 in β-octyl glucoside suggests that CD44 may reside in lipid microdomains that are resistant to Triton X-100 solubilization.
DISCUSSION:

Characterization of CD44 Binding to HA in Lymphoid Cells:

HA Binding by CD44 Expressed by Lymphoid Cell Lines

CD44 is a widely expressed adhesion receptor that binds to hyaluronan in a regulated manner. The focus of this thesis was to analyze different CD44 expressing cell lines and to try to determine why some cell lines can bind hyaluronan (HA), why some cannot, and why some can be induced to bind. All cell types tested could bind HA following activation with a CD44 specific monoclonal antibody but exhibited differences in their HA binding capacities in their normal state. These results suggest that different regulatory mechanisms of CD44/HA binding occurs in different cell lines. In lymphoid cells CD44/HA binding is highly regulated and diverse. The two fibroblast cell lines tested did not bind Fl-HA unless CD44 was activated by the IRAWB14.4 antibody. Whether or not the fibroblast cell lines could bind to immobilized HA is currently unknown. To address the regulation of CD44 various lymphoid cell lines were used that expressed CD44 and displayed differences in their ability to bind HA. Analysis of CD44 associations in these cell lines has provided insight into CD44 regulation with respect to HA binding since it seems that CD44 is not regulated by cytoskeletal associations but may be regulated by other proteins in certain situations. Fibroblast cell lines were also used to elucidate possible cytoskeletal associations since the fibroblast cytoskeleton is more easily discerned than the lymphoid cytoskeleton.

The lymphoid cell lines used in these experiments provide a model to study HA binding by CD44. Each cell line expressed high levels of CD44 and has distinct characteristics with respect to HA binding (Lesley et al. 1992). Comparison of CD44/HA binding between a wild type and a cytoplasmic deletion transfectant supports an important role for the cytoplasmic domain in maintaining HA binding by wild type CD44 in this cell line since the absence of the cytoplasmic domain decreases HA binding.
Interestingly, not all CD44 molecules that contain the cytoplasmic domain will bind HA as evidenced by the cell lines expressing full length CD44 that cannot bind HA (Figure 1.2 D and F). The role of the cytoplasmic domain, when CD44 is expressed in the transfected cell line, may be to maintain the extracellular domain in a conformation optimal for HA binding. Maintenance of the optimal HA binding conformation may result from an association of the cytoplasmic domain with a cytoplasmic or membrane associated protein. The cytoplasmic domain may be differentially phosphorylated which could regulate HA binding by the wild type transfectant. Phosphorylation may determine if cytoplasmic molecules will associate with the cytoplasmic domain to elicit some control. It may result in an alteration in the extracellular domain to permit HA binding by CD44. The cytoplasmic domain may be responsible for controlling the mobility of CD44 within the cell membrane to allow aggregates of CD44 to bind one HA molecule. WT(3).1 has been shown to bind immobilized HA (Lesley et al. 1992). CD44, when binding to immobilized HA, could be in a different conformation since in this situation the cells are lying on a flat surface as opposed to free in solution where binding to soluble HA could be more difficult and require greater ligand affinity. The exact role of the cytoplasmic domain is currently unknown. The cytoplasmic domain has been shown to be important for localizing CD44 within the lateral membrane of MDCK cells (Neame and Isacke 1992). It has also been shown to be important for melanocyte mobility on an HA substrate (Thomas et al. 1992). Thus the cytoplasmic domain is important for CD44 function.

Incubation of the wild type transfectant with an antibody that induces CD44 binding to HA (IRAWB14.4) slightly increased HA binding by this cell line. It also significantly increased HA binding by the cytoplasmic deletion transfectant (Figure 1.2 B and C). Antibody induced activation has also been described for the integrins GPIIb-IIIa (O'Toole et al. 1991) and LFA-1 (van Kooyk et al. 1991).

The activating antibody, IRAWB14.4, enhanced CD44 binding to HA in the
transfected cell lines as well as in the other wild type cell lines tested. Possible mechanisms of antibody-induced HA binding include a change in conformation and/or a change in distribution of CD44 to increase HA binding (Lesley et al. 1993).

WH(4).1 was clonally selected to express high levels of CD44 and was found to bind low variable levels of HA. WB(3).6 was selected to bind relatively high levels of HA. Both cell lines bound more HA when incubated with PMA for at least 16 hours (Hyman et al. 1991). These results suggest that a regulatory factor, a new isoform of CD44 or more CD44 molecules are synthesized by the cell lines to increase HA binding by CD44. One possible reason why WH(4).1 cannot bind HA might be due to a negative regulatory factor acting on CD44. Alternatively, a positive factor expressed in WB(3).6 may allow CD44 to bind HA. These factors may be either repressed or induced, respectively, following incubation with PMA. Alternatively, the ability of WB(3).6 to bind HA could result from this cell line being in a more advanced state of activation than WH(4).1. WH(4).1 assumes this activation state following incubation with PMA where HA binding increases. Different states of activation could result in different cytosolic or membrane associated proteins enhancing CD44's ability to bind HA. These associations may positively regulate CD44 to allow the extracellular domain to assume a conformation more suited to HA binding. Another mechanism that might increase HA binding by CD44 is the association of CD44 molecules in the membrane to create CD44 aggregates. These aggregates might possess greater avidity towards HA. Aggregate formation may involve associations of the extracellular domain of different CD44 molecules to enhance HA binding. CD44 mobility within the cellular membrane may not be a factor in this process. This explanation is supported by experiments involving the activating antibody (IRAWB14.4) that can increase HA binding in both cell lines at 0°C, a temperature where membrane mobility is reduced (Figure 1.2). Additional species of CD44 are expressed by WH(4).1 that have a molecular mass higher than the standard hematopoietic form which is 85-95 Kd. These higher molecular mass species might explain the variable HA binding by WH(4).1 if they have their own potential to bind HA.
bind HA. Following PMA incubation the level of expression of all the species increases. Increased levels of expression of the CD44 variants could account for the increased HA binding. The insensitivity of the immunoprecipitation data does not allow accurate determinations of quantitative changes in any of the species expressed since the level of expression of all the species increases dramatically (Figure 1.4). Interestingly, when the increase in CD44 expression is compared between the Western blot (Figure 1.4) and FACS analysis (Figure 1.7) it appears that there is a far greater increase in CD44 expression as detected by the Western blot analysis. This suggests that there is a portion of CD44 present inside the cell that is detected by the western blot that would not be detected by FACS analysis since FACS analysis only measures surface expression.

WB(3).6 expresses only one CD44 species of about 95Kd and its expression does increase following PMA incubation (data not shown).

RAW253 is an immature B-cell line that does not express cell surface IgM. CD44 expressed in this immature cell line does not have the potential to bind HA unless induced by the activating antibody. Comparison of this B-cell line with a more mature B-cell line (WEHI253) demonstrated that CD44 binding to HA increased in the more mature cellular environment. This suggests that CD44/HA binding may be dependent upon cellular activation. This result is consistent with the results of Murakami (Murakami et al. 1991) and Lesley (Lesley et al. 1990) where cells could be induced to bind HA as a result of cellular activation. CD44 expressed by RAW253 has a slightly higher molecular mass than CD44 expressed by the T-cell lines; a difference that could be due to slight differences in CD44 glycosylation. CD44 expressed by elicited macrophages was shown to have a slightly higher molecular mass than CD44 expressed by resting macrophages (Camp et al. 1991). This difference was shown to be due to differential glycosylation. Glycosylation of CD44 may contribute to the difference in HA binding observed for RAW253 if glycosylation sterically hinders the HA binding site. Interestingly this B-cell line expresses the same CD44 allele that is expressed by the wild type transfected T-cell line, TFX591.2. This further demonstrates the differences in CD44
regulation when expressed in different cellular environments.

Specificity of CD44 Binding Fl-HA:

CD44 is the sole HA receptor on the lymphoid cell lines tested. Titration of CD44/HA binding by WB(3).6 showed that HA binding approaches saturation. This suggests that there are a limited number of receptors on the cell surface able to bind HA. Furthermore, this binding was abolished by KM201 a CD44 monoclonal antibody that inhibits CD44/HA binding. This further indicates that CD44 is the principle receptor for HA on these cell lines. Not only is CD44 the principle HA receptor on these cell lines, but results with IRAWB14.4, the activating antibody which further increases HA binding in all cell lines within seconds, indicates that even in cell lines with high HA binding, binding is not at its maximal level. This suggests that not all of the CD44 molecules on the cell surface are binding HA. The antibody recruits more CD44 molecules to bind HA to cause the increased HA binding either by increasing CD44 affinity to HA or by altering the distribution of CD44 to allow more CD44 molecules to bind HA. It is interesting that not all of the CD44 molecules would bind HA. Perhaps this reserves an 'emergency' binding capacity for times when more HA binding is needed to facilitate migration or adhesion. This suggests that the antibody-induced activation overrides a control that normally prevents CD44 positive cells from binding their maximal levels of HA. The antibody may be responsible for causing CD44 aggregation to allow for the increase in HA binding. This aggregation may not involve actual membrane mobility but merely a localization of the extracellular domains of CD44 on the membrane surface. Indeed, it has been shown that IRAWB14.4's inducing ability requires divalent antibody binding (Lesley et al. 1993). IRAWB14.4 may change the conformation of CD44 to induce more molecules to bind HA. Unactivated CD44-expressing cells are incapable of eliciting the changes made by the activating antibody. Explanations for the differences in CD44/HA binding in the cell lines that will be discussed include cytoskeletal associations, differential glycosylation, phosphorylation
or cellular activation resulting in the transcription of a novel regulatory protein, different CD44 isoforms or more CD44 molecules to contribute to the increased HA binding.

**CD44 Expression and Fl-HA Binding by 2 Strains of Murine T-cells:**

The event of activation in determining CD44/HA binding was evaluated by comparing the lymphoid cell lines to normal murine T-cells. Results showed that in cells exhibiting an untransformed and unactivated state, CD44 does not bind HA unless induced with the activating antibody. CD44 expressed on naive lymph node T-cells do not bind HA. Thus, CD44's binding may increase later in development as a result of cellular activation. Interestingly, the pattern of CD44 expression on the murine T-cells resembled the pattern of HA binding following antibody induction. However, a direct relationship between CD44 expression and antibody-induced HA binding was not established. Immature T-cells isolated from the two murine species showed diversity in CD44 expression and similarity in their inability to bind HA. Why T-cells from different murine species display different patterns of CD44 expression is not clear, but it is clear that inactive T-cells do not bind HA. The role of activation was shown for splenic B cells that would not bind HA unless induced for three days with IL-5 (Murakami et al. 1991). These results provide evidence for the variation of CD44 regulation in different cellular environments and suggest a role for activation to increase HA binding.

Disruption of filamentous actin in normal lymph node T-cells allowed for the assessment of the role of filamentous actin in regulating CD44/HA binding. The cytoskeleton of normal T-cells is sensitive to cytochalasin D (CD) depolymerization as evidenced by the ability of CD to prevent PMA induced aggregation which is dependent on an intact cytoskeleton (data not shown). Treatment of normal lymph node T-cells with CD to depolymerize filamentous actin did not increase CD44/HA binding, thus filamentous actin does not seem to be responsible for the inability of CD44 to bind HA in the normal lymph node T-cells. CD also did not alter CD44/HA binding in the T-
cell lines, although one cannot conclusively assess whether or not CD is disrupting actin in the T-cells or if the cytoskeleton is not already perturbed in these cells. The association of actin with CD44 in the cell lines was further assessed by evaluating the Triton X-100 solubility of CD44 in these cell lines.

**Triton X-100 Solubility of CD44 in Relation to F1-HA Binding Ability:**

Western blot analysis compared Triton X-100 insoluble versus soluble CD44 from whole cell lysates of; WT(3).1, TFX591.2, WH(4).1 +/- PMA, WB(3).6 and RAW253. Triton X-100 insoluble CD44 may be cytoskeletonally linked and therefore under the control of cytoskeletal components. Two different HA binding T-cell lines displayed variability in Triton X-100 solubility - TFX591.2 and WH(4).1+PMA. TFX591.2 expressed a Triton X-100 soluble CD44 population and WH(4).1+PMA expressed a Triton X-100 soluble and insoluble CD44 population. This suggests that cytoskeletal links are not a determining factor governing CD44 binding to HA since CD44 that was or was not cytoskeletonally associated could bind HA. It may be that CD44 associating with the cytoskeleton following PMA activation of WH(4).1 may cause the increased HA binding, this possibility cannot be ruled out. WH(4).1+PMA and the B-cell line, RAW253 both contained Triton X-100 insoluble CD44 but WH(4).1+PMA could bind HA while RAW253 could not. This suggests that there is no simple correlation between cytoskeletal linkages and the control of CD44/HA binding.

The results with WH(4).1 where HA binding increased following activation with PMA for 16 hours alluded to the fact that protein synthesis is involved to effect an increase in HA binding in this cell line. To address the role of a newly synthesized protein to increase CD44/HA binding the effects of PMA on WH(4).1 were further evaluated using protein synthesis inhibitors.
PMA - Inducible HA Binding by CD44 is Negatively Affected by Protein Synthesis Inhibitors;

One outcome of prolonged PMA treatment to cause the increased HA binding seen in WH(4).1 could include an increase in protein synthesis. This possibility was addressed by co-incubating the cells with PMA and one of four known inhibitors of protein synthesis. The protein synthesis inhibitors prevented PMA induced HA binding by this cell line. The fact that all four inhibitors resulted in the same effect and the common function of all four inhibitors is to prevent protein synthesis, provides assurance that the effect seen here is indeed due to the inhibition of synthesis of a new protein that regulates CD44/HA binding. Furthermore, the fact that the effects of cycloheximide were reversible showed that this inhibitor did not have a deadly side effect on the cells that may cause the increased HA binding. The identity of the protein synthesized was not determined. Possible candidates include new isoforms of CD44 that have greater binding potentials for HA. More CD44 molecules could be produced to increase the number of CD44 molecules on the cell surface which would increase the chances of HA being bound by CD44. A regulatory protein may be produced that enhances CD44/HA binding. This protein may associate with the cytoplasmic domain to alter the extracellular domain so it takes on a conformation optimal for HA binding. This protein may also cause CD44 molecules to aggregate within the cellular membrane to increase the local concentration of CD44 to increase the avidity of CD44 to HA. A novel regulatory protein has been described (cell adhesion regulator (CAR)) that was shown to increase the adhesion of cells transfected with this cDNA to bind collagen type I (Pullman and Bodmer 1992). Binding to collagen type I was inhibited by co-incubation of the cells with antibodies against α2 and β1 integrins. These investigators hypothesized that this intracellular protein may couple the intracellular domain of adhesion molecules to the cytoskeleton in a phosphorylation dependent manner. A similar protein could be involved in CD44 regulation.
Another possible outcome of prolonged PMA incubations include the downregulation of PKC. Preliminary results (not shown) using an inhibitor of PKC, Calphostin C, showed that HA binding was unaltered in the cell line when treated with this inhibitor. Although PKC inhibition was not confirmed in the cell line, these initial results suggest that PKC downregulation does not alter HA binding in this cell line.

The cell lines described above provide a model displaying the diversity of HA binding by CD44. CD44 regulation is not uniform in the different cell lines. Some cell lines are affected positively by PMA while others are not. These findings suggest that a regulatory factor is synthesized in some cell types but not in others to enhance HA binding by CD44. A similar effect may be brought about by increased expression of CD44 or expression of a different isoform of CD44 to increase HA binding. The importance of the cytoplasmic domain is clear. The cytoplasmic domain may mediate conformational changes in CD44 as a result of associations with regulatory factors. It may also control the localization of CD44. One CD44 variant that does not bind HA when expressed by the immature B-cell line, RAW253, binds HA when expressed in the T-cell line TFX591.2 (Figure 1.2 B and F). Perhaps a factor is absent in RAW253 that is present in TFX591.2 that causes an increase in HA binding by CD44. Alternatively, RAW253 may express a negative regulatory factor that prevents CD44 from binding HA in this cell line. The higher molecular weight of CD44 expressed in RAW253 suggests that CD44 is modified in this cell line, and this modification may result in the decreased HA binding by CD44.

**Characterization of CD44 Binding to HA in Fibroblast Cells:**

The FL-HA binding by fibroblast cell lines was studied and the cytoskeletal associations of CD44 evaluated. Fibroblast cell lines have been shown to bind HA in the form of soluble [³H]-HA and to have cytoskeletallly associated CD44 (Lacy and Underhill 1987). Since the fibroblast cell lines have a more distinct cytoskeleton than the lymphoid cell lines, these cells may provided a better model to assess possible
cytoskeletal associations as well as possible correlation's with HA binding.

CD44 expressed by NIH 3T3 or L-fibroblast cells do not bind high levels HA unless first incubated with the activating antibody. This difference in HA binding between CD44 expressed in NIH 3T3 fibroblasts and L-cells as compared to SV 3T3 fibroblast is due to the regulation of CD44 in the different fibroblasts since SV 3T3 cells were shown to bind soluble \(^{3}\)H-HA (Culty et al. 1992). Fibroblast cells produce HA (Toole 1989), and since this polymer is intended to be unbound and free in the extracellular matrix it is not surprising that cells producing HA do not bind HA. Another possibility is that HA is bound by a different HA receptor such as the receptor described by Turley (1989) even though this receptor has not been shown to be present on NIH 3T3 or L-fibroblast cells. Perhaps CD44 expressed by these cells binds to a different as yet unknown ligand or HA binding by these cells is highly regulated.

Another possibility is that CD44 expressed by fibroblast cells may have HA already bound to them and can therefore no longer bind exogenous HA. This is probably not the case since treatment of the fibroblast cells with hyaluronidase, an enzyme that degrades HA, does not increase HA binding (data not shown). Glioblastoma cells were shown to bind HA following treatment with hyaluronidase (Asher and Bignami 1992).

Furthermore, the activating antibody can increase HA binding in NIH 3T3 and L-fibroblast cells. If the HA binding region were occupied in these cell lines the antibody should not be able to increase HA binding. Furthermore, NIH 3T3 cells could bind low levels of Fl-HA. Another possibility is that the HA polymers in our sample are not large enough to allow high affinity binding by these cell lines, since CD44 binds more strongly to large HA polymers than to smaller ones (Toole 1989, Underhill 1989).

Interestingly, a different fibroblast cell line (SV 3T3 cells) will take up and degrade HA (Culty et al. 1992). This again shows the diversity of CD44/HA binding in different cell lines.
The Effect of Glycosaminoglycans on Fl-HA Binding by CD44 Expressed by L-cells:

A very interesting result was found with the L-cells lacking the glycosaminoglycan (GAG) chondroitin sulphate (CS). L-cells expressing CD44 but lacking (CS) will not bind HA in the presence of the activating antibody. This suggests that CS is necessary to allow the activating antibody to increase HA binding by CD44. CS is a negatively charged repeating disaccharide whose presence may alter the charge of the cell surface. Alteration of the cell surface charge may affect potential ionic charge interactions to permit HA binding by CD44 in the presence of CS. CS present on the L-cells as part of a different proteoglycan may regulate CD44 on these cells. Antibody-induced activation may involve a redistribution of CD44 on the cell surface. The lack of CS on this proteoglycan may affect the mobility of CD44 preventing the increased binding to HA. This is probably not the case since the distribution of CD44 was unaffected in L-cell membranes lacking GAG's either before or after Triton X-100 solubilization. A more likely explanation is the presence of a proteoglycan on the L-cells that associates with CD44 to increase its potential to bind HA. This protein may alter the conformation of CD44 to maintain it in a form ready to bind HA. CS may be an important functional part of this protein allowing it to regulate CD44. Its absence would prevent antibody-induced HA binding. The role of the antibody is to carry out a later step of a regulatory process to induce CD44/HA binding. Although CD44 molecules do exist that contain CS attachments this is not the case for CD44 expressed by the L-cells. CD44 expressed by these cells, including the ones lacking the GAG's, are all of a similar molecular weight that is lower than the molecular weight of the CD44/CS molecule (data not shown). Another finding in this study further supports the requirement of GAG's on the cell surface for antibody-induced binding of CD44 to HA. GAG attachment on the surface of normal L-cells was prevented by the addition of xyloside. Xyloside effectively decreases the amount of GAG's present on the cell surface. Following treatment of the L-cells with this sugar derivative the antibody induced HA
binding by CD44 decreased. Thus, GAG's - specifically CS - assist the activating antibody to increase HA binding by CD44.

**Elucidation of CD44 Associations with Cytoskeletal Components in Fibroblast Cells:**

CD44 associations with the cytoskeleton were assessed in the fibroblast cell lines, NIH 3T3 and L-cells. CD44 expressed by these fibroblast cell lines was partially Triton X-100 insoluble. Neame et al. also found CD44 to be partially Triton X-100 insoluble in NIH 3T3 cells (Neame and Isacke 1993). The insolubility of CD44 in NIH 3T3 cells was not a result of phosphorylation or association of the cytoplasmic domain with the cytoskeleton. Triton X-100 insolubility is a method used to assess cytoskeletal associations. The partial Triton X-100 insolubility of CD44 suggests a cytoskeletal link in these cell lines. Previous work has shown a link between CD44 and the cytoskeleton, whether it be through actin (Lacy and Underhill 1987) or ankyrin (Kalomiris and Bourguignon 1989). Triton X-100 insolubility is not a conclusive method to determine cytoskeletal associations since proteins that are not cytoskeletonally linked are insoluble in Triton X-100, for example, Thy-1 and MHC Class I. Assessment of CD44 Triton X-100 solubility was used to determine possible associations of CD44 with other known cytoskeletal components such as actin, vimentin and keratin in NIH 3T3 cells. If a strong association exists between CD44 and these proteins then CD44 should co-localize with the proteins following solubilization with Triton X-100.

**Assessment Of CD44 Associations with the Cytoskeletal Protein Actin, or Intermediate Filaments Vimentin or Keratin in NIH 3T3 Cells:**

CD44 distribution did not resemble that of actin, vimentin or keratin either before or after Triton X-100 solubilization. Therefore, CD44 does not appear to co-localize with any of these proteins in NIH 3T3 cells. Previous reports from Lacy and Underhill described an association of CD44 with actin in SV 3T3 fibroblast cells (Lacy
and Underhill 1987). Carter et al described a correlation between CD44 with vimentin, but not actin in WI-38 normal human lung fibroblasts (Carter and Wayner 1988). These results again display the inherent diversity of CD44 regulation in different cell types. CD44 may have cytoskeletal associations in some cells and perhaps different, more elusive associations in others.

The lacy pattern displayed by CD44 following Triton X-100 solubilization was also described for annexin II (Neame and Isacke 1993). Annexin II was shown to partially co-localize with spectrin (Glenney 1986). The distribution of annexin II was not tested but the distribution of spectrin and ankyrin was attempted. The importance of a possible CD44/annexin II association will provide insight into the regulation of CD44 in 3T3 cells. Possible inhibition of annexin II function will conclusively determine if this protein is causing the distribution of CD44 and if this association regulates HA binding by CD44 in this cell line. The role of annexin II is currently unknown but it belongs to a class of Ca$^{2+}$-dependent phospholipid binding proteins. It is thought to be involved in mediating aggregation of phospholipid vesicles as well as being involved in regulating membrane trafficking such as exocytosis (Johnstone et al. 1992). It is possible that an association of CD44 with annexin II may result in its sequestration to lipid microdomains.

Since Triton X-100 solubility does not provide conclusive evidence for an association of CD44 with the cytoskeleton, a direct link with actin was evaluated by disrupting filamentous actin with cytochalasin D.

**Direct Assessment of Filamentous Actin Regulating CD44 Distribution or Triton X-100 Insolubility:**

To further assess the role of filamentous actin associating with CD44 in NIH 3T3 cells cytochalasin D (CD) was used to depolymerize actin. Disruption of filamentous actin by CD did not alter the distribution of CD44 before or after Triton X-100.
solubilization. The solubility of CD44 was also not affected to any significant extent. The ability of CD44 to bind HA was unaltered in cells treated with CD. These results show that CD44 does not associate with nor is its HA binding regulated by filamentous actin that makes up the framework of the NIH 3T3 cytoskeleton. This is not surprising since CD44 did not correlate with actin distribution in NIH 3T3 cells so the disruption of filamentous actin should not effect CD44 distribution or function in this cell line.

It seems that CD44 does not associate with the cytoskeleton in NIH 3T3 cells. This then turned attention to the L-cell line that has many of the same characteristics as the NIH 3T3 cells. CD44 is insoluble in Triton X-100 and CD44 does not bind HA unless induced with the activating-antibody. The L-cell lines deficient in chondroitin sulphate (CS) and heparan sulphate (HS) allowed for the elucidation of possible associations of CD44 with these glycosaminoglycans.

Assessing the Role of the Glycosaminoglycans Heparan Sulphate or Chondroitin Sulphate in the Triton X-100 Insolubility or Distribution of CD44 in L-cells:

Triton X-100 insolubility does not necessarily correlate with cytoskeletal associations. The possibility that glycosaminoglycans (GAG's) were responsible for CD44 Triton X-100 insolubility was addressed. Three L-cell lines containing either heparan sulphate and chondroitin sulphate, lacking heparan sulphate or lacking chondroitin sulphate were examined. CD44 was Triton X-100 insoluble in all three L-cell lines. Its distribution was unaffected by the presence or absence of the GAG's. These results show that CD44 is not Triton X-100 insoluble as a result of possible associations with GAG's. The lacy pattern displayed by CD44 was not a result of the presence of the glycosaminoglycans.

It seems that CD44 does not associate strongly with molecules on the outside of the cell that would effect CD44's distribution. In order to determine what associations might be responsible for the distribution of CD44 in the membrane, the distribution of
CD44 was compared to the distribution of other membrane associated molecules with known interactions.

Assessment of CD44 Associations with a GPI linked protein, Thy-1, or a Protein with no Known Cytoskeletal Associations in NIH 3T3 cells;

Solubilization of NIH 3T3 cells with Triton X-100 resulted in a distinct lacy distribution of CD44 not mimicked by Thy -1 or MHC Class I as assessed by immunofluorescent staining. A different non ionic detergent, β-octyl glucoside, solubilizes proteins from membranes that resist Triton X-100 solubilization (Brown and Rose 1992). This detergent solubilized CD44 and Thy-1 quite effectively but did not disrupt filamentous actin. β-octyl glucoside replaces phospholipid/protein interactions within the membrane as well as providing a hydrophilic surface for charged residues from adjacent proteins (Roth et al. 1991). Since β-octyl glucoside resembles phospholipid and glycolipids in that all three structures have a hydrophobic carbon chain and a polar head group, it is not surprising that β-octyl glucoside can displace lipids and effectively solubilize membrane proteins with strong lipid associations. Associations disrupted by β-octyl glucoside include GPI linked proteins localized to detergent resistant membrane vesicles. This suggests that this detergent can completely disperse proteins localized to membrane lipid microdomains (Brown 1993). The GPI linked protein Thy-1 is solubilized by this detergent but not by Triton X-100. This provides further support for the ability of this detergent to solubilize Triton X-100 resistant proteins that reside in lipid vesicles (Thomas and Samelson 1992). Placental alkaline phosphatase, another GPI linked protein, was shown to be solubilized by β-octyl glucoside but not Triton X-100 (Brown and Rose 1992). CD44 is also efficiently solubilized by β-octyl glucoside suggesting that CD44 may also reside in lipid microdomains or be retained in the Triton X-100 insoluble fraction by hydrophobic interactions. This would also explain CD44's resistance to Triton X-100 solubilization and could perhaps regulate CD44's ability to bind HA. The role of CD44 within these
microdomains is not clear. It is interesting that CD44 is largely Triton X-100 soluble in the lymphoid cells but insoluble in fibroblast cells. CD44 localized within the lipid microdomains of fibroblast cells may be more Triton X-100 resistant than lipid domains residing within lymphoid cells. This difference could be attributed to the different cellular environments of the lipid domains, fibroblast versus lymphoid, or the different protein to lipid ratios between the different cell types (Brown 1993).

Conclusion

The results of this thesis show the inherent diversity of CD44/HA binding when expressed in different cell lines. It has been demonstrated that CD44/HA binding in the lymphoid cell lines is not regulated by cytoskeletal associations as assessed by Triton X-100 solubility. In one lymphoid cell line, it has been shown that newly synthesized proteins, possibly membrane bound or cytoplasmic proteins, may be involved in regulating CD44/HA binding. Perhaps these proteins interact with CD44 and induce a conformational change in the protein. This conformational change may result in CD44 now being able to bind HA. The involvement of regulatory proteins that increased HA binding in some cell lines is possible. Identification of these proteins is the next step and will provide further insight into the regulation of this adhesion molecule.

CD44 does not associate with actin, vimentin, or keratin in NIH 3T3 fibroblast cells. CD44 also does not directly associate with GAG's since the lack of GAG's does not affect CD44 distribution. CD44 may be regulated somehow by chondroitin sulphate since the lack of this GAG prevents antibody-induced activation of CD44 expressed by chondroitin sulphate deficient L-cells. NIH 3T3 cells may have lipid microdomains that regulate CD44 distribution and perhaps CD44 function.

It was confirmed that CD44 is the principle receptor for HA on both the lymphoid and fibroblast cell lines studied. It was shown that HA binding is regulated independently of the cytoskeleton in lymphoid cells, NIH 3T3 cells and L- fibroblast
It will be interesting to determine further the molecular associations of CD44, and to determine which of these associations regulate HA binding.
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