

Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning

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SUMMARY

CD44 is a multifunctional adhesion protein that acts as a major receptor for the hygroscopic extracellular matrix component, hyaluronan. This receptor-ligand binding directly mediates at least some of the cell-cell and cell-matrix interactions ascribed to CD44. Other interactions involving CD44 may be modulated indirectly by its ability to bind growth factors and thereby to promote cell attachment. During vertebrate development, multiple cases of hyaluronan involvement in cell proliferation, cell migration and histogenesis have been documented. In addition, there is evidence suggesting a central role for cell surface glycoproteins and proteoglycans in mediating the action of polypeptide growth factors involved in tissue patterning. In view of this, we undertook to investigate expression of the CD44 protein during postimplantation mouse embryogenesis. Between 9.5 and 12.5 days of embryonic development, the predominant form of CD44 protein corresponds to the hyaluronan-binding CD44H form. However, species with a higher M_r were also detected, implying that CD44

isoforms generated by alternative splicing of *CD44* RNA are employed in normal development. Further, we used mouse embryos to perform whole-mount immunohistochemistry and examine the temporal and spatial distribution of this glycoprotein. CD44 is expressed at high levels in the heart, somites and condensing limb-bud mesenchyme at critical stages of morphogenesis. These sites correlate with regions where hyaluronan has been demonstrated to regulate morphogenetic events. Of novel interest, however, is the high expression of CD44 in regions that do not correlate with sites of known hyaluronan-mediated developmental events. These include instructive epithelia participating in epithelial-mesenchymal cell interactions such as the apical ectodermal ridge of the developing limb bud and the odontogenic placodes of the presumptive upper and lower jaws.

Key words: CD44, cell adhesion molecule, hyaluronan, mouse embryogenesis, instructive epithelium

INTRODUCTION

The hyaluronan receptor, CD44, is an integral plasma membrane glycoprotein credited with functions involving cell-cell and cell-matrix adhesion. Physiological processes in which this protein has been implicated include lymphocyte homing to Peyer's patches and the high endothelial venules of peripheral lymph nodes, T cell activation, cell migration, metastatic spread and wound healing (rev. Haynes et al., 1989, 1991; Underhill, 1992). Originally identified in NIH 3T3 fibroblasts (Hughes and August, 1981), CD44 was subsequently shown to be expressed by a range of haematopoietic cells (Trowbridge et al., 1982), by adult tissues including the lung, brain, smooth muscle, connective tissue and glia (Flanagan et al., 1989; Picker et al., 1989),

and by proliferating epithelial cells (Alho and Underhill, 1989). The predominant CD44 form in cells of the haematopoietic system has an approximate M_r of $80-90 \times 10^3$ (Trowbridge et al., 1982) and has accordingly been denoted the haematopoietic form, CD44H. CD44H is comprised of a protein core of $37 \times 10^3 M_r$ (Stamenkovic et al., 1989), the extracellular domain of which is extensively modified by *N*- and *O*-linked glycosylation. Sequence analysis of *CD44* cDNAs from different species (Goldstein et al., 1989; Idzerda et al., 1989; Nottenburg et al., 1989; Stamenkovic et al., 1989; Zhou et al., 1989; Aruffo et al., 1990; Wolffe et al., 1990; Bosworth et al., 1991; Gunthert et al., 1991; Tavernor et al., 1993) demonstrates a homology in the extracellular amino terminus with cartilage link and proteoglycan core proteins and a highly conserved cytoplasmic

domain which is phosphorylated on serine residues (Isacke et al., 1986; Carter and Wayner, 1988; Neame and Isacke, 1992).

The wide diversity of CD44 functions may in part reflect its ability to bind the hygroscopic glycosaminoglycan, hyaluronan (Aruffo et al., 1990; Culty et al., 1990; Lesley et al., 1990; Miyake et al., 1990). Hyaluronan is synthesized at the cell surface as an unbranched high molecular weight polysaccharide composed of several thousand repeated *N*-acetyl-D-glucosamine and D-glucuronic acid disaccharide units. It is an abundant component of many extracellular matrices and interstitial spaces (Underhill, 1989), where it can support cell attachment (Aruffo et al., 1990; Miyake et al., 1990), cell migration (Turley, 1989) and cell proliferation (Alho and Underhill, 1989). It has long been recognised that there are high concentrations of hyaluronan in the early embryo (Morriss and Solursh, 1978; Poelmann et al., 1990;

van Straaten et al., 1990; Brown and Papaioannou, 1993) and multiple cases of its involvement in histogenesis during vertebrate development have been documented (rev. Toole, 1990, 1991). For example, in submandibular epithelia, the presence of hyaluronan in the basal lamina is required for the maintenance of salivary gland lobular morphology (Banerjee et al., 1977) while, in the inner ear, basal deposition of hyaluronan is required to propel semicircular canal formation (Haddon and Lewis, 1991). Hyaluronan can also generate cell-free spaces by its ability to expand when hydrated and thus prevent cells from aggregating and differentiating. An example of such a process is the migration of stromal cells into the developing cornea (Toole and Trelstad, 1971).

More recently, *CD44* cDNAs have been isolated from a number of different tissues that have revealed the presence of splice variants, where multiple combinations of 10 exons

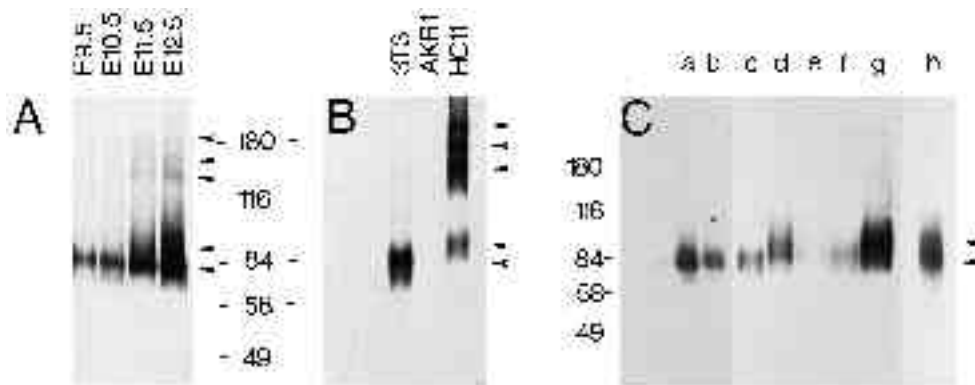


Fig. 1. Immunoblot analysis of CD44 protein expression in mouse embryos and cell lines. Protein extracts were prepared from (A) E9.5, E10.5, E11.5 and E12.5 mouse embryos, (B) 3T3, AKR1 and HC11 murine cell lines, (C) dissected embryonic parts as follows: a, E11.5 heart, b, E12.5 heart, c, E9.5 embryo minus heart, d, E11.5 embryo minus heart, e, E11.5 embryo minus heart, liver and limb buds, f, E12.5 head, g, E12.5 embryo minus head, h, E12.5 liver. 10 μ g

(panel B) or 20 μ g (panels A,C) of protein was loaded and subjected to immunoblot analysis using the IM7 anti-CD44 mAb followed by an HRP-conjugated anti-rat Ig. Blots were developed using the ECL system and exposed to X-ray film for 20 minutes (A), 5 minutes (B) and 60 minutes (C) except panel h, which was exposed for 1 minute. Arrowheads in each panel indicate CD44 forms of M_r 80, 100, 130, 160, 180 $\times 10^3$ (A); 80, 100, 130, 160, 200 $\times 10^3$ (B); 80, 100 $\times 10^3$ (C). Size markers are $10^3 M_r$.

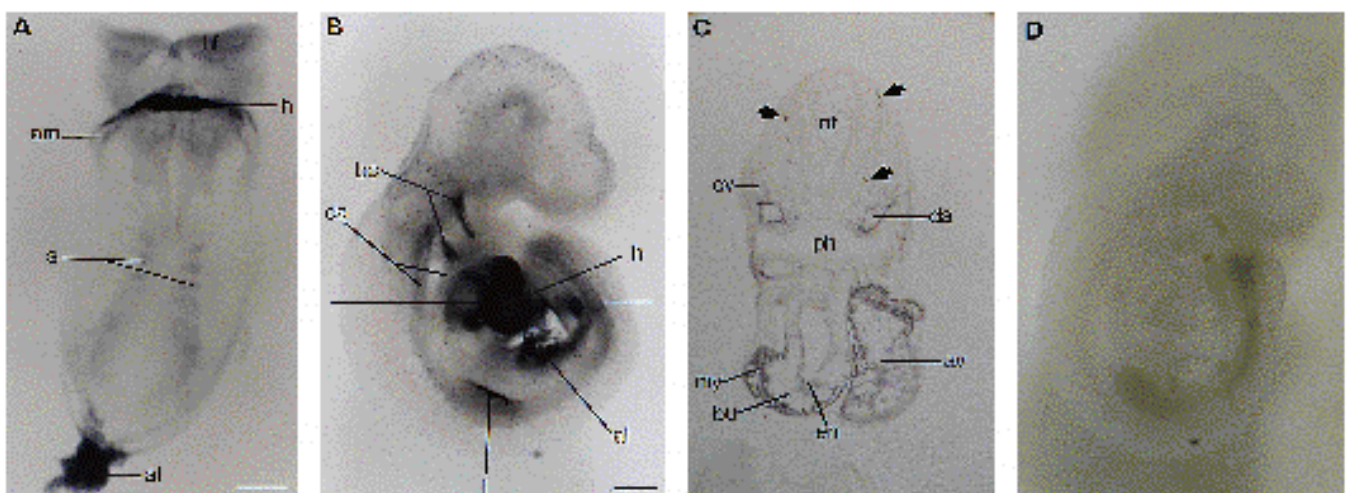


Fig. 2. Distribution of CD44 protein in mouse embryos using whole-mount immunohistochemistry. Whole-mount immunohistochemistry of (A) an E8.25 embryo and (B-D) E9.5 embryos; embryos were incubated with the IM7 anti-CD44 mAb (A,B) or no mAb (D), followed by an HRP-conjugated anti-rat Ig as described in Materials and Methods. The solid line on panel B indicates the transverse section seen in panel C. (C) A transverse wax section through the heart as shown by the line in panel B. Solid arrowheads indicate dispersed CD44-positive cells. al, allantois; am, amnion; av, atrio-ventricular canal; bc, branchial cleft; bu, bulbus cordis; cv, cardinal vein; da, dorsal aorta; en, endocardium; h, heart; hf, head fold; l, forelimb bud; my, myocardium; nt, neural tube; ph, pharynx; s, somite. Scale bar, 200 μ m.

may be inserted into the region encoding the extracellular membrane proximal domain (Brown et al., 1991; Dougherty et al., 1991; Gunthert et al., 1991; Stamenkovic et al., 1991; Cooper et al., 1992; He et al., 1992; Jackson et al., 1992; Screaton et al., 1992; Tolg et al., 1993). In some cases, altered functions have been assigned to such novel isoforms. For example, a rat CD44 variant, pMeta-1, confers metastatic potential to non-metastasising pancreatic tumour cells (Gunthert et al., 1991). By contrast, a different isoform found in epithelial cells, CD44E, can abrogate the hyaluronan-mediated binding of B cells to high endothelial venules (Stamenkovic et al., 1991), but has no effect on tumour formation and metastasis (Sy et al., 1991).

Although the importance of the CD44-hyaluronan interaction has been demonstrated in a number of *in vitro* model systems (Aruffo et al., 1990; Miyake et al., 1990), it is still not known if during development (a) the plethora of events associated with the regulation of hyaluronan production are mediated by CD44, (b) CD44 can function other than as a hyaluronan receptor, or (c) variants generated as a result of alternative RNA splicing, some of which are reported to have a reduced hyaluronan-binding capacity, are employed.

For these reasons, we undertook to investigate whether multiple CD44 forms are expressed during postimplantation mouse embryogenesis and to examine the temporal and spatial distribution of the CD44 protein.

MATERIALS AND METHODS

Immunoblotting

Cultured cells were lysed in sample buffer (Laemmli, 1970) without β -mercaptoethanol or bromophenol blue, sonicated for 10 seconds and denatured by heating to 95°C for 5 minutes. Whole embryos or dissected embryonic parts were removed from TO strain mice, rinsed once in PBS and then homogenized in 1 ml of 5 mM HEPES pH 7.4, 2 mM MgCl₂ (lysis buffer) with 10 strokes in a Dounce homogeniser. Nuclei and large cellular debris were removed by centrifugation for 5 minutes at 6,300 *g* and the membranes were collected by centrifugation for 5 minutes at 13,000 *g* in an Eppendorf microfuge. The membrane pellet was resuspended in sample buffer without β -mercaptoethanol and bromophenol blue, sonicated and denatured as described above. The protein content of each sample was assayed using a Pierce BCA kit, bromophenol blue was added and 10 or 20 μ g of each sample

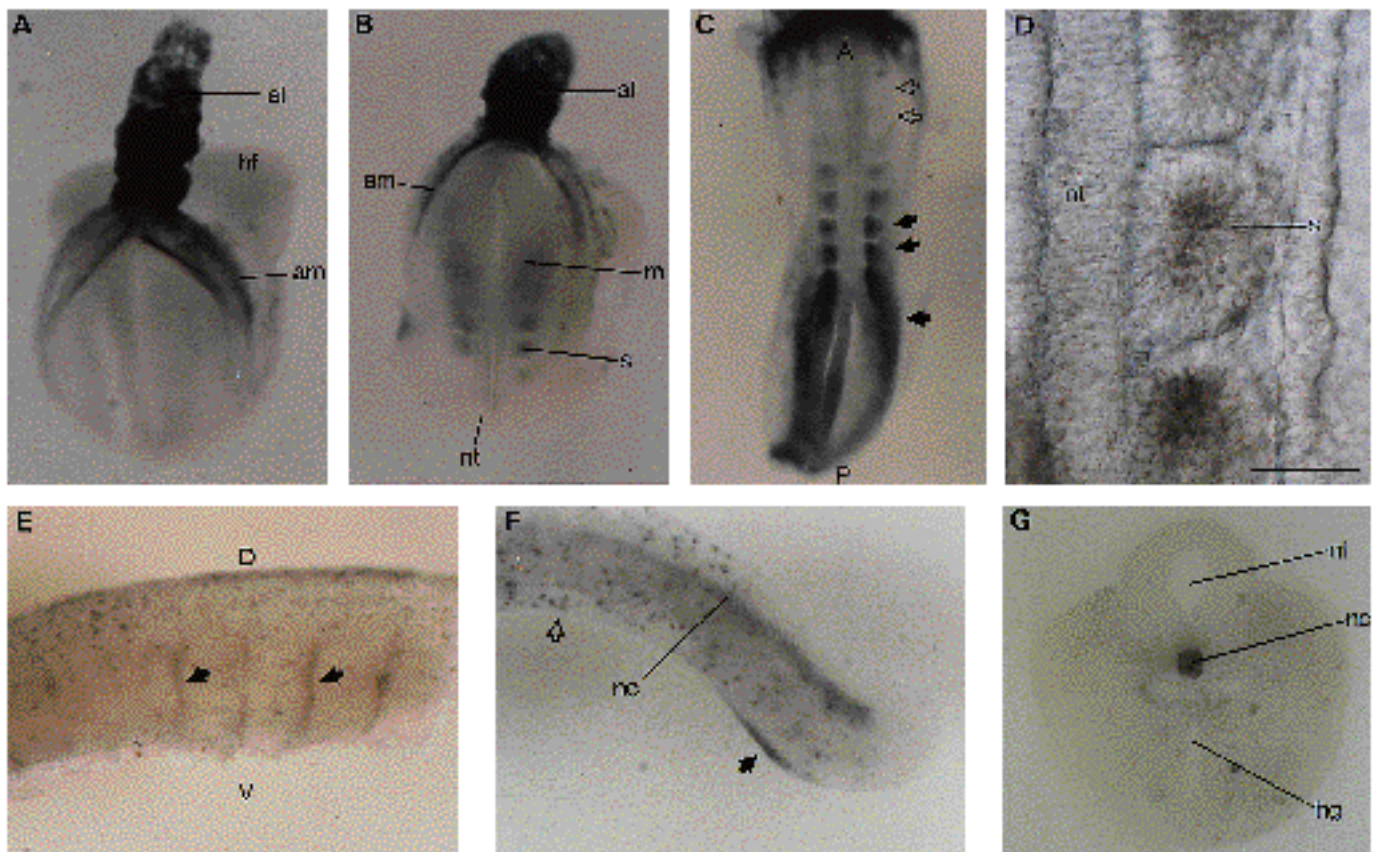


Fig. 3. Distribution of CD44 protein in the somites and notochord. CD44 protein was detected in whole-mount E8.25 to E10.5 embryos using mAb IM7. (A) E8.0 embryo viewed from posterior ventral surface. (B) E8.25 embryo viewed from posterior ventral surface. (C) E8.5 embryo viewed from the dorsal surface. Open arrowheads indicate lack of CD44 expression in matured somites; solid arrowheads indicate CD44 protein in newly formed somites and in the presomitic mesoderm. (D) Somitic region from an E8.75 embryo viewed with Nomarski optics. (E) Lateral view of the trunk region between the forelimb and hindlimb buds of an E10.5 embryo. Solid arrowheads indicate CD44-positive intersegmental arteries. (F) E10.5 embryo tail bud. Solid arrowhead indicates a patch of ventral ectodermal cells expressing CD44 protein. Open arrowhead indicates position of the transverse slice shown in panel G. (G) Transverse slice of an E10.5 embryo tailbud from the position indicated in panel E. al, allantois; am, amnion; hf, head fold; hg, hindgut diverticulum; m, presomitic mesoderm; nc, notochord; nt, neural tube; s, somite; A, anterior; D, dorsal; P, posterior; V, ventral. Scale bar, 50 μ m.

was resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto Hybond-C extra nitrocellulose membrane (Amersham). Nitrocellulose membranes were blocked with PBS containing 5% low-fat milk powder and 0.2% Tween 20 for 20 minutes. To detect the CD44 protein, blots were incubated with a rat anti-CD44 monoclonal antibody (mAb), IM7.8.1 (Trowbridge et al., 1982), at a 1:50 dilution of the hybridoma supernatant in fresh blocking solution for 45 minutes at room temperature. The blots were washed for 30 minutes with TBST (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.05% Tween-20), incubated for 45 minutes with 0.1 µg/ml horseradish peroxidase (HRP)-conjugated anti-rat Ig (Jackson ImmunoResearch) and washed with TBST for 30 minutes. HRP anti-rat Ig was detected using an enhanced chemiluminescence (ECL) system (Amersham Life Sciences) with exposure to X-ray film (Fuji XR) as indicated in the figure legend.

Chondroitin ABC lyase digestion

20 µg membranes from E9.5-E11.5 embryos were resuspended in a buffer containing 40 mM Tris-HCl, pH 8.0, 40 mM sodium acetate, pH 7.0 and 0.01% BSA in a total volume of 100 µl. 10 mU chondroitin ABC lyase (ICN Flow) in 20 µl 20 mM Tris-HCl (pH 8.0)/0.1% BSA were added and the membranes were incubated at 37°C for 1 hour. In parallel samples, chondroitin ABC lyase was omitted. Membranes were collected by centrifugation for 5 minutes at 13,000 g in an Eppendorf microfuge, resuspended in sample buffer and subjected to immunoblotting using the mAb IM7. Confirmation was made that the enzyme remained active in the presence of membrane preparations using the manufacturer's recommended assay.

Whole-mount immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (Agar Scientific), washed in PBS, dehydrated by incubation for 5 minutes each in 25%, 50%, 75% methanol in PBS and 100% methanol and then stored at -20°C until required. Embryos were bleached in 6% hydrogen peroxide in methanol for 1 hour at room temperature, rehydrated through the reverse series of methanol dilutions in PBS and blocked by incubation in PBMT (PBS plus 2% skimmed milk powder and 0.1% Triton X-100) twice for 1 hour each. Embryos were then incubated with PBMT containing 10% foetal calf serum and a 1:50 dilution of mAb IM7 hybridoma supernatant overnight at 4°C followed by five 1 hour washes in PBMT. The primary antibody was detected by incubation in PBMT containing 1.6 µg/ml HRP-conjugated anti-rat Ig overnight at 4°C followed by washing as described above. For the enzyme reaction, the embryos were washed for 20 minutes in PBT (PBS plus 0.1% Triton X-100), incubated in PBT containing 0.08% NiCl₂ and 250 µg/ml diaminobenzidine (Sigma) for 10 minutes and finally hydrogen peroxide was added to 0.03%. When the desired colour was reached, the reaction was stopped by rinsing the embryos 3-4 times in PBT. Embryos were viewed with incident light using a Nikon SMZ-U dissecting microscope or with Nomarski optics using a Leitz Aristoplan microscope.

Where indicated, stained embryos were prepared for embedding by dehydration for 5 minutes each in 25%, 50%, 60%, 75%, 85% and 95% methanol in PBT. Embryos were rotated in a 1:1 mix of 95% methanol and infiltration solution (Historesin Embedding Kit, Reichert-Jung) for 1 hour, in infiltration solution for 3 hours and embedded. Alternatively, stained embryos were dehydrated to 100% methanol, cleared in chloroform overnight and embedded in paraffin wax. 10 µm (plastic) or 8 µm (wax) sections were cut, mounted in DPX mountant (BDH) and viewed with phase-contrast and bright-field optics using a Nikon Microphot-FX microscope.

Immunocytochemistry of sectioned embryos

Unstained embryos were embedded in paraffin wax after dehydra-

tion through graded methanols to 100%. 10 µm sections were cut and subjected to immunohistochemistry using mAb IM7 or mAb F4/80 (Austyn and Gordon, 1981). Primary antibody was detected using a Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturers instructions.

Neural tube explant culture

Anterior neural tubes from E9.0 embryos, with the forebrain and spinal cord below the hindbrain cut away, were removed by pancreatin/trypsin digestion followed by manual microdissection. Explants were cultured on fibronectin-coated plastic tissue culture dishes in a 1:1 mixture of DME and Ham's F12 medium supplemented with 1% FCS, 1 µg/ml insulin, 5 µg/ml transferrin and 10 ng/ml TGF- β , cultured for 14 hours at 37°C, fixed and subjected to an immunohistochemical analysis with mAb IM7 as described above.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described using digoxigenin-labelled riboprobes (Conlon and Rossant, 1992). *CD44* cDNA (Nottenburg et al., 1989) was linearised with *Nae*I and transcribed with T3 RNA polymerase and *tek* cDNA (Dumont et al., 1992) was linearised with *Kpn*I and transcribed with T7 RNA polymerase.

RESULTS

Immunoblot analysis of CD44 protein expression in mouse embryos and cell lines

Immunoblot analysis of E9.5-E12.5 embryo samples using the IM7 anti-CD44 mAb demonstrates that the predominant CD44 species expressed has an approximate M_r of 80×10^3 (Fig. 1A) and co-migrates with the CD44H form. The relative abundance of this $80 \times 10^3 M_r$ species increases with the age of the embryo. In E11.5 and E12.5 embryos, a CD44 form of approximately $100 \times 10^3 M_r$ is detected and, in addition, longer exposure of the immunoblots reveals the presence of low abundance forms of approximately 130, 160 and $180 \times 10^3 M_r$ (Fig. 1A). Parallel analysis of three murine cell lines demonstrates that Swiss 3T3 fibroblasts express only the $80 \times 10^3 M_r$ CD44H species as has been previously documented (Hughes and August, 1981), and, as expected, no protein is detected in the CD44-negative T lymphoma cell line, AKR1 (Trowbridge et al., 1982). By contrast, in a mammary epithelial cell line, HC11 (Ball et al., 1988), abundant CD44 species with approximate M_r s of 100, 130, 160 and 200×10^3 are observed (Fig. 1B).

To discern whether the appearance of higher M_r CD44 forms is associated with the development of distinct embryonic structures, embryos were dissected and subjected to immunoblot analysis (Fig. 1C). Embryonic hearts dissected from E9.5-E12.5 embryos predominantly express the $80 \times 10^3 M_r$ CD44 species (lanes a,b; data not shown). In the remainder of E9.5 embryos (lane c) and the remainder of the E10.5 embryos (data not shown), only the $80 \times 10^3 M_r$ species is detected. At E11.5, the pattern of CD44 expression in the forelimb and hindlimb buds (data not shown), liver and the rest of the embryo (lanes d,e) is similar in that all samples express both the 80 and $100 \times 10^3 M_r$ forms. After E11.5, the liver accounts for a large proportion of the increased levels of CD44 expression observed at these ages (Fig. 1A; lanes d-g). In these crude dissections, it is not

clear whether the less abundant 130, 160 and $180 \times 10^3 M_r$ CD44 forms (Fig. 1A) are localized to discrete regions of the embryo.

To assess whether any of the CD44 forms detected in the embryo have chondroitin sulphate additions, we carried out chondroitin ABC lyase digestion of embryo samples from E9.5-E11.5 and analysed the digestion products by immunoblotting. No alteration in M_r was observed in any of the samples studied (data not shown).

Detection of CD44 protein in mouse embryos using whole-mount immunohistochemistry

To detect those regions that express CD44 in the postimplantation mouse embryo, whole-mount immunohistochemistry was performed using the IM7 anti-CD44 mAb. At E8.25, the amnion, allantois, heart and developing somites express high levels of CD44 (Fig. 2A). By E9.5 these tissues continue to express CD44 but, in addition, strong staining is also observed in the developing limb-bud ectoderm, the branchial clefts and in a population of cells dispersed throughout the embryo (Fig. 2B). No CD44-specific staining is detected in parallel control experiments when the primary antibody is omitted (Fig. 2D). A DIG-labelled antisense *CD44* riboprobe was used to detect *CD44* transcripts in E8.5-E10.5 embryos and it was found that the pattern of *CD44* mRNA expression correlates well with that of the CD44 protein (data not shown).

CD44 protein expression in the developing cardiovascular system

From the earliest observable stage of heart development, CD44 is expressed by presumptive cardiogenic cells located above the primitive foregut invagination. By E8.25, high levels of CD44 are expressed in the fused endothelial tubes and the surrounding muscular epimyocardium (Fig. 2A) that constitute the early embryonic heart. At E9.5, the developing myocardium expresses CD44 abundantly and a low level of expression is detected in the endocardium (Fig. 2C). From E10.5, the heart continues to express CD44 (Figs 1C, 6A) with the endocardial cushions exhibiting very weak immunostaining for CD44 (data not shown). In addition to the heart, the arterial system displays high CD44 protein expression in the E9.5-E12.5 embryos examined. For example, CD44 protein is detected in the endothelial angioblasts lining the wall of the dorsal aortae (Figs 2C, 5A), in the intersomitic arteries (Fig. 3E), in the branchial arch arteries (Figs 5I, 6C) and in the umbilical artery (data not shown). Some blood cells present in the branchial arch arteries (Fig. 5I) also express CD44. By contrast, no CD44 protein is detected in the venous system at any stage of development studied (Fig. 2C).

CD44 protein expression in the somites and notochord

Axis development is associated with the formation of mesodermal segments, the somites, which give rise to components of the axial skeleton, musculature and skin. At E8.0 (1-2 somites), no CD44 is detected in the somitic region (Fig. 3A). By E8.25 (5-6 somites), CD44 is observed in the presomitic mesoderm and in the most recently formed somites (Fig. 3B). At E8.5, (7-8 somites), a gradient of

CD44 expression has been established. The highest expression levels are seen in the presomitic mesoderm and in the most posterior somites, with the levels of expression diminishing towards the anterior of the embryo and the most mature somites no longer expressing CD44 (Fig. 3C). During maturation, the protein associates with the sclerotome in the central regions of the positive somites and the outer somitic regions are CD44-negative (Fig. 3D). By E9.5, the intersomitic arteries are clearly CD44-positive (Fig. 3E).

The notochord is a mesodermal structure located in the midline. Initially the notochord is derived from the anterior region of the primitive streak but, from E9.5 onwards, it is derived from the tailbud. Prior to tailbud formation, no CD44 is detected in the notochord but, at E10.5, the notochord in the tail expresses high levels of CD44 protein (Fig. 3F,G). In addition to CD44 expression in the notochord, a patch of CD44-positive cells is also present on the ventral aspect of the tailbud (Fig. 3F). Previously, this tissue has been defined as the ventral ectodermal ridge (VER), lying at approximately 250 μm from the tip of the tail (Gruneberg, 1956).

CD44 protein expression during limb development

Limbs develop from the Wolffian ridges, strips of lateral plate mesoderm extending the length of the embryo. Localized regions of mesoderm adjacent to somites 7-13 and somites 27-31 thicken, heralding the formation of the forelimb and hindlimb buds, respectively. CD44 is initially present in the ectoderm surrounding the limb-bud mesenchyme from the earliest stage of limb-bud outgrowth. At stage 1-2 (Wanek et al., 1989), CD44 protein is localized to the ectoderm on the ventral side of the limb bud (Fig. 4A). CD44 expression is not detected on the dorsal side (Fig. 4B). At stage 2-3, CD44 is expressed in the apical ectodermal ridge (AER), which forms as an ectodermal thickening at the apex of the limb bud (Fig. 4C). From stage 3 to the latest studied (stage 8), this expression in the AER is maintained (Fig. 4D,E,F) but, by stage 8, CD44 is also expressed in the condensing limb-bud mesenchyme prior to the cartilage-generating chondrogenic process (Fig. 4F).

CD44 protein expression in the branchial region

The face and throat form largely as a result of the development of the branchial arches. CD44 protein first appears in this region at E8.5 as a patch in the endoderm of the developing first pharyngeal pouch adjacent to the developing first branchial arch (data not shown). By E9.5, CD44 is expressed between the first and second and between the second and third branchial arches (Fig. 5A). There is no CD44 in the branchial arch mesenchyme. Sectioning of embryos demonstrates that, at E9.5, the CD44 observed in the first branchial cleft is localized to the endoderm of the branchial membrane, with expression extending partially into the ectoderm of the second branchial arch (Fig. 5D,E). Later in development, the domain of CD44 expression is expanded such that at E10.5 the protein is localized to the endoderm surrounding all the pharyngeal pouches and in the ectoderm around the posterior side of the second branchial arch and surrounding the third and fourth branchial arches (Fig. 5F-I). CD44 is also observed in the cells lining the branchial arch arteries (Fig. 5I).

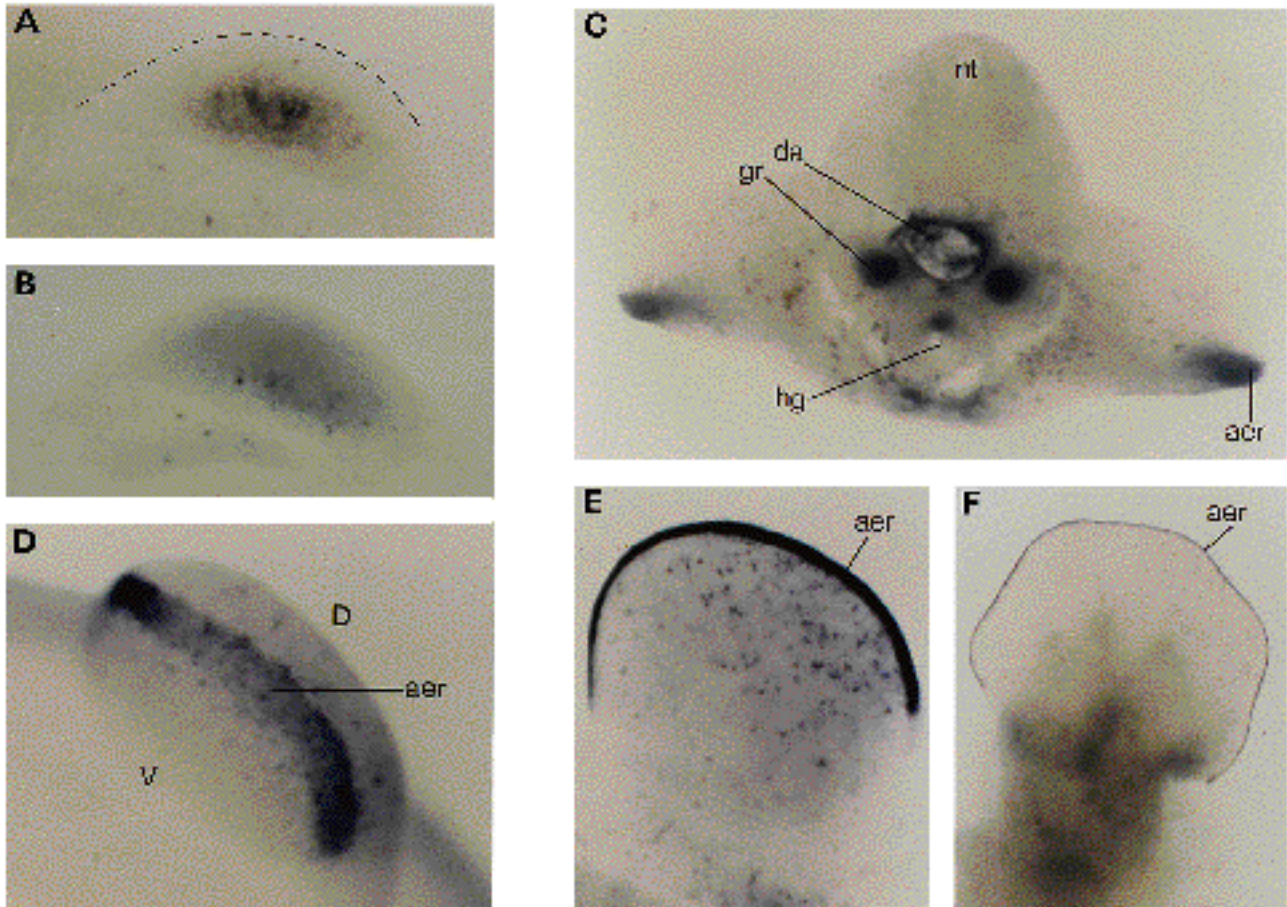


Fig. 4. Distribution of CD44 protein during limb development. CD44 protein was detected in whole-mount E9.5 to E12.5 embryos using mAb IM7. (A) Ventral surface of a forelimb bud of an E9.5 embryo (limb bud stage 1-2), where the dotted line indicates the distal boundary of the limb bud. (B) Dorsal surface of a forelimb bud of an E9.5 embryo (limb bud stage 1-2). (C) Transverse slice cut through the hindlimb region of an E10.5 embryo (limb bud stage 2-3) viewed from the anterior side. (D) Hindlimb bud (limb bud stage 2-3) of an E10.5 embryo viewed from the apex of the AER. (E) Forelimb bud (limb bud stage 4) of an E10.5 embryo viewed from the ventral side. (F) Hindlimb bud (limb bud at stage 8) of an E12.5 embryo viewed from the dorsal side. aer, apical ectodermal ridge; da, dorsal aorta; gr, genital ridge; hg, hindgut; nt, neural tube; D, dorsal; V, ventral.

CD44 protein expression in developing craniofacial tissue

At E10.5-10.75, CD44 is expressed in the ectoderm of the nasolacrimal groove (Fig. 6A) and posterolateral rim of the developing nasal pit (Fig. 6C). CD44 expression is also observed in the odontogenic placodes. These are regions of the opposing ectodermal epithelium overlying the maxillary and mandibular prominences of the first branchial arch (Fig. 6D).

CD44 protein expression in a dispersed cell population

From E9.0-E9.5 onwards, a large number of CD44-positive cells arise in the mesenchyme of the embryo, appearing as a scattered population first in the head region and then throughout the embryo including the limb buds and tail (Figs 2-6). Some of these cells have a stellate morphology and many have cytoplasmic CD44 (Fig. 5F-I).

In transverse section, a proportion of these cells can be seen in pathways suggestive of migratory neural crest (Fig.

2C). However, in cultured anterior neural tube explants from E9.0 embryos, no intensely immunostained CD44-positive dispersed cells were detected (data not shown). CD44-positive cells are also present in the liver (Fig. 7C) and visceral yolk sac (Fig. 7D,E), which suggests that the CD44-positive scattered cells may be derived from the primitive haematopoietic system. A comparative immunohistochemical analysis of E10.5 embryo sections was performed using mAb IM7 and mAb F4/80 which detects an epitope on macrophages (Austyn and Gordon, 1981). This analysis demonstrated that only a small number of F4/80-positive dispersed cells were present in the regions populated by relatively abundant CD44-positive cells (data not shown). To address whether CD44-positive cells present in the roofplate of the hindbrain and subectodermally in the region of the forebrain (Fig. 7A,B) could represent a population of angioblasts, comparative whole-mount in situ hybridization using *CD44* and *tunica interna endothelial cell kinase (tek)* riboprobes was performed. This analysis demonstrated similar distributions of the two mRNAs in arterial walls

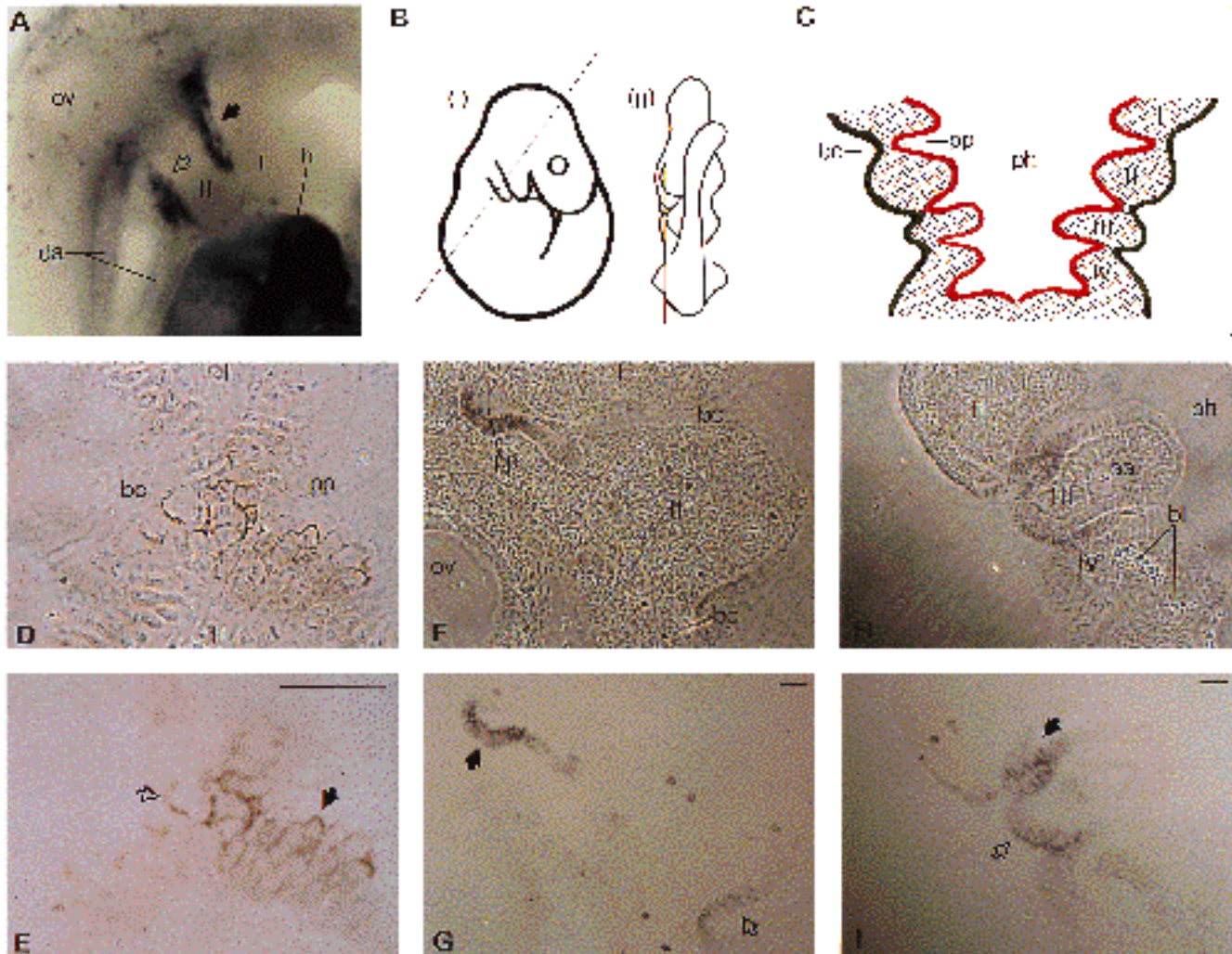


Fig. 5. Distribution of CD44 protein in the branchial apparatus. CD44 protein was detected in whole-mount E9.5 and E10.5 embryos using mAb IM7. (A) E9.5 embryo, neck region. Solid arrowhead indicates CD44 expression in the first branchial cleft, open arrowhead indicates CD44 expression in the second branchial cleft. (B,i) schematic diagram (taken from Kaufman, 1992) to show the plane of section for plates D, E, H and I. (B,ii) Schematic diagram to show the plane of section for plates F and G. (C) Schematic diagram of a coronal section through the branchial region of an E10.5 embryo showing the arrangement of the three tissue layers where ectoderm is indicated by a solid black line, mesenchyme is indicated by stippled shading and endoderm is indicated by a solid red line. Branchial arches are numbered from anterior to posterior direction using Roman numerals. Stained E9.5 embryos (D,E) and E10.5 embryos (F-I) were embedded in plastic and sectioned as described in the Materials and Methods. (D) Phase-contrast photomicrograph of a coronal section through the right first branchial cleft and pharyngeal pouch, as indicated in panel Bi. (E) Bright-field photomicrograph of same region as shown in panel D. Solid arrowhead indicates strong expression in the endoderm of the first pharyngeal pouch. Open arrowhead indicates weak CD44 expression in the ectoderm of the second branchial arch. (F) Phase-contrast photomicrograph of a sagittal section through the branchial region of an E10.5 embryo, as indicated in panel Bii. (G) Bright-field photomicrograph of the same region as shown in panel F. Solid arrowhead indicates CD44 expression in the endoderm of the first pharyngeal pouch. Open arrowhead indicates CD44 expression in the ectoderm surrounding the posterior part of the second branchial arch. (H) Phase-contrast photomicrograph of a coronal section through the second, third and fourth branchial arches of an E10.5 embryo, as indicated in panel Bi. (I) Bright-field photomicrograph of same region as shown in panel H. Solid arrowhead indicates CD44 expression in the endoderm of the second pharyngeal pouch. Open arrowhead indicates CD44 expression in the ectoderm surrounding the third branchial arch. aa, branchial arch artery; bc, branchial cleft; bl, blood cells; da, dorsal aorta; h, heart; ov, otic vesicle; ph, pharynx; pp, pharyngeal pouch. Scale bar, 50 μ m.

(data not shown), but failed to detect single dispersed cells expressing *tek* mRNA.

DISCUSSION

CD44 is now widely recognised as a principal receptor for

hyaluronan, although the converging lines of study that led to this conclusion have also elucidated a variety of other functions in which CD44 participates. Further, it has recently become apparent that different isoforms may be generated from the *CD44* gene by alternative RNA splicing and some of these forms can have altered binding affinities for hyaluronan. Distinct CD44 forms may be diagnostic of

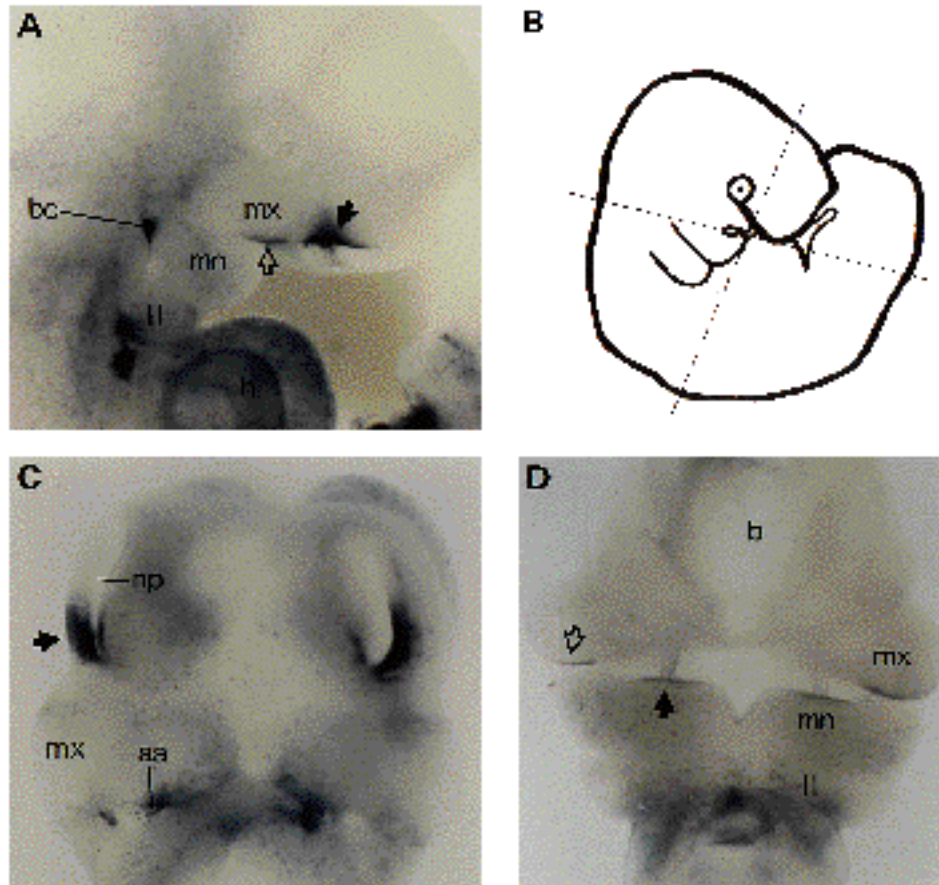


Fig. 6. Distribution of CD44 protein in craniofacial tissue. CD44 protein was detected in whole-mount E10.5 and E10.75 embryos using mAb IM7. (A) E10.75 embryo, lateral view of head and upper trunk. Solid arrowhead indicates CD44 expression in the ectoderm of the nasolacrimal groove, open arrowhead indicates CD44 expression in the upper jaw odontogenic placode. (B) Schematic diagram (taken from Kaufman, 1992) of an E10.5 embryo indicating the planes in which the embryos were cut to generate the views shown in panels C (solid line) and D (dotted line). (C) E10.5 embryo; a transverse cut was made along the solid line shown in panel B between the maxillary and mandibular components of the first branchial arch, the portion comprising the forebrain and midbrain was viewed from the posterior side. Solid arrowhead shows CD44 staining in the ectoderm surrounding the posterolateral rim of the nasal pit. (D) E10.5 embryo; a cut was made along the dotted line shown in panel B to remove the forebrain and the branchial region was viewed

from the front. Staining in the odontogenic placodes of the intact mandibular (solid arrowhead) and maxillary (open arrowhead) components of the first branchial arch is indicated. The second branchial arch is indicated by Roman numerals. aa, branchial arch artery; b, brain cavity; bc, branchial cleft; h, heart; mn, mandibular component of the first branchial arch; mx, maxillary component of the first branchial arch; np, nasal pit.

specialized cell types in the developing embryo, and may be switched on to participate in the normal functioning of specific tissues in the embryonic or adult animal. Arch et al. (1992) have shown, for example, that a metastatic splice variant of CD44 is expressed by lymphocytes when they are challenged with antigen.

Using a mAb that recognises all of the forms of CD44, we have demonstrated that the predominant CD44 form expressed in E9.5-E12.5 mouse embryos has a M_r of approximately 80×10^3 , which corresponds to the hyaluronan-binding CD44H species (Fig. 1). The abundance of this CD44H form increases with the age of the embryo. Higher M_r CD44 species are observed in E11.5 and E12.5 embryos. From others' work, it has been established that some of the high M_r CD44 forms are modified by chondroitin and/or heparan sulphate additions but such additions are not apparent on the 80×10^3 M_r CD44H form (Jalkanen et al., 1988; Brown et al., 1991; Camp et al., 1991; Lokeshwar and Bourguignon, 1991). It has also been demonstrated that increases in the M_r correlate with the presence of splice variants, the extra M_r not being solely due to glycosaminoglycan modification of the CD44H form (Stamenkovic et al., 1991; Gunthert et al., 1991; Hofmann et al., 1991; He et al., 1992). Our immunoblot analysis of chondroitin ABC lyase-

digested E9.5-E11.5 samples showed no detectable M_r change in any CD44 form (data not shown). We postulate that the higher M_r species in the E11.5 and E12.5 embryos are generated as a result of alternative splicing and not large chondroitin sulphate additions. However, they may contain low or heterogeneous levels of chondroitin sulphate or other glycosaminoglycans.

Correlation of CD44 expression with hyaluronan-mediated developmental events

The response of a cell to a hyaluronan-rich extracellular matrix depends on both the unique hydration properties of hyaluronan (rev. Comper and Laurent, 1978) and its association with hyaluronan-binding proteins (rev. Toole, 1990). Such matrices support cell proliferation, cell migration and changes in cell shape, processes pivotal to embryogenesis (rev. Toole, 1990, 1991). The co-distribution of CD44 and hyaluronan has been observed in some adult tissues such as the crypts of Lieberkuhn and the dermal skin layer (Alho and Underhill, 1989), suggesting that such an interaction may be important at least in regions that undergo active cell proliferation. Our data demonstrate that a proportion of those sites where CD44 is expressed, the heart, somites and

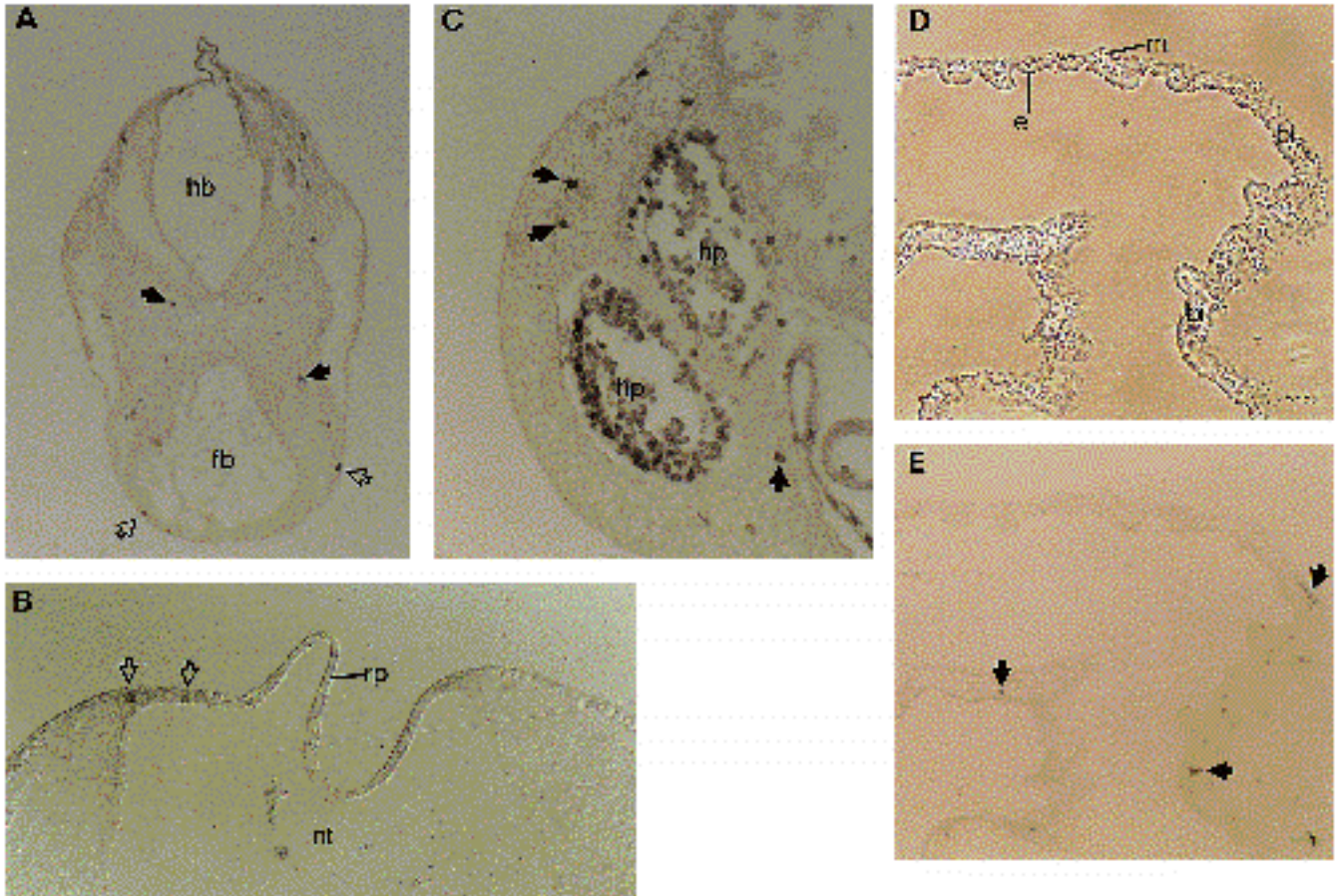


Fig. 7. Distribution of CD44 protein in a dispersed cell population. Embryos were either immunostained, embedded in wax and sectioned (A,C) or embedded in wax, sectioned and immunostained (B,D,E). (A) Transverse section through the head of an E9.5 embryo. Solid arrowheads indicate CD44-positive dispersed cells deep within the mesenchyme. Open arrowheads indicate CD44-positive dispersed cells subectodermally. (B) Transverse section through the hindbrain of an E10.5 embryo showing the roofplate region at high magnification. Open arrowheads indicate CD44-positive cells present in the roofplate of the hindbrain. (C) Sagittal section through the foetal liver at E10.5 which contains a large number of CD44-positive cells. Solid arrowheads indicate CD44-positive dispersed cells deep within the mesenchyme. (D) Phase-contrast photomicrograph of a section through the visceral yolk sac of an E10.5 embryo, (E) Bright-field photomicrograph of same region as shown in panel D. Solid arrowheads indicate CD44-positive cells within the blood islands. bi, blood island; e, endoderm; fb, forebrain; hb, hindbrain; hp, hepatic primordium; m, mesoderm; nt, neural tube; rp, hindbrain roofplate.

limb-bud mesenchyme (Figs 2, 3, 4), overlap with sites of hyaluronan-mediated morphogenesis.

The endocardial and myocardial layers of the heart are separated by cardiac jelly, a substance rich in glycosaminoglycans including hyaluronan (Gessner et al., 1965). At different stages of heart development, hyaluronan secretion and hyaluronidase activity are differentially regulated (Orkin and Toole, 1978). The data presented here demonstrates that the heart predominantly expresses the $80 \times 10^3 M_r$ CD44 form at all developmental stages studied, the form extensively characterized as a principal hyaluronan receptor (Aruffo et al., 1990; Culty et al., 1990; Lesley et al., 1990; Miyake et al., 1990; Stamenkovic et al., 1991;). Hyaluronan is also present in the developing somites, notochord and neural tube (Kvist and Finnegan, 1970a; Copp and Bernfield, 1988). During differentiation of the myotome and aggregation of the sclerotome, the abundance of hyaluronan increases (Kvist and Finnegan, 1970b) and the subsequent migration of the sclerotomal mass to the notochord is facilitated by the existence of a hyaluronan-rich matrix (Solursh

et al., 1979). The strict temporospatial regulation of CD44 expression at sites of hyaluronan-mediated morphogenesis strongly implies a role for this receptor-ligand interaction in regulating cell-matrix adhesion. In addition, there is evidence that CD44 can regulate levels of hyaluronan by a process of receptor-mediated internalization (Culty et al., 1992; Hall and Miyake, 1992). In this respect, it is interesting that in the developing limb-bud mesenchyme the expression of CD44 (Fig. 4) correlates both temporally and spatially with the removal of hyaluronan from the matrix during the condensation of prechondrogenic and premyogenic centres (Knudson and Toole, 1987).

A CD44-positive dispersed cell population

From E9.0-E9.5 until at least E12.5, a population of strongly CD44-positive cells are detected dispersed throughout the embryo, first appearing in the head and then both subectodermally and deep within the mesenchyme. The distribution of these CD44-positive dispersed cells observed in Fig. 2 raised the possibility that a proportion may represent late-

exiting neural crest cells. An immunohistochemical analysis of cultured E9.0 anterior neural tube explants (data not shown) showed no intensely immunostained cells. Therefore either our culture system is unable to support the formation of the CD44-positive dispersed cell phenotype in neural explants or these cells normally arise from outside the neural tube.

CD44 is known to be expressed in foetal thymocytes and lymphocytes (Lesley et al., 1985; Horst et al., 1990) and we show that cells expressing CD44 are present in abundance in the liver at E10.5 (Fig. 7) and, as early as E9.5, CD44-positive cells are detected in circulating blood (data not shown). These latter cells most likely derive from the blood islands in the visceral yolk sac and enter the foetal circulation via the vitelline duct. In addition, a population of macrophages has recently been described, present in the mesenchyme and liver from E10.5 and proposed to function in phagocytosis, particularly during tissue modelling (Morris et al., 1991). CD44-positive macrophages have also been proposed to function in the uptake of hyaluronan during lung development (Underhill et al., 1993) and, indeed, a process of receptor-mediated hyaluronan internalization may explain the cytoplasmic CD44 observed in some cells. An immunohistochemical analysis with an antibody that detects an epitope on macrophages, F4/80, revealed only a small number of immunostained cells, compared with the abundant CD44-positive cells (data not shown). This suggests that the large majority of the CD44-positive dispersed cells are not macrophages.

A further possibility for the identity of the CD44-positive dispersed cells is that they are endothelial cell precursors, angioblasts. Consistent with this idea is the presence of CD44-positive cells in the blood islands of the yolk sac, in the endothelial lining of the arteries and in the hindbrain and forebrain (Figs 2, 7). Recently it has been demonstrated that a novel tyrosine kinase gene, *tunica interna endothelial cell kinase (tek)*, is expressed by the endothelial cell lineage (Dumont et al., 1992). By in situ hybridization, we have observed similar distributions of *tek* and *CD44* mRNAs in arterial walls, although this method did not readily identify single cells (data not shown). Therefore the hypothesis that the CD44-positive dispersed cell population consists of migratory angioblasts remains to be substantiated.

A role for CD44 in instructive epithelia

Perhaps the most original results deriving from these studies are those showing localization of CD44 to specialized regions of instructive epithelia, which function to direct correct tissue outgrowth through reciprocal epithelial-mesenchymal cell interactions. Such structures include the morphologically distinct AER, which transmits growth factor signals to promote limb outgrowth in the limb buds (rev. Hinchcliffe and Johnson, 1980; Niswander and Martin, 1992), and the odontogenic placodes in the presumptive jaws, which are critical for the initiation of tooth development (Lumsden, 1988). Morphogenesis in these regions is not known to require hyaluronan and CD44 may act here by an alternative mechanism other than its capacity to bind this ligand. CD44 is expressed throughout the AER (Fig. 4) and in the odontogenic placodes of the upper and lower jaws (Fig. 6). In these regions, CD44 may act as an adhesion

molecule to maintain the integrity of the epithelium via heterotypic interactions with other cell surface proteins (St. John et al., 1990) or by binding to a matrix ligand such as collagen (Carter and Wayner, 1988; Faassen et al., 1992) or fibronectin (Jalkanen and Jalkanen, 1992). A more exciting possibility is that CD44 is playing a role in growth factor signalling. This idea is supported by the observation that CD44 can bind cytokines in vitro (Tanaka et al., 1993) and that members of the FGF, TGF- and Wnt families of signalling molecules are expressed in instructive epithelia (Gavin et al., 1990; Lyons et al., 1990; Jones et al., 1991; Niswander and Martin, 1992). It will be interesting to determine whether specific CD44 forms are expressed in instructive epithelia, or indeed in any discrete embryonic region.

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