Distribution of hyaluronate and hyaluronate receptors in the adult lung

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Summary

In the present study, we have examined the distribution of both hyaluronate receptors and hyaluronate in adult hamster lung. The receptor for hyaluronate is a transmembrane glycoprotein of Mr 85 000 that interacts with actin filaments and is thought to mediate many of the effects that hyaluronate has on cell behaviour, such as cell-to-cell adhesion and migration. It was localized histochemically with a monoclonal antibody, designated as K-3, which specifically binds to the receptor. Hyaluronate was detected by a biotinylated form of cartilage proteoglycan, which binds with high affinity and specificity to hyaluronate. At the light-microscopic level, both hyaluronate and its receptor were present on the basolateral surfaces of bronchial and bronchiolar epithelium, suggesting that these two components interact with each other. This interaction may be important for maintaining the attachment of the epithelium to the basement membrane. In addition, hyaluronate was present in the adventitial regions of large pulmonary blood vessels. Receptors for hyaluronate were also evident on the surfaces of pulmonary macrophages, as demonstrated by the fact that K-3 immunoreactive cells coincided with cells positive for non-specific esterase, a characteristic feature of macrophages. The receptors on pulmonary macrophages probably mediate the effects that hyaluronate has on these cells with respect to cell-to-cell adhesion and migration.

Key words: hyaluronate, hyaluronate receptor, lung, histochemistry, macrophage.

Introduction

Hyaluronate, a widely distributed glycosaminoglycan, has been shown to play an important role in various biological processes, such as mediating cell-to-cell adhesion (Pessac & Defendi, 1972; Shannon et al. 1980; Shannon & Love, 1980; Underhill & Dorfman, 1978), directing the migration of cells during morphogenesis (Toole, 1981; Toole et al. 1980), modulating the behaviour of endothelial cells (Astrupunk, 1986; Feinstein & Beebe, 1983; West et al. 1985), and influencing the activity of inflammatory cells (Ahlgren & Jarstrand, 1984; Forrester & Balazs, 1980; Forrester & Wilkinson, 1981; Hakansson et al. 1980; Hakansson & Venge, 1985). These events may be mediated through receptors for hyaluronate that are present on the cell surface. This laboratory has previously identified the hyaluronate receptor as an integral membrane glycoprotein of 85 000 Mr (Underhill et al. 1985) that appears to be associated either directly or indirectly with actin filaments in the cytoskeleton (Lacy & Underhill, 1987).

In a recent study, we identified a monoclonal antibody designated K-3 (K-3 MAb), which specifically binds to the hyaluronate receptor on hamster cells and blocks its interaction with [3H]hyaluronate (Tarone et al. 1984; Underhill et al. 1987). Since then, we have used a biotinylated form of this antibody (b-K-3 MAb) to examine the distribution of the receptor in a variety of tissues of the adult hamster. While most of the tissues showed some degree of staining with the K-3 MAb, lung tissue contained by far the most prominent staining. For this reason, we decided to concentrate our efforts on lung tissue, and identify the specific cell types containing the hyaluronate receptors.

In addition to localizing the hyaluronate receptors, we also examined the distribution of hyaluronate in lung tissue. To do this, we prepared a biotinylated form of the hyaluronate-binding complex from cartilage proteoglycan (b-PG), similar to that described by
Ripellino et al. (1985). Control experiments showed this b-PG was a very sensitive and specific probe for staining hyaluronate.

With these staining techniques, both hyaluronate and its receptor were found to be present in the basal and lateral regions of bronchial and bronchiolar epithelium. This coupled expression of the hyaluronate receptor and hyaluronate at the epithelial cell–matrix interface suggests that these two components must interact with each other. This interaction could play a role in attaching epithelial cells to the basement membrane and in maintaining their polarized morphology.

The hyaluronate receptor was also present on the surfaces of pulmonary macrophages. This observation was particularly interesting in view of the fact that these cells are responsible for maintaining the integrity of lung tissue. Pulmonary macrophages destroy bacteria as well as other pathogens, and thereby maintain a sterile environment in the lungs (Herscovitz, 1985). Furthermore, pulmonary macrophages play a major role during the inflammation and wound-healing processes (Hunt et al. 1984). The presence of hyaluronate receptors on these cells is consistent with the possibility that hyaluronate can modify the behaviour of pulmonary macrophages in both normal and diseased states of the lung (Galindo et al. 1975; Hallgren et al. 1985; Love et al. 1979; Poncin et al. 1986; Shannon et al. 1980; Shannon & Love, 1980).

Materials and methods

Cell lines and culture conditions

The rat fibrosarcoma (RFS) and baby hamster kidney (BHK) cell lines have been described (Goldberg & Toole, 1984; Underhill et al. 1987). These cells were routinely grown on 100-mm plastic tissue culture dishes (Corning) in Dulbecco's modified Eagle's medium (Grand Island Biological, Grand Island, NY) containing high levels of glucose (4.5 g l⁻¹) supplemented with either 10% foetal calf serum (Grand Island Biological) or NuSerum (Collaborative Research, Lexington, MA) and antibiotics (100 units l⁻¹ penicillin, 100 μg l⁻¹ streptomycin). The cells were grown in a 5% CO₂-95% air atmosphere.

Preparation of biotinylated proteoglycan (b-PG)

Bovine nasal cartilage (Pel-Freez, Rogers, AR) was shredded with a Sure-Form blade, mixed with 10 vol. of 4 M guanidinium·HCl, 0.5 M-sodium acetate, pH 5.8, and shaken overnight at 4°C. This mixture was then centrifuged (10 000 g, 45 min, 4°C) and the supernatant was further clarified by passage through filter paper (Whatman, chromatography paper no. 1) on a Buchner funnel. This extract was thoroughly dialysed against distilled water and then lyophilized. This crude extract served as starting material for further purification of the proteoglycan.

In some cases, the proteoglycan complex was treated with trypsin in order to produce smaller fragments, which would be better able to penetrate tissue sections. The following procedure was modified from that of Tengblad (1979). The crude extract (1-6 g) was dissolved in 50 ml of 0.1 M-N₂H₂—2-hydroxyethylpiperazine-N₂-2-ethanesulphonic acid (Hepes), 0.1 M-sodium acetate, pH 7.3, by stirring overnight. To this solution was added 0.8 mg of trypsin (type III, Sigma, St Louis, MO) and the mixture was incubated at 37°C. After 2 h, the proteolytic activity was stopped by the addition of 1 mg of soybean trypsin inhibitor (Sigma). The protein concentration of this solution was determined using the Coomassie Blue staining reagent (Bio-Rad, Richmond, CA).

Both intact and trypsin-treated preparations of the proteoglycan were coupled to biotin using a modification of the protocol of Updyke & Nicolson (1986). For this, the pH of the solution was adjusted to 8.0 with NaOH and, for every mg of protein 0.1 mg of sulphosuccinimidyl 6-(biotinamide)-hexanoate (Pierce, Rockford, IL) was added to the solution, which was maintained at room temperature for 30 min.

The hyaluronate-binding fraction was then purified by affinity chromatography on hyaluronate covalently coupled to Sepharose (hyaluronate-Sepharose), which was prepared according to the methods of Tengblad (1979). For affinity chromatography, samples of both intact and trypsinized, biotinylated proteoglycan were briefly dialysed against 4 M guanidinium·HCl, 0.5 M-sodium acetate, pH 5.8, and then mixed with 100 ml of hyaluronate-Sepharose that had been equilibrated in the same buffer. The samples were then dialysed overnight against 9 vol. of distilled water at 4°C in order to permit binding of the proteoglycan to the insolubilized hyaluronate. The hyaluronate-Sepharose was packed into a chromatography column and then washed with 100 ml of 1.0 M- NaCl, followed by a 400 ml linear gradient from 1.0 M to 3.0 M- NaCl. The material bound to the column was eluted by the addition of 4.0 M-guanidinium·HCl, 0.5 M-sodium acetate, pH 5.8. Fractions of 2.5 ml were collected and the protein content of each was determined by the Coomassie Blue assay. The fractions containing significant amounts of protein were pooled, and dialysed against 0.15 M- NaCl. The resulting preparations of both intact and trypsinized b-PG were mixed with an equal volume of glycerol and stored at -20°C. Under these conditions, the binding activity appeared to be quite stable. The yield from this procedure was approximately 1 mg b-PG for every 10 g of cartilage starting material. While both the intact and trypsinized b-PG gave similar results with respect to staining, we use the trypsinized preparation exclusively in this study, for histochemical localization.

The preparations of b-PG were analysed by SDS–polyacrylamide gel electrophoresis (PAGE). For this, both the intact and trypsinized b-PG were mixed with 10 vol. of ethanol and the precipitated proteins were collected by centrifugation (9000 g, 5 min). The resultant pellets were dissolved in 20 μl of sample buffer containing β-mercaptoethanol and placed in boiling water for 3 min, as recommended by Laemmli (1970). The samples were electrophoresed on a 10% SDS–polyacrylamide gel and subsequently stained with Coomassie Blue (Lacy & Underhill, 1987).

Specificity of the b-PG binding to hyaluronate

RFS cells were subcultured into a 24 multiwell culture dish (Falcon, Oxnard, CA) such that there were 1×10⁵ cells in
each well. After growing for one day, the cells were washed three times with phosphate-buffered saline (Uenderhill & Dorfman, 1978), and then fixed for 1 h in 2-0 % glutaraldehyde (Polyscience, Warrington, PA) in phosphate-buffered saline. The cells were washed five times (1 ml/well) for a total of 15 min with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) (Uenderhill & Dorfman, 1978) before further processing.

The b-PG (trypsin-treated) was diluted to 2 #g ml⁻¹ in a solution of 10 % calf serum in CMF-PBS. To determine the degree of non-specific binding, samples of the b-PG solution were mixed with varying concentrations of hyaluronate (type I, Sigma), testicular hyaluronidase (type IV, Sigma) and Streptomyces hyaluronidase (Sigma). Each of these solutions was then placed in duplicate wells (200 µl/well) containing the fixed RFS cells and the multwell culture dish was shaken at room temperature for 1 h. Following this, the cells were washed, and then incubated with a soluble substrate for peroxidase (200 µl/well) consisting of 1 mm-2,2-azino-di(3-ethylbenzothiazoline sulphonic acid) in 0-1 M-citrate, pH 4-2, containing 0-03 % H$_2$O$_2$. The reaction was allowed to proceed for approximately 5 min and was then terminated by the addition of 1 ml of 0-5 M-HCl. The A$_{416}$ of each sample was determined.

**Preparation of the biotinylated K-3 monoclonal antibody (b-K-3 MAb)**

A clone of the K-3 hybridoma was isolated that produced IgG, and then grown in the peritoneal cavity of Balb/c mice. The ascites fluid was collected and centrifuged to remove cells and particulate material (900 g, 3 min). The K-3 MAb was purified by affinity chromatography on Protein A—Sepharose C1-4B (Sigma), using a commercially available buffer system (MonoPure, Pierce). To optimize the binding of the K-3 MAb, the ascites fluid was mixed with 2 vol. of the binding buffer (MonoPure) prior to application to the column. The material specifically eluted from the column was extensively dialysed against CMF-PBS followed by 0-15 M-NaCl. Analysis of this material by SDS-PAGE revealed no protein bands other than the light and heavy chains of IgG.

The purified antibody was then biotinylated using a modification of the method described by Updyke & Nicolson (1986). For this, the concentration of the K-3 MAb was adjusted to 1-0 mg ml⁻¹ in 0-1 M-Hepes buffer, pH 8-0 (diluted from a stock solution of 1-0 M-Hepes, pH 8-3). To each ml of this solution was added 0-2 ml of 1 mg ml⁻¹ sulphosuccinimidyl 6-(biotinamide)hexanoate in 0-1 M-Hepes, pH 8-0. The reaction was allowed to proceed for 30 min at room temperature, and then the solution was thoroughly dialysed against CMF-PBS followed by 0-15 M-NaCl. The protein content of each sample was determined using the Coomassie Blue reagent of Bio-Rad and then frozen at -90 °C in 1-ml samples. This preparation is referred to as b-K-3 MAb.

As a control for the b-K-3 MAb, the IgG fraction from whole mouse serum (Sigma) was isolated and biotinylated in an identical fashion to that described above (b-mIgG).

The concentration of sulphosuccinimidyl 6-(biotinamide) hexanoate reagent used in the procedure described above was determined from a series of experiments designed to optimize the binding of peroxidase-conjugated streptavidin. Varying amounts of the sulphosuccinimidyl 6-(biotinamide)hexanoate reagent were mixed with a set amount of the K-3 MAb. The resulting preparations were then tested in a modified enzyme-linked immunosay, using fixed BHK cells as a source of antigens. The preparation giving the highest response corresponded to 0-2 #g of sulphosuccinimidyl 6-(biotinamide)hexanoate per mg of antibody protein.

**Cryosectioning of lungs, liver, brain, testis, uterus and spleen**

Adult Syrian hamsters (Charles River) were used throughout this study. The tissues were dissected out and fixed with 3 % formaldehyde in the presence and absence of 1 % cetylpyridinium chloride, dissolved in 60 mm-sucrose in phosphate-buffered saline. After 2 h at room temperature, the tissue was then infused with 0-3 % formaldehyde and 0-32 M-sucrose in CMF-PBS at 4 °C overnight. The fixed tissues were frozen in liquid nitrogen and subsequently mounted on microtome specimen holders. Frozen sections of 8 #m were cut and placed on gelatin-subbed glass slides for histochemical labeling and light-microscopic observations.

**Staining of sections with b-PG and b-K-3 MAb**

To inhibit endogenous peroxidase activity, the frozen sections were hydrated in CMF-PBS and then immersed for 10 min in each of the following solutions: (1) 70 % ethanol, (2) 100 % methanol, 0-1 % H$_2$O$_2$, (3) 70 % ethanol and (4) CMF-PBS (Wood, 1984). The sections were then incubated with the primary probe dissolved in 10 % calf serum, CMF-PBS. The b-K-3 MAb and its b-mlgG control, were used at a concentration of 4-8 #g ml⁻¹, while the b-PG (trypsinized) was used at a concentration of 1-2 #g ml⁻¹. Sections incubated with only streptavidin-horseradish peroxidase served as an appropriate control for endogenous biotin, which was consistently absent. The controls for the b-PG consisted of the identical solution to which was added hyaluronate (100 #g ml⁻¹) or Streptomyces hyaluronidase (20 units ml⁻¹). The primary probes were incubated with the tissue sections for 1 h at room temperature. The sections were quickly washed (10 times, CMF-PBS), and then incubated for 15 min with a 1:500 dilution of peroxidase-conjugated streptavidin in 10 % calf serum, CMF-PBS. After additional washes with CMF-PBS, the sections were incubated with an insoluble substrate for peroxidase. This consisted of 3-amino-9-ethyl carbazole prepared according to the method described by Graham et al. (1965). The progress of the reaction was monitored by microscopic examination at low power, and after appropriate colour development (generally 5-10 min for the b-PG and 20-60 min for the b-K-3 MAb and b-mIgG) the sections were washed, and mounted in a 1:1 (v/v) mixture of glycerol and PBS.

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Histochemical stain for non-specific esterase

To stain lung sections for alveolar macrophages, we used the non-specific esterase assay described by Dannenberg & Suga (1981). The substrate for this was prepared by mixing 0.5 ml of a 10 mg/ml−1 naphthol AS-D acetate (Sigma) solution in dimethylformamide with 50 ml of 0.1 M-Tris·HCl, pH 6.0, followed by the addition of 10 mg of fast garnet GBC (Sigma). The resulting solution was passed through a 0.45 μm Millipore filter and added directly to the frozen sections. The progress of the reaction was monitored by microscopic examination and terminated after sufficient colour development (generally 4–6 h). Some sections were further processed for staining with the b-K-3 MAb as described above. The reaction product of the non-specific esterase had a purple to red colour, which could be easily distinguished from the red colour produced as the reaction product of peroxidase on 3-amino-9-ethyl carbazole.

Results

Specific staining of hyaluronate with b-PG

For the histochemical localization of hyaluronate, we used a biotinylated form of the proteoglycan complex isolated from cartilage. This hyaluronate binding probe was prepared by extracting bovine nasal cartilage with 4 M-guanidinium·HCl to release the proteoglycan complex (Tengblad, 1979), which was then treated with trypsin and coupled to biotin (Updyke & Nicolson, 1986). This material was then purified by affinity chromatography on hyaluronate-Sepharose (Tengblad, 1979). Fig. 1B shows the analysis of trypsinized b-PG by SDS–PAGE. This preparation consists of a diffuse band between 70 000 and 80 000 Mr, which represents the hyaluronate-binding fragment of the proteoglycan monomer, and a band at 43 000 Mr, which corresponds to one of the link proteins (Tengblad, 1979). In contrast, the intact b-PG (i.e. non-trypsinized), shown in Fig. 1A, consists of the proteoglycan monomer, which is at the top of the gel, and the link proteins at 45 000 and 50 000 Mr (Tengblad, 1979). Trypsin treatment appears to result in the loss of one of the link proteins (Fig. 1B).

To test the specificity of the b-PG, we examined its ability to bind to cell surface hyaluronate under controlled conditions. For this, RFS cells, which produce a particularly large coat of hyaluronate (Goldberg & Toole, 1984), were subcultured at equal density into a multiwell culture dish and allowed to grow overnight. These cells were fixed with glutaraldehyde to stabilize the cell surface hyaluronate, and then incubated with the biotinylated proteoglycan followed by peroxidase-conjugated streptavidin. After washing, the relative amounts of peroxidase attached to the fixed cells was determined by the addition of a soluble substrate. As shown in Fig. 2A, the binding was completely inhibited by the addition of relatively small amounts of free hyaluronate. In a similar fashion, binding was also inhibited by the addition of testicular hyaluronidase (Fig. 2B) as well as Streptomyces hyaluronidase (Fig. 2C), which specifically degrades hyaluronate (Ohya & Kaneko, 1970). These characteristics were also reflected in the histochemical staining of frozen sections. In all of the sections we examined, the staining was completely abolished by the addition of hyaluronate or hyaluronidase. These results demonstrate that the b-PG probe binds specifically to hyaluronate.

It is important to note that the staining characteristics of the b-PG can be influenced by several factors. The first factor is whether or not the hyaluronate is freely exposed in the tissue section. For example, the b-PG reacts poorly with hyaluronate present in cartilage,
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which is presumably masked by proteoglycan monomers that sterically hinder the access of the biotinylated probe. A second factor in determining the staining is whether the hyaluronate is lost from the section during processing. Some tissues, such as the vitreous body of the eye, contain large amounts of free hyaluronate (e.g. not associated with protein), which may be lost from the tissue during normal fixation procedures. In a number of studies cetylpyridinium chloride has been added to the fixative solution in order to prevent the loss of these glycosaminoglycans (Ripellino et al. 1985). However, in our study, we found that cetylpyridinium chloride had little or no effect on the staining pattern of hyaluronate in lung. In this case, the hyaluronate is presumably retained by a core protein (Green & Underhill, 1987; Mikuni-Takagaki & Toole, 1981), or by hyaluronate binding proteins such as proteoglycans (Schmidt et al. 1982), which are immobilized by aldehyde fixation alone.

Localization of hyaluronate in adult lung

In the adult hamster lung, hyaluronate was evident in the perivascular zones of large blood vessels and basement membrane regions of bronchial and bronchiolar epithelium (Fig. 3A). This staining was specific for hyaluronate, since it was completely eliminated by the addition of *Streptomyces* hyaluronidase (Fig. 3B) or exogenous hyaluronate (data not shown). The perivascular hyaluronate was particularly prominent in the adventitia (Fig. 3C,D,F), and occasionally extended to regions immediately beneath the endothelium (Fig. 3C). Far less hyaluronate was apparent in regions surrounding smaller blood vessels and capillaries.

Hyaluronate was not universally present in perivascular regions of all tissues. For example, it was largely absent (or greatly reduced) from regions surrounding blood vessels of adult liver, spleen, brain and testis (data not shown). However, it was quite prominent in developing blood vessels of the chick embryonic yolk sac.

In the adult hamster lung, hyaluronate was also consistently observed in regions surrounding respiratory passageways, where it was present in the basal regions (Fig. 3E) and occasionally along the lateral surfaces of the epithelium (Fig. 3F). It was particularly prominent in the interstitium immediately between large blood vessels and air passageways (Fig. 3A,F). However, hyaluronate was either greatly reduced in, or absent from, the interstitium of terminal respiratory pathways and interalveolar septums.

Again, hyaluronate did not appear to be a universal component of all basement membranes, since it was absent in those regions of liver, spleen and testis. However, it was prominent in the basement membrane region of the adult salivary gland (data not shown).

Distribution of the hyaluronate receptor

To examine the distribution of the hyaluronate receptor in hamster lung tissue, we used the b-K-3 MAb in conjunction with peroxidase-conjugated streptavidin. The level of background staining was determined with an equivalent amount of b-mIgG purified from mouse serum.

In the adult hamster lung, the hyaluronate receptor was found on the basolateral surface of epithelium lining bronchi and larger bronchioles (Fig. 4). The distribution of the hyaluronate receptor in this epithelium was quite reminiscent of that of hyaluronate, which was also located on the basal and lateral surfaces.
Fig. 3. The distribution of hyaluronate in frozen sections of lung as shown by b-PG staining. A. Hyaluronate was observed surrounding a bronchiole (bo) and a large pulmonary blood vessel (bv). Hyaluronate was consistently present in connective tissue immediately between large blood vessels and respiratory passageways (A, asterisk). B. The staining with b-PG was completely eliminated by incubating the sections with *Streptomyces* hyaluronidase (B) or hyaluronate (Fig. not shown). The positions of the endothelium (en) and the bronchiole epithelium (ep) are indicated. C–D, F. The hyaluronate surrounding blood vessels was prominent in the adventitia and was occasionally observed immediately adjacent to the endothelium (C, arrow). Only minor and sporadic staining of hyaluronate was observed in the surrounding alveolar interstitium (C, is). E–F. Hyaluronate was also present in the basement membrane regions of bronchioles and on the lateral surfaces of the epithelial cells (F, arrowheads). However, it was greatly reduced in or absent from the interalveolar septum (E, arrow). Bars, 50 μm.

of the epithelial cells. This co-localization of hyaluronate and its receptor at the epithelial cell–matrix interface suggests that these components may be important in maintaining the attachment and polarization of the epithelium. In contrast, only weak staining was observed in the interstitium near blood vessels and staining was totally absent in terminal respiratory pathways, such as alveolar ducts and sacs.

Other adult tissues examined for the hyaluronate receptor included the adult hamster brain, testis, liver, heart and uterus. In comparison with the lungs, there was far less staining apparent in these tissues, with the
exception of the uterus, which showed some staining on basal and lateral surfaces of the epithelium. Only a modest amount of staining was apparent in the brain, while no staining was detected in the heart and testis.

**Hyaluronate receptors on the surfaces of pulmonary macrophages**

In addition to staining the bronchial epithelium of the lungs, the b-K-3 MAb also stained the surfaces of a select group of large cells with a distinctively round morphology. These cells were the most prominent staining components of the lung tissue and had no counterparts in the liver, heart or testis (data not shown). These cells were widely distributed throughout the lung tissue, being present as individual cells in the alveolar region (Fig. 5A) as well as aggregates of cells in the lumen of respiratory passageways (Fig. 5C–D).

On the basis of these characteristics, it seemed likely that these K-3 immunoreactive cells could be pulmonary macrophages, which show a similar type of distribution. To test this possibility, sections of lung were stained for non-specific esterase activity, which is a characteristic feature of macrophages (Dannenberg & Suga, 1981). As shown in Fig. 6A, the distribution of esterase positive cells was very similar to that of the K-3 immunoreactive cells (compare with Fig. 6C). At higher power, it was apparent that most of the esterase staining was cytoplasmic in nature (Fig. 6B), while the b-K-3 MAb stained mainly at the periphery of the cell, presumably at the level of the plasma membrane (Fig. 6D). To determine if the two procedures were staining the same cell, sections of lung tissue were first stained for non-specific esterase activity and subsequently stained with the b-K-3 MAb. As shown in Fig. 6E, in most if not all cases the two techniques did indeed stain the same cell type. At higher power (Fig. 6F), both the cytoplasmic staining of esterase and...
Fig. 5. The localization of the hyaluronate receptor in lung tissue as determined by immunohistochemical staining with the b-K-3 MAb. A. Histochemical localizations; and B, phase-contrast photomicrograph of hyaluronate receptors on cells (arrows) found near interalveolar septum (arrowheads) subdividing the alveolar spaces (a). C. Aggregates of immunoreactive cells (m) were present in lumina of bronchioles (bo); and D, attached to the epithelium (ep). Bar, 50 μm.

the peripheral staining of the b-K-3 MAb were apparent in the same cell. It should be noted that, while all esterase positive cells were found to stain with the K-3 antibody, not all K-3 positive cells contained significant amounts of esterase activity. This may be due to variations in the amount of esterase associated with pulmonary macrophages, or alternatively, the b-K-3 MAb may be staining cells in addition to pulmonary macrophages.

Discussion

Four major conclusions can be inferred from this study. First, hyaluronate is present in the perivascular regions of large blood vessels, but greatly reduced in that of smaller blood vessels and capillaries. Second, hyaluronate is apparent in the basement membranes of epithelium lining bronchi and bronchioles. Third, receptors for hyaluronate are evident on the basal and lateral surfaces of bronchial and bronchiolar epithelium. And finally, hyaluronate receptors are present on pulmonary macrophages. Each of these findings will be discussed in greater detail in the following sections.

The first major finding of this study was that hyaluronate was present in the perivascular regions of large pulmonary blood vessels. It was most prominent in the adventitia, and occasionally extended into the subendothelial regions of the intima. This pattern of hyaluronate distribution in perivascular regions was not observed in other types of tissues such as testis, liver, spleen or kidney. However, it is probably not unique to the lung vascular tissue, since other studies have demonstrated that complexes of hyaluronate and proteoglycans are present in the wall of the aorta (Schmidt et al. 1982) and smaller blood vessels (Ausprouk, 1986). Indeed, the formation of such complexes may be the mechanism by which the hyaluronate is retained in these tissues.

While the significance of hyaluronate in these locations is unclear, several studies have shown that hyaluronate can modulate the behaviour of endothelial cells. For example, native hyaluronate has been shown to inhibit the vascularization of developing limb buds (Feinberg & Beebe, 1983), whereas its degradation
Fig. 6. Co-localization of non-specific esterase staining and b-K-3 immunoreactive cells in lung tissue. A–B. Non-specific esterase localization of pulmonary macrophage as viewed under low and high magnification, respectively. Note that most of the staining is cytoplasmic in nature. C–D. The b-K-3 MAb immunoreactive cells are shown under low and high magnification, respectively. The staining appears to be restricted to the peripheral region of the cell adjacent to the plasma membrane. E–F. Both non-specific esterase and b-K-3 MAb staining patterns are localized to the same cell types as shown under low and high magnification, respectively. Note both cytoplasmic and peripheral staining. Bars, 20 μm.
products stimulate angiogenesis in chorioallantoic membranes (West et al. 1985). It is possible that the hyaluronate in the adult tissue serves to stabilize the vasculature.

The second major finding of this study was that hyaluronate was particularly prominent in the basement membranes of bronchi and bronchioles, where it was observed to extend from the basal regions to the lateral surfaces of the epithelial cells. Hyaluronate did not appear to be a universal component of all basement membranes, since it was not observed in basement membranes of testis or liver. However, it was apparent in the basement membrane region of the adult hamster salivary glands. These results are consistent with those of Bernfield & coworkers (1984), who demonstrated that hyaluronidase-sensitive glycosaminoglycans (presumably hyaluronate) were present in certain regions of the epithelial basal lamina of developing salivary glands. Indeed, they found that these glycosaminoglycans were present in the clefts of growing and branching lobules but absent from the tips. This particular arrangement of the glycosaminoglycans suggested that they might be involved in the morphogenetic process of lobulation. Similarly, the continued presence of hyaluronate in the basement membrane of differentiated lung tissue may also influence tissue remodelling and repair.

Several studies have shown that there is a marked increase in the amount of hyaluronate present in bronchoalveolar lavage fluid during inflammatory responses (Galindo et al. 1975) and in certain diseased states (sarcoidosis) of the lungs (Hallgren et al. 1985). It is quite possible that this released hyaluronate is derived from the hyaluronate present in the spaces surrounding the respiratory passageways. Perhaps the release of the hyaluronate results from a breakdown of the epithelium, which normally represents a physical barrier between the hyaluronate and the lumen of the air passageways.

The third major finding of this study was that receptors for hyaluronate were present on the basal and lateral surfaces of the epithelial cells lining bronchi and larger bronchioles. In contrast, the staining of the epithelium in terminal bronchioles and alveolar tissue was either greatly reduced or absent. The staining pattern of hyaluronate receptors on the basolateral surfaces of bronchiolar epithelium was quite similar to that of hyaluronate. This close association makes it extremely likely that the cell-surface receptors interact with the hyaluronate in the underlying basement membrane. This interaction could be important in maintaining the attachment between the epithelial cells and the basement membrane. In addition, this interaction also may be partly responsible for maintaining the correct polarity of the epithelial cells, since the receptors provide a transmembrane link between hyaluronate and the cytoskeleton (Lacy & Underhill, 1987). This is consistent with the notion that the surfaces of cells and their cytoskeletons are influenced by the interaction with extracellular matrix components via transmembrane receptors (Hay, 1986).

The final observation made in this study was that hyaluronate receptors were present on the surfaces of pulmonary macrophages. The intensity of staining of pulmonary macrophages was significantly greater than that observed on macrophages of other tissues. For example, no staining was apparent in the liver, which contains a population of macrophages (Kupffer's cells). Thus, the presence of hyaluronate receptors on macrophages may be a distinguishing feature of a certain subpopulation of macrophages, including those present in the lungs.

The presence of hyaluronate receptors on the surfaces of pulmonary macrophages is consistent with several earlier studies. Shannon and his coworkers (Shannon et al. 1980; Shannon & Love, 1980) have shown that hyaluronate can induce the aggregation of both alveolar and peritoneal macrophages. Furthermore, hyaluronate has been shown to mediate the macrophage disappearance reaction (Love et al. 1979; Shannon et al. 1980), which is considered to be a form of delayed-type hypersensitivity. This reaction occurs when an antigen is injected into the peritoneal cavity of a previously sensitized animal, which results in a decrease in the number of peritoneal macrophages that can be recovered from subsequent washes of the cavity. This phenomenon appears to be mediated by sensitized T-cells, which release factors causing the mesothelial cells to increase their production of hyaluronate. This hyaluronate, in turn, causes macrophages to clump together and stick to the epithelium, thus making them seem to disappear from the washes. This aggregation is presumably mediated by the interaction of hyaluronate with hyaluronate receptors on the peritoneal macrophages. The macrophage disappearance reaction is mimicked by intraperitoneal injections of hyaluronate for a period of 24 h. However, over a longer period of time (i.e. 3 days), the hyaluronate induces a dose-dependent mobilization of inflammatory cells and doubles the population of peritoneal macrophages (Ponzin et al. 1986).

The hyaluronate receptors may also mediate the effects of hyaluronate on cell migration. Shannon & Love (1980) have shown that relatively high concentrations of hyaluronate inhibit the migration of peritoneal macrophages. Presumably, the interaction between hyaluronate and the receptors on the surfaces of the macrophages interferes with the reorganization of actin filaments that is necessary during cell migration.
The functional role of hyaluronate receptors on pulmonary macrophages is still an open question. We would like to speculate that these receptors may be important in recruiting the macrophages to the lung tissue. According to this hypothesis, a subpopulation of monocytes present in the blood has receptors for hyaluronate on their surfaces. When these cells are carried by the blood to the lungs, they come in contact with the hyaluronate present in the perivascular region. This interaction may cause the cells to adhere to the walls of the blood vessels and then stimulate them to migrate into the parenchyma. Such a situation would account for the presence of the hyaluronate receptor on a restricted population of macrophages and for the fact that hyaluronate is so prominent in pulmonary blood vessels.

The presence of hyaluronate in the basement membranes of lung tissue may provide a defence mechanism against air- and blood-borne substances. It is possible that at sites of lung infection relative large amounts of hyaluronate could be generated, in much the same way that antigens stimulate hyaluronate production in the peritoneal cavity. Indeed, high concentrations of hyaluronate have been shown to inhibit the migration of macrophages (Shannon & Love, 1980), which may serve to immobilize them at sites of infection. Fragments of hyaluronate could also act as a chemoattractant agent for resident macrophages in lung interstitium. This may be analogous to the situation in which fragments of elastin have been shown to attract macrophage precursors to diseased sites in pulmonary emphysema (Hunninghake et al. 1981; Senior et al. 1984). Clearly, further research is necessary to clarify the role of hyaluronate and hyaluronate receptors in lung physiology.

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