Assembly of a chondrocyte-like pericellular matrix on non-chondrogenic cells

Role of the cell surface hyaluronan receptors in the assembly of a pericellular matrix

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Summary

In this study, we have examined the capacity of various cell types, which express cell surface hyaluronan receptors, to organize a chondrocyte-like pericellular matrix when given chondrocyte-derived extracellular matrix macromolecules exogenously. The assembly of a pericellular matrix was visualized by a particle exclusion assay. Without the addition of exogenous macromolecular components, none of the cell types studied exhibited significant pericellular matrices extending from their plasma membranes. However, upon the addition of high molecular weight hyaluronan in combination with aggregating cartilage proteoglycan monomers, large pericellular matrices were formed within two hours of incubation. No pericellular matrices were formed if these macromolecular components were added separately at equivalent concentrations or if the components were added in the presence of hyaluronan hexasaccharide, a competitive inhibitor of hyaluronan interaction with cell surface hyaluronan receptors. Fully assembled pericellular matrices could also be displaced by the subsequent addition of hyaluronan hexasaccharides. Nonliving, glutaraldehyde-fixed cells, which retained functional hyaluronan receptors, maintained the capacity to assemble pericellular matrices with exogenous components, in serum-containing or serum-free medium. Cells that did not express hyaluronan receptors were not capable of organizing such pericellular matrices when incubated with these exogenous components. These findings suggest that cells expressing hyaluronan receptors have a significant capacity to organize their immediate extracellular environment via hyaluronan–hyaluronan receptor interactions. Possible physiological functions for this type of matrix organizing capacity are discussed.

Key words: hyaluronan, hyaluronan receptors, chondrocyte, pericellular matrix, particle exclusion assay.

Introduction

Several cell types in culture, including embryonic and adult chondrocytes, have been shown to exhibit large pericellular matrices or 'coats' extending from their plasma membrane by as much as one cell diameter (Clarris and Fraser, 1968; Knudson and Toole, 1985a; Knudson and Kuettner, 1990; Underhill and Toole, 1982). These pericellular matrix halos are readily visualized by a particle-exclusion assay whereby small particles (in our studies these particles are formalin-fixed red blood cells) are added to cells in low-density culture. Upon settling, the particles are excluded from a distinct area or zone surrounding the cells, clearly defining the boundary of the pericellular matrix. This assay represents the most useful method of visualizing the full extent of a cell's pericellular matrix, since conventional staining of the matrix by histochemical or immunohistochemical techniques often leads to significant collapse of this structure (Clarris and Fraser, 1968; Knudson and Toole, 1985a).

On chondrocytes this pericellular matrix is composed predominantly of chondroitin sulfate-rich proteoglycan (termed 'aggrecan'), although its composite structure appears dependent on the presence of hyaluronan (Knudson and Toole, 1985a). Treatment of these cells with Streptomyces hyaluronidase, an enzyme that specifically degrades hyaluronan, completely removes the pericellular matrix within 30 min. After addition of fixed red blood cells to the hyaluronidase-treated cultures, no excluded area around the cells is observed and the particles now abut the plasma membrane directly (Knudson and Toole, 1985a; Knudson and Kuettner, 1990). Other extracellular matrix macromolecules are undoubtedly resident within this pericellular matrix but have not as yet been fully characterized.

These same chondrocytes that display an extensive pericellular matrix also express specific, saturable, high-affinity hyaluronan binding proteins or receptors on their cell surface (Knudson and Toole, 1987; McCarthy and Toole, 1989). The chondrocyte receptors have similar properties to hyaluronan receptors as reported on many other cell types (Nemec et al. 1987; Underhill and Toole, 1979; Orkin et al. 1985; Green et al. 1988). One important property shared by all of these cell surface hyaluronan
receptors is the minimum size of a hyaluronan oligosaccharide required to compete effectively for the binding of native hyaluronan to its receptor, which is a hyaluronan hexasaccharide. This property is helpful in differentiating the specific binding of hyaluronan to the cell surface receptor from the specific aggregation of hyaluronan with other extracellular matrix macromolecules such as cartilage proteoglycan and link protein, which require a minimum hyaluronan oligosaccharide sequence of 10–12 monosaccharides for competition (Solursh et al. 1986; Kimura et al. 1979; Hascll and Heinegaard, 1974). We have shown previously that the assembly of a pericellular matrix on chondrocytes, utilizing endogenous macromolecules, can be blocked by the addition of hyaluronan hexasaccharides (Knudson and Kuettner, 1990; Knudson et al. 1991). In the presence of the hexasaccharides, the displaced matrix macromolecules are recovered intact in the culture medium of the chondrocytes (Knudson and Kuettner, 1990; Knudson et al. 1991). A fully assembled matrix on chondrocytes, utilizing endogenous macromolecules, is also displaced by the addition of hyaluronan hexasaccharides (Knudson and Kuettner, 1990). Therefore, the chondrocyte pericellular matrix not only contains hyaluronan as an integral part of its meshwork but is, in fact, anchored to the cell surface via interaction between hyaluronan and hyaluronan receptors. These hyaluronan receptors thus have the capacity to assemble and organize the extracellular components within the pericellular matrix.

Many other cell types express similar hyaluronan receptors but do not exhibit significant pericellular matrices (Nemec et al. 1987; Underhill and Toole, 1979; Underhill and Toole, 1982). For instance, the human urinary bladder carcinoma cell line, HCV-29T, expresses saturable, high-affinity, high-specificity cell surface hyaluronan receptors (Nemec et al. 1987), but does not elaborate an extensive pericellular matrix. The function of the hyaluronan receptors present on the HCV-29T cells is not known but, unlike chondrocytes (Knudson and Toole, 1987), the receptors are not occupied by endogenous hyaluronan (Nemec et al. 1987), which they synthesize at only very low levels (Knudson and Knudson, 1990; Pauli and Knudson, 1988). Nevertheless, the hyaluronan receptors on these cells may have a similar capacity to chondrocytes for organizing a chondrocyte-like pericellular matrix given the appropriate extracellular matrix macromolecules exogenously. We therefore conducted experiments to determine if the addition of exogenous hyaluronan alone, aggregating proteoglycan monomers alone, or hyaluronan in combination with aggregating proteoglycan, to non-chondrogenic cells, which express hyaluronan receptors but are matrix-deficient, would result in the assembly of a pericellular matrix. The data described in this study demonstrate that these cells have this capacity to form such pericellular matrices and that this matrix is indeed anchored to the cell surface via interaction between hyaluronan and hyaluronan receptors.

Materials and methods

Cell lines and culture conditions

The human urinary bladder carcinoma cell lines, HCV-29T (Nemec et al. 1987) and HU-466 (Pauli and Knudson, 1988); simian virus-transformed mouse 17T fibroblasts, SV-3T3 (Underhill et al. 1982); rat fibrocoma cells (Underhill et al. 1982); and B-16 mouse melanoma cells (Biwas, 1988), have been described. The non-invasive human urinary bladder papilloma, RT-4, was obtained from the American Type Culture Collection (Rockville, MD). Low-passage bovine aortic endothelial cells were a gift from Dr B. Pauli (Department of Pathology, Cornell University College of Veterinary Medicine, Ithaca, NY), and were similar to cells previously described by his laboratory (Pauli and Lee, 1986).

These cells were tested by us and found to be Factor VIII positive by immunofluorescence (data not shown). All cells were routinely grown in 75 cm² or 175 cm² tissue culture flasks (Falcon) in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) containing 4.5 g/l glucose supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin solution (Sigma) and 2 mm glutamine (Gibco BRL, Grand Island, NY), at 37°C in a humidified incubator supplied with 5% CO₂/95% air. Cells from near-confluent monolayers were dissociated with a 0.25% trypsin/0.05 M EDTA solution (Sigma) and 1x10⁶ cells ml⁻¹ plated into 35-mm tissue culture dishes (Falcon) in complete medium for assay.

In some experiments, HCV-29T cells were plated in 35-mm dishes as described above, allowed to attach for 18 h at 37°C, rinsed 3 times in PBS and then exposed to a solution of 1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in phosphate-buffered saline, pH 7.4 (PBS), for 3 min at room temperature. The cells were then rinsed 3 times with PBS containing 1% bovine serum albumin (Sigma), over a 60 min time interval. These fixed HCV-29T cells were used in similar matrix assembly experiments to those in which the live, non-fixed cells were used.

Particle exclusion assay

The particle exclusion assay method followed a protocol described previously (Knudson and Toole, 1986a). Briefly, horse red blood cells (MG Scientific, Buffalo Grove, IL) were fixed in 1.5% formaldehyde in calcium- and magnesium-free PBS overnight at room temperature, washed extensively and stored in CMF-PBS with antibiotics at 4°C. For assay, the culture medium of a 35-mm dish of cells to be tested was removed and replaced with a 750 μl suspension of fixed red blood cells (10⁶ ml⁻¹) in PBS containing 0.1% BSA. The particles were allowed to settle for approximately 10 min. The cells were then observed and photographed on an Olympus IMT-2 inverted phase-contrast microscope or a Nikon Optiphot inverted phase-contrast microscope equipped with Hoffman optics.

Morphometric analysis of matrix to cell area ratio

Photographs of randomly selected cells (20–30 for each condition), printed at the same magnification, were analyzed using a Summa Sketch Plus digitizing graphics tablet (Summagraphics, Fairfield, CT) interfaced with an IBM-compatible microcomputer and utilizing a Sigma Scan software program (Jandel Scientific, Corte Madera, CA). A coat:cell ratio, defined as the ratio of the area delimited by the perimeter of the pericellular matrix to the area delimited by the plasma membrane, was determined by tracing the matrix and cell perimeters on photomicrographs placed on the digitizing tablet (Knudson and Toole, 1986a). If no detectable pericellular matrix was present the coat:cell ratio was 1.0.

Preparation and addition of exogenous macromolecules

Large aggregating chondroitin sulfate proteoglycan monomer derived from Swarm rat chondrosarcoma and proteoglycan monomers derived from adult bovine articular or nasal cartilages were gifts from Drs J. Kimura and E. Thomas (Department of Biochemistry, Rush-Presbyterian-St Luke's Medical Center, Chicago, IL), respectively. The proteoglycans (A1DI purified fractions) were incubated with 0.67 unit ml⁻¹ Streptomyces hyaluronidase (Type IX, Sigma) for 4 h at 37°C in order to remove small concentrations of hyaluronan found within these preparations. The proteoglycans were recovered following this treatment, by overnight precipitation at 4°C with 3 volumes of cold 10% ethanol containing 1.3% potassium acetate or carried through another desalting cassette chloride equilibrium centrifugation. The precipitated proteoglycan pellet was air-dried and redissolved in tissue culture medium. For the centrifuged
Enzymatic removal of rat fibrosarcoma pericellular containing hyaluronan, proteoglycan, hyaluronan plus proteoglycan binding assays

Hyaluronan oligosaccharides were prepared by an exhaustive digestion of high molecular weight hyaluronan with bovine testicular hyaluronidase. Briefly, a 0.5 mg ml⁻¹ solution of hyaluronan in 0.1 M sodium acetate, 0.05 M sodium chloride (Knudson et al. 1984) was incubated with 150 USP/TFU units ml⁻¹ testicular hyaluronidase (Type I-S, Sigma) for 24 h at 37°C. The digestion mixture was heat-inactivated by boiling for 10 min, adjusted to pH 7.2 and filter sterilized. Alternatively, purified hyaluronan hexasaccharides were prepared following partial enzymatic digestion (Kimura et al. 1979; Hasse1 and Heinegard, 1974). High molecular weight hyaluronan was first digested with papain, dialyzed against 0.5 M sodium acetate, precipitated twice with 95% ethanol, redissolved in 0.1 M sodium acetate, 0.15 M NaCl, 0.001 M EDTA, pH 5.0, and digested for 16 h with testicular hyaluronidase (172 USP/TFU hyaluronidase mg⁻¹ hyaluronan). Following a 10 min heat inactivation at 100°C, the hyaluronan oligosaccharides were then separated on a 2.5 cm x 118 cm column of Bio-gel P30, eluted in 0.5 M pyridinium acetate, pH 6.5 (Solursh et al. 1980). The hexasaccharide peak was pooled (oligosaccharides from 2-14 monosaccharides were well resolved) and lyophilized to dryness, redissolved in distilled water and lyophilized again. We determined that the exhaustive digestion mixture, before column chromatography, containing inactivated enzyme plus hyaluronan tetra- and hexasaccharides, had a proportional capacity to inhibit pericellular matrix assembly as the column-purified hyaluronan hexasaccharide. A control solution of buffer containing heat-inactivated enzyme alone had no effect on the cells. Therefore, the tetra-/hexaasccharide mixture was used in most of these studies.

The concentrations of exogenous macromolecules added to cells used in these studies were those previously determined as optimal for pericellular matrix assembly on chondrocytes (Knudson et al. 1991). Medium from cells grown in low-density culture for 24 h in 35-mm dishes was removed and replaced with 2.0 ml fresh serum-supplemented medium containing: (a) no additional components; (b) 2.0 mg ml⁻¹ proteoglycan monomer; (c) 12 μg ml⁻¹ hyaluronan; (d) 2.0 mg ml⁻¹ proteoglycan monomer plus 12 μg ml⁻¹ hyaluronan; or (e) 2.0 mg ml⁻¹ proteoglycan monomer, 12 μg ml⁻¹ hyaluronan, and 100 μg ml⁻¹ hyaluronan tetra-/hexasaccharides. Cells were allowed to incubate at 37°C in the humidified CO₂ incubator for 2-24 h. Following this incubation, the medium was removed and the cultures were assayed by the particle exclusion method as described above.

**Enzymatic removal of rat fibrosarcoma pericellular matrix**

Medium from rat fibrosarcoma cells plated at low density in 35-mm dishes was removed and replaced with 2.0 ml fresh medium containing 0.5 units of Streptomyces hyaluronidase (Type IX, Sigma). Following incubation with the hyaluronidase for 1 h at 37°C, the cell cultures were washed twice with Hank's balanced salt solution followed by the addition of medium alone, or medium containing hyaluronan, proteoglycan, hyaluronan plus proteoglycan or hyaluronan plus proteoglycan and hyaluronan tetra-/hexasaccharides, as described above. Following 2h of incubation with these solutions, the medium was removed and the cells assayed by the particle exclusion method.

**Hyaluronan binding assays**

Saturation binding assays were performed on live, intact cells as described previously by Nemec et al. (1987). Briefly, cells were released from monolayer cultures by treatment with 10 mM EDTA in CMF-PBS, centrifuged and resuspended in PBS. Cells were aliquoted into duplicate bovine serum albumin-coated microcentrifuge tubes containing varying concentrations of ³H-hyaluronan or ³H-hyaluronan plus 250 μg/ml non-labeled hyaluronan (added to determine non-specific background binding), in a total volume of 0.5 ml containing 1 x 10⁶ cell/ml. The ³H-hyaluronan ligand was purified from the conditioned medium of rat fibrosarcoma cells labeled with ³H-hexacarote as described previously (Knudson and Toole, 1987; Nemec et al. 1987). The ³H-labeled hyaluronan was of high molecular weight (voided on Sephacryl CL-2B), free of containing glycosaminoglycans (by DEAE chromatography) and had a specific activity of 61300 cpm μg⁻¹. Cells plus ³H-hyaluronan were incubated for 90 min at high temperature with gentle shaking. Following this incubation, cells were pelleted by centrifugation at 500 g, washed twice with 5xPBS, dissolved in 0.5% sodium dodecyl sulfate, mixed with scintillation cocktail (Ecocint; ICN, Costa Mesa, CA) and counted. Specific binding was determined by subtraction of background binding.

**Results**

**Assembly of a chondrocyte-like pericellular matrix around human HCV-29T bladder carcinoma cells**

The HCV-29T cell line was derived from an invasive human urinary bladder transitional cell carcinoma. We have previously reported that these HCV-29T cells express high numbers of unoccupied, specific binding sites or 'receptors' for hyaluronan (Nemec et al. 1987), yet synthesize and secrete only low levels of endogenous hyaluronan (Knudson and Knudson, 1990). When placed in low-density culture for 12-24 h the cells attach, spread on the substrate, and assume a somewhat heterogeneous morphology, from round to polygonal. As shown in Fig. 1A and B, using the particle-exclusion assay, no pericellular matrices were detectable around the cells. This feature appeared independent of the length of time in culture (data not shown). Incubation of these cells for 24 h in the presence of exogenously added hyaluronan alone (Fig. 1C and D), or large hyaluronan-aggregating chondroitin sulfate proteoglycan alone (Fig. 1E and F) did not result in pericellular matrix formation. However, if the HCV-29T cells were incubated for 24 h in the presence of both hyaluronan and proteoglycan monomers, an extensive pericellular matrix was assembled around the cells (Fig. 1G and H). A morphometric analysis of these data is given in Table 1. Matrix formation in the presence of these components occurred within 2h, with maximal matrix assembly established after 18h (data not shown). Additionally, pericellular matrix assembly was observed on HCV-29T cells in the presence of hyaluronan and proteoglycan monomers derived from bovine articular (cartilage ratio=2.75±0.40) or, bovine nasal (2.47±0.22) cartilages, similar in size to the matrices formed with hyaluronan and rat chondrosarcoma proteoglycan (Table 1). These data suggest that the formation of an extensive pericellular matrix, with the capacity to exclude fixed red blood cells, can be obtained with a minimum of two extracellular matrix macromolecules: namely, hyaluronan and an aggregating proteoglycan.

To determine the mode of interaction of the pericellular matrix with the HCV-29T cell surface, hyaluronan tetra-/hexasaccharides were introduced into the hyaluronan plus proteoglycan mixture. As shown in Fig 2A, the presence of the hyaluronan hexasaccharides prevented the assembly of pericellular matrices (see also Table 1). These data suggest that the mechanism of pericellular matrix assembly by these cells is similar to that of cells derived from embryonic tibial chondrocytes (Knudson and Kuecettner, 1980), i.e. the matrix is anchored to the cell surface via specific hyaluronan receptor sites. Additionally, a fully assembled pericellular matrix, formed on HCV-29T cells following 24h incubation in the presence of exogenous hyaluronan and proteoglycan (Fig. 1G), could be displaced within 3h by the subsequent addition of
Fig. 1. Assembly of a pericellular matrix with exogenous matrix macromolecules on HCV-29T cells. (A, C, E and G) Phase-contrast photomicrographs; (B, D, F and H) corresponding cells visualized via Hoffman optics. Medium from HCV-29T cells grown in low-density culture for 24 h was removed and replaced with either: fresh medium as a control (A and B); medium containing hyaluronan (C and D); medium containing proteoglycan (E and F); or medium containing hyaluronan plus proteoglycan (G and H). Following these additions, cells were incubated for 24 h, test or control media were removed and the cells assayed by the particle exclusion method. Only cells incubated in the presence of both hyaluronan and proteoglycan exhibited significant pericellular matrices (G and H). Bar, 50 μm.
Table 1. Morphometric analysis of matrix to cell area ratios

<table>
<thead>
<tr>
<th>Cell-type</th>
<th>Control</th>
<th>HA</th>
<th>PG</th>
<th>HA/PG</th>
<th>HA/PG/HA/HA$_{4-s}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-29T</td>
<td>1.03±0.03</td>
<td>1.03±0.05</td>
<td>1.28±0.11</td>
<td>3.47±0.97</td>
<td>1.04±0.02</td>
</tr>
<tr>
<td>SV-3T3</td>
<td>1.04±0.03</td>
<td>1.02±0.05</td>
<td>1.19±0.07</td>
<td>2.86±0.39</td>
<td>1.02±0.02</td>
</tr>
<tr>
<td>HU-456</td>
<td>1.02±0.02</td>
<td>1.01±0.05</td>
<td>1.01±0.03</td>
<td>2.29±0.36</td>
<td>1.01±0.03</td>
</tr>
<tr>
<td>RT-4</td>
<td>1.02±0.03</td>
<td>1.01±0.01</td>
<td>1.01±0.02</td>
<td>1.01±0.02</td>
<td>1.01±0.02</td>
</tr>
<tr>
<td>B-16</td>
<td>1.04±0.04</td>
<td>1.04±0.07</td>
<td>1.02±0.02</td>
<td>1.00±0.04</td>
<td>1.04±0.08</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>1.05±0.05</td>
<td>1.03±0.05</td>
<td>1.07±0.06</td>
<td>2.69±0.45</td>
<td>1.06±0.06</td>
</tr>
</tbody>
</table>

Values represent the mean ratio of the area delineated by the pericellular matrix to the area delineated by the plasma membrane. Error values represent the 95% confidence range from the mean, n=20–30 cells. Control, medium alone; HA, medium containing hyaluronan; PG, medium containing proteoglycan; HA/PG, medium containing added hyaluronan and proteoglycan; HA/PG/HA$_{4-s}$, medium containing hyaluronan, proteoglycan and hyaluronan tetra-/hexasaccharides.

hyaluronan hexasaccharides (Fig. 2C). Medium alone for 3h had no effect on the size of the assembled matrix (Fig. 2B). Therefore the pericellular matrix is initially assembled and subsequently maintained via anchorage to these hyaluronan binding sites.

To exclude the possibility that addition of exogenous proteoglycan and hyaluronan induced the synthesis of an endogenous component(s) required for matrix assembly, hyaluronan and proteoglycan were added to glutaraldehyde-fixed HCV-29T cells. We have shown previously that the cell surface hyaluronan binding sites are stable to mild fixation in glutaraldehyde (Nemec et al. 1987). Following 24 h of incubation of the fixed HCV-29T cells with optimal concentrations of hyaluronan and proteoglycan, a pericellular matrix again assembled, with a coat:cell ratio of 3.57±0.77. This matrix was equivalent in size to matrices assembled on live HCV-29T cells (Table 1). Pericellular matrices on fixed cells did not form without the addition of the exogenous macromolecules (1.02±0.03), or when hyaluronan and proteoglycan were added in the presence of excess hyaluronan tetra-/hexasaccharides (1.00±0.02). Like the live cells, fully assembled matrices on fixed cells could also be displaced by subsequent incubation with hyaluronan tetra-/hexasaccharides (1.02±0.02). Additionally, the fixed HCV-29T cells were able to assemble a pericellular matrix (2.54±0.31) when incubated in serum-free medium containing exogenous hyaluronan and proteoglycan. Therefore, serum components are also not required for matrix assembly.

In order to determine the stability or persistence of these pericellular matrices formed from exogenous components, HCV-29T carcinoma cells were incubated with optimal concentrations of hyaluronan plus proteoglycan for 24 h, rinsed extensively and then incubated in fresh culture medium minus the exogenous macromolecules. After various chase times, cell cultures were assayed by the particle exclusion assay for persistence of pericellular matrix in the absence of the exogenous components. As shown in Table 2, the pericellular matrix on live cells, which had a mean coat:cell ratio of approximately 2.7 at the start of the chase experiment, showed no reduction in coat:cell ratio at 3 h (see also Fig. 2B), a reduced but still Fig. 2. The effect of hyaluronan hexasaccharides on the assembly and maintenance of HCV-29T cell pericellular matrices. (A) Medium from HCV-29T cells grown in low-density culture for 24 h was removed and replaced with medium containing hyaluronan, proteoglycan and excess hyaluronan tetra-/hexasaccharides, incubated for 24 h and then assayed by the particle exclusion method. No pericellular matrix assembled under these conditions. (B and C) HCV-29T cell medium was replaced with medium containing hyaluronan plus proteoglycan and incubated for 24 h in order to establish pericellular matrices around the cells. This medium was then removed and replaced with either medium alone as a control (B), or medium containing an excess of hyaluronan tetra-/hexasaccharides (C). The cells were incubated for an additional 3 h, and then assayed by the particle exclusion method. The hyaluronan tetra-/hexasaccharides displaced the pericellular matrix (C). Cells in medium alone, representing non-specific shedding into the culture medium, retained the matrix (B). Bar, 50 μm.

*Fig. 2. The effect of hyaluronan hexasaccharides on the assembly and maintenance of HCV-29T cell pericellular matrices. (A) Medium from HCV-29T cells grown in low-density culture for 24 h was removed and replaced with medium containing hyaluronan, proteoglycan and excess hyaluronan tetra-/hexasaccharides, incubated for 24 h and then assayed by the particle exclusion method. No pericellular matrix assembled under these conditions. (B and C) HCV-29T cell medium was replaced with medium containing hyaluronan plus proteoglycan and incubated for 24 h in order to establish pericellular matrices around the cells. This medium was then removed and replaced with either medium alone as a control (B), or medium containing an excess of hyaluronan tetra-/hexasaccharides (C). The cells were incubated for an additional 3 h, and then assayed by the particle exclusion method. The hyaluronan tetra-/hexasaccharides displaced the pericellular matrix (C). Cells in medium alone, representing non-specific shedding into the culture medium, retained the matrix (B). Bar, 50 μm.*

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significant pericellular matrix at 6 h and total absence of matrix at later time points. The pericellular matrix on fixed, nonliving HCV-29T cells showed a more gradual loss of coat size (Table 2). Therefore, the HCV-29T pericellular matrix assembled from exogenous matrix macromolecules does not depend on continuous exposure to high concentrations of the soluble macromolecules in order to be maintained. Nevertheless, these data also support the suggestion that these cells cannot, on their own, synthesize the macromolecules required for maintenance of the matrix and any turnover or shedding of exogenously added components cannot be replaced.

Correlation of matrix assembly with expression of hyaluronan receptors on other cell types
In order to test further the hypothesis that cell surface hyaluronan receptors are essential for assembly and organization of pericellular matrices, hyaluronan and proteoglycan were added to several cell types, which were either positive or negative for expression of hyaluronan receptors. Cell surface hyaluronan-specific receptors on SV-3T3 cells have been extensively reported (Underhill and Toole, 1979; Toole, 1981). In addition, Toole et al. (personal communication), at Tufts University in Boston, using a newly developed monoclonal antibody directed against the hyaluronan receptor present in embryonic chick brain, have described the immunohistological localization of hyaluronan receptors on bovine capillary endothelial cells. To characterize the other cell types, direct hyaluronan binding assays were performed. As shown in Fig. 3, only the HU-456 cells (and the HCV-29T cells used as a positive control) exhibited specific, saturable binding of \( ^{3} \text{H} \)-labeled hyaluronan. No binding was detected on the RT-4 or B-16 cells.

These cells were next studied for their capacity to assemble pericellular matrices via exogenous macromolecules. As shown in Table 1, like the HCV-29T cells, without addition of exogenous macromolecules none of the cell cultures tested exhibited endogenous pericellular matrices. However, upon addition of hyaluronan and proteoglycan, the SV-3T3 and HU-456 tumor cells, as well as the normal, low-passage bovine aortic endothelial cells, assembled pericellular matrices with coat:cell ratios similar to the HCV-29T cells. No matrices were observed on the RT-4 or B-16 cells. Also, no matrix assembly was observed on any of the cell cultures in the presence of hyaluronan alone, proteoglycan alone or hyaluronan, proteoglycan and excess hyaluronan tetra-/hexasaccharides. Thus only cells that exhibited expression of hyaluronan receptors had the capacity to assemble pericellular matrices via exogenous macromolecules.

Other, non-chondrogenic cells such as fibroblasts naturally exhibit endogenous hyaluronan-dependent pericellular matrices in culture (Clarris and Fraser, 1968; Knudson and Toole, 1985a). In order to determine whether these cells can also utilize exogenous macromolecules for matrix assembly, hyaluronan and proteoglycan were added to rat fibrosarcoma cells stripped of their endogenous matrix. The large pericellular matrices of rat fibrosarcoma cells (Fig. 4A) were removed via a brief treatment with Streptomyces hyaluronidase. Following the enzymatic removal of this pericellular matrix, regrowth of an endogenous pericellular matrix required approximately 8 h of incubation (data not shown). However, upon addition of exogenous hyaluronan plus proteoglycan, a pericellular matrix assembled within 2 h (Fig. 4C), at which time no endogenous matrix was present in control cultures (Fig. 4B). Additionally, the inclusion of hyaluronan tetra-/hexasaccharides along with the hyaluronan plus proteoglycan mixture, prevented pericellular matrix assembly via the exogenous macromolecules (Fig. 4D). These fibroblast-like cells can therefore assemble similar pericellular matrices using either endogenous or exogenous macromolecules. The data also suggest that these matrices are anchored via hyaluronan receptors, the expression of which on these cells has been controversial (Underhill, 1988).

Discussion
Extensive pericellular matrices, of varying thicknesses,
Fig. 4. Assembly of a pericellular matrix with exogenous macromolecules on *Streptomyces* hyaluronidase-stripped rat fibrosarcoma cells. Rat fibrosarcoma cells, grown in low-density culture for 24 h, exhibit an extensive endogenous pericellular matrix (A). This matrix can be stripped from the cell surface by a 1 h treatment at 37°C with medium containing *Streptomyces* hyaluronidase. The hyaluronidase-treated cells were then washed and incubated with either fresh medium alone (B); medium containing containing hyaluronan and proteoglycan (C); or medium containing hyaluronan, proteoglycan and excess hyaluronan tetra-/hexasaccharides (D). Following these additions, cells were incubated for an additional 2 h and then assayed by the particle exclusion method. In this time frame, no endogenous pericellular matrix was assembled (B). However, cells incubated with exogenous hyaluronan and proteoglycan for 2 h assembled a pericellular matrix (C). Pericellular matrix assembly in the presence of hyaluronan oligosaccharides was blocked (D). Bar, 50 μm.

have been visualized surrounding a wide variety of eukaryotic cells in culture (reviewed by Toole, 1981; also Clarris and Fraser, 1968; Knudson and Toole, 1985; Knudson and Kuettnner, 1990; Underhill and Toole, 1982). The principal feature known concerning the structural composition of these pericellular matrices is that they are dependent on hyaluronan. Clarris and Fraser (1968) demonstrated that the pericellular matrix surrounding synovial fibroblasts was not sensitive to treatment by ribonuclease, deoxyribonuclease, neuraminidase, EDTA or dilute trypsin, but was highly sensitive to various purified hyaluronidases (though the hyaluronidases used were not specific for hyaluronan). Subsequent studies using *Streptomyces* hyaluronidase demonstrated that hyaluronan was indeed an essential structural component of these matrices (Toole, 1981; Knudson and Toole, 1985a). Goldberg and Toole (1982) further showed that inhibition of hyaluronan synthesis by treatment of fibrosarcoma cells with monensin also inhibited formation of detectable pericellular matrices. Underhill and Toole (1982) suggested that the size of the coat may even be proportional to the content of cell surface hyaluronan. In subsequent studies, however it was observed that cells of embryonic limb mesenchyme could turn on, or off, the expression of pericellular matrices without change in the levels of their cell surface hyaluronan, suggesting that other components are also required for matrix formation (Knudson and Toole, 1985a). Indeed, it was suggested earlier that these matrices were too thick to be composed entirely of hyaluronan (Toole, 1981) and most likely represented secondary interactions between hyaluronan and other molecules such as proteoglycans, fibronectin or proteoglycan-collagen complexes, none of which have been fully characterized in terms of their capacity to be organized into a coat structure. Therefore, the first conclusion from our present results is that the formation of a pericellular matrix, which can exclude particles or cells, can be obtained with as few as two components, the addition of exogenous hyaluronan and an aggregating-type proteoglycan. Preliminary work (C. Knudson, unpublished data) has further shown that the proteoglycan component must also be structurally intact. No pericellular matrix was formed when exogenous hyaluronan was added in combination with: (1) chondroitinase ABC-digested proteoglycan, (2) reduced and alkylated proteoglycan, (3) the proteoglycan hyaluronan-binding domain, or (4) the chondroitin sulfate-rich domain of the proteoglycan.

A second conclusion of this study is that the ability to assemble a pericellular matrix requires the expression of cell surface hyaluronan binding sites or 'receptors'. The pericellular matrix that is organized around the cells used in this study (i.e. HCV-29T, SV-3T3, HU-456 and bovine aortic endothelial cells) appears, to be anchored to the cell surface via interactions between hyaluronan and hyaluronan receptors. This conclusion was drawn because the
pericellular matrices could be prevented from assembling, or displaced from the cells once assembled, via the addition of an excess of hyaluronan hexasaccharides, which are competitive inhibitors of hyaluronan receptors. Similar observations have been made concerning the assembly of pericellular matrices on embryonic chick chondrocytes and adult chondrocytes from a rat chondrosarcoma cell line (Knudson and Kuettner, 1990; Knudson et al. 1991). Cells such as the B-16 mouse melanoma or RT-4 non-invasive bladder papilloma, which did not express hyaluronan binding sites, did not assemble a pericellular matrix when presented with exogenously added hyaluronan plus proteoglycan. The capacity to assemble a pericellular matrix, given the appropriate exogenous components, appears indicative of hyaluronan receptor expression and may therefore serve as a simple, indirect assay for the detection of hyaluronan receptors.

The in vivo presence or function of extensive pericellular matrices that surround many cell types in culture is not well established. Clearly, the hyaluronate-dependent pericellular matrices around chondrocytes correlates well with their primary cellular function: to generate and organize an extracellular matrix capable of withstanding load forces. The degree of extracellular matrix surrounding chondrocytes in vivo also seems to correlate well with the expression of pericellular matrices in vitro, suggesting that these structures do in fact exist in vivo (Knudson and Toole, 1985a).

The physiological function of pericellular matrices surrounding other cell types is less well understood. Early work in this area suggested that such matrices may serve as a protective barrier around cells. For example, human white blood cells were observed to be excluded from a zone surrounding cultured synovial fibroblasts (Clariss and Fraser, 1988), in a fashion similar to the exclusion of red blood cells used in the particle exclusion assays. McBride and Bard (1979) demonstrated that T-lymphocytes were similarly excluded from a zone surrounding fibrosarcoma cells in culture. This exclusion apparently inhibited their lymphocyte-mediated cytolysis, which returned after removal of the pericellular matrix with hyaluronidases. Thus, extensive pericellular matrices may function to repel or exclude other cells, conferring a degree of protection from cyotoxic cell types. It has also been proposed that extensive pericellular matrices reduce interaction between homologous cells, in other words, modulate cell-cell aggregation (Underhill, 1982; Knudson and Malecki, 1987).

In considering whether these structures exist in vivo, the source of the extracellular macromolecules required for the formation of these pericellular matrices must also be considered. Certainly chondrocyte-derived aggregating proteoglycans are not present at high concentrations in other distant connective tissues. It is interesting to consider that the KBV-29T chondrosarcoma cells (Knudson and Knudson, 1990; Pauli and Knudson, 1988) and the bovine aortic endothelial cells (Knudson et al. 1988) have the capacity to stimulate the synthesis of matrix macromolecules by neighboring cell types such as fibroblasts. It is therefore possible that the function of this stimulation of synthesis may be to provide the matrix components required for the formation of a pericellular matrix around the tumor or endothelial cells. Can fibroblasts produce the required matrix-forming macromolecules, that is, hyaluronan plus an aggregating proteoglycan? In preliminary studies, we observed that the addition of 25% conditioned medium isolated from rat fibrosarcoma cells to HCV-29T cells for 24 h resulted in the assembly of a prominent pericellular matrix (coat:cell ratio = 2.23±0.52) after 24 h. The exact composition of the macromolecules present in this conditioned medium is still under investigation. It is known that these cells synthesize copious amounts of hyaluronan (Underhill and Toole, 1982). However, our model would imply that a fibroblast-derived proteoglycan, capable of aggregating with hyaluronan, must also be present. Other non-cartilage-specific proteoglycans such as versican (Johannsson et al. 1975; Zimmerman and Roulaht, 1989), as well as glial hyaluronan binding protein (Perides et al. 1989) and hyaluronectin (Delpech et al. 1982), have also been reported to interact strongly with hyaluronan (or proposed to interact via sequence homology to cartilage link proteins). It remains to be seen whether any of these molecules, in association with hyaluronan, can promote the assembly of pericellular matrices around matrix-deficient cells expressing hyaluronan receptors. Nonetheless, it is interesting to note that during the infiltration of basal cell carcinomas, part of the intense stromal response at the advance of the invading tumor cells includes the elevated deposition of hyaluronectin, synthesized by the stromal fibroblasts (Delpech et al. 1982). This hyaluronectin, along with hyaluronan, may serve as an exogenous component for pericellular matrix formation around the infiltrating neoplastic cells or migrating endothelial cells involved in neovascularization.

References


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(Received 12 December 1990 – Accepted 27 February 1991)