

## SPECIFIC ENDOCYTOSIS AND CATABOLISM IN THE SCAVENGER ENDOTHELIAL CELLS OF COD (*GADUS MORHUA* L.) GENERATE HIGH-ENERGY METABOLITES

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### Summary

The catabolic fate of circulating hyaluronan and the proteoglycan chondroitin sulphate (CSPG) was studied in the Atlantic cod (*Gadus morhua* L.). Distribution studies using radio-iodinated ligand demonstrated that CSPG was rapidly eliminated from the blood by the endocardial endothelial cells (EECs) of the heart atrium and ventricle. The presence of excess amounts of hyaluronan or CSPG inhibited uptake of [<sup>125</sup>I]hyaluronan into cultured atrial EECs (aEECs) by 46 % and 84 %, respectively. Neither formaldehyde-treated serum albumin (FSA) nor mannose inhibited this uptake. The presence of excess amounts of CSPG and hyaluronan inhibited uptake of [<sup>125</sup>I]CSPG by 90 % and 42 %, respectively, suggesting that aEECs express a specific hyaluronan binding site that also recognizes CSPG. FSA inhibited endocytosis of [<sup>125</sup>I]CSPG by 65 %, indicating that CSPG is also recognized by the scavenger receptor. Approximately

17 % and 57 % of added [<sup>125</sup>I]hyaluronan and 15 % and 65 % of the added [<sup>125</sup>I]CSPG were endocytosed after 1 and 24 h, respectively. High-performance liquid chromatographic analyses of the spent medium after endocytosis of hyaluronan and CSPG serglycin labelled biosynthetically with <sup>3</sup>H in the acetyl groups identified labelled the low-molecular-mass degradation products as [<sup>3</sup>H]acetate, indicating that aEECs operate anaerobically. These findings suggest that acetate released from cod EECs following catabolism of endocytosed hyaluronan and CSPG represents a high-energy metabolite that may fuel cardiomyocytes.

Key words: hyaluronan, chondroitin sulphate, endocytosis, endothelium, endocardium, hyaluronan receptor, Atlantic cod, *Gadus morhua*, energy metabolism.

### Introduction

Mammalian sinusoidal liver endothelial cells (LECs) represent a major part of the reticuloendothelial system (Smedsrød et al., 1990). These cells have an extensive capacity to endocytose and degrade soluble physiological, and foreign blood-borne, macromolecular waste substances. To fulfil this function, mammalian LECs express five major types of specific endocytosis receptor: (i) the hyaluronan receptor (which eliminates major matrix polysaccharides and proteoglycans such as hyaluronan and chondroitin sulphate); (ii) the collagen  $\alpha$ -chain receptor (which eliminates collagen  $\alpha$ -chains or physiologically denatured collagen); (iii) the scavenger receptor (which eliminates negatively charged macromolecules such as the amino-terminal propeptides of types I and III procollagen, physiologically and artificially modified macromolecules such as atherogenic advanced glycation end products (Smedsrød et al., 1997) and oxidized low-density lipoproteins); (iv) the mannose receptor (which eliminates the carboxy-terminal propeptide of type I procollagen, lysosomal enzymes and tissue plasminogen activator); and (v) the Fc receptor (which eliminates IgG–antigen complexes). Studies performed

recently in our laboratory with animal species from all seven principal vertebrate classes suggest that all vertebrates carry a population of specialized endothelial cells that express at least two of the five LEC receptors involved in the scavenging of circulating soluble waste macromolecules, namely the collagen  $\alpha$ -chain receptor and the scavenger receptor (Smedsrød et al., 1999). Since the specialized vertebrate scavenger type of endothelial cells are located in the liver of mammals, birds, reptiles and amphibians, whereas all other vertebrates studied carry these cells in the kidney, heart or gills, the term ‘scavenger endothelial cells’ (SECs) was proposed to make it possible to discuss these cells and their unique scavenger functions in all vertebrates without having to refer to the particular organ in which they are located.

The endocardial endothelial cells (EECs) lining the sinusoid-like trabeculae of both heart chambers represent the SECs of the Atlantic cod (*Gadus morhua* L.). These cells in cod carry three of the five major endocytosis receptors found in mammalian LECs: (i) the collagen  $\alpha$ -chain receptor (Koren et al., 1997; Smedsrød et al., 1995); (ii) the mannose receptor

(T. Sørensen, unpublished results); and (iii) the scavenger receptor (Sørensen et al., 1998).

Hyaluronan and the proteoglycan chondroitin sulphate (CSPG) aggrecan are important structural components of the extracellular matrix of all higher animals, with the largest amounts being found in soft connective tissue (Laurent and Fraser, 1992). In mammals, disposal of hyaluronan occurs at three main levels: (i) locally, in the tissues where hyaluronan is synthesized and (ii) in the lymph nodes after displacement from the tissues; (iii) hyaluronan that is not eliminated by the lymph nodes enters the bloodstream (Fraser et al., 1997). The majority (85–90%) of the blood-borne hyaluronan of mammals is removed by the liver (Fraser et al., 1983; Fraser et al., 1986), with the remainder being removed by the spleen (Fraser et al., 1981), kidneys (Bentsen et al., 1986) and bone marrow (Fraser et al., 1983). Studies in our laboratory on the turnover of hyaluronan in the Atlantic cod show that hyaluronan deposited in peripheral tissues is transferred quantitatively to the major blood circulation, from where it is efficiently eliminated by endocytosis in EECs (Sørensen et al., 1997). This suggests that EECs represent the major site of uptake and degradation of all hyaluronan released from the tissues in cod.

The removal of hyaluronan from the blood in mammals is mediated by an endocytic cell surface receptor on LECs that also recognizes CSPG (McCourt et al., 1999; Smedsrød et al., 1984). After internalization, hyaluronan is subjected to lysosomal degradation, yielding glucuronic acid and *N*-acetylglucosamine. The acetyl groups released from *N*-acetylglucosamine are then transformed to acetate, whereas the pyranose rings are degraded to lactate (Smedsrød et al., 1984). Both lactate and acetate are released from the LECs and oxidized by the hepatocytes to CO<sub>2</sub> and H<sub>2</sub>O (for a review on hyaluronan catabolism, see Smedsrød, 1991).

In the present study, we demonstrate that the cod EECs represent the major site of clearance of both hyaluronan and CSPG from the blood. Following endocytosis, these substances are subjected to endosomal/lysosomal degradation *in vitro*. The low-molecular-mass degradation products released from the cells after endocytosis of hyaluronan and CSPG labelled with <sup>3</sup>H in the acetyl groups are quantitatively identified as [<sup>3</sup>H]acetate. We suggest that this product may serve as a fuel enabling the cardiomyocytes to produce ATP for contraction.

## Materials and methods

### Animals

Fish for *in vivo* studies, Atlantic cod (*Gadus morhua* L.) (0.1–1 kg), were obtained from Tromsø Aquaculture Station (Norwegian Institute of Fisheries and Aquaculture Ltd, Tromsø, Norway). The fish were kept in plastic tanks (2000 l) supplied with running sea water (at 8 °C). All fish were adapted to the test conditions for at least 1 week before the experiments started and fed a commercial diet daily and during the adaptation and experimental periods. For cell isolation, hearts from net-pen captured Atlantic cod (1–4 kg) were used. These

fish were kept in large dip nets in the sea (3–10 °C) and fed a commercial diet.

### Chemicals and media

High-molecular-mass hyaluronan (Healon), Sephadex G-25 (PD-10 disposable column), Sepharose S-300, Q-Sepharose Fast Flow and Superose 6 HR 10/30 were obtained from Pharmacia (Uppsala, Sweden). Carrier-free Na<sup>125</sup>I was purchased from the Institute of Energy Technology (Kjeller, Norway). Sodium [<sup>3</sup>H]acetate was obtained from NEN Life Science Products, Inc. (Boston, MA, USA). The liquid scintillation cocktail Ultima Gold™ was purchased from Packard Instruments (Groningen, Netherlands). Leibovitz 15 (L-15) medium was obtained from Gibco (Grand Island, NY, USA), adjusted to 380 mosmol l<sup>-1</sup> using 0.5 mol l<sup>-1</sup> NaCl and supplemented with 0.33 g l<sup>-1</sup> glucose and 0.05 g l<sup>-1</sup> gentamycin. Foetal calf serum and heparin were purchased from Hyclone (Logan, UT, USA) and Novo Nordisk (Copenhagen, Denmark), respectively. Human serum albumin (HSA) and trypsin (1:250) were purchased from Octapharma (Wien, Austria) and Difco (Detroit, MI, USA), respectively. Chondroitinase ABC was obtained from Seikagaku Kogyo Corp. (Tokyo, Japan). Blue dextran was purchased from Pharmacia (Uppsala, Sweden). All other chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

### Ligands for endocytosis studies

Formaldehyde-treated serum albumin (FSA) was prepared as described by Mego et al. (Mego et al., 1967). Hyaluronan-amine (*M<sub>r</sub>* 3×10<sup>6</sup>) was generously given by Dr Paul H. Weigel (University of Oklahoma, USA). Metabolically labelled [<sup>3</sup>H]hyaluronan with a specific activity of 4.2×10<sup>5</sup> disintegrations min<sup>-1</sup> μg<sup>-1</sup> (*M<sub>r</sub>* 3.85×10<sup>6</sup>) was a kind gift from Dr Robert Fraser (Department of Biochemistry, Monash University, Clayton, Australia). Bovine CSPG was a kind gift from Dr Håkan Pertoft (Uppsala, Sweden). Biosynthetically labelled CSPG serglycin was prepared as described below.

### Preparation of <sup>125</sup>I-labelled ligands

Iodination of hyaluronan-amine and CSPG was performed with carrier-free Na<sup>125</sup>I in a direct reaction using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce, Rockford, IL, USA) as oxidizing agent, according to instructions provided by the manufacturer. Free iodine was removed from the solution by gel filtration on a PD-10 column eluted with phosphate-buffered saline (PBS). The specific activities of [<sup>125</sup>I]hyaluronan and [<sup>125</sup>I]CSPG were 6.61×10<sup>6</sup> cts min<sup>-1</sup> μg<sup>-1</sup> and 1×10<sup>6</sup> cts min<sup>-1</sup> μg<sup>-1</sup>, respectively. Radioactivity was measured in a Packard gamma counter (Packard Instruments, Groningen, Netherlands).

### Preparation of biosynthetically labelled [<sup>3</sup>H]CSPG serglycin

The CSPG serglycin produced by the monocytic cell line THP-1 was biosynthetically labelled with sodium [<sup>3</sup>H]acetate. In brief, THP-1 cells (Tsuchiya et al., 1980) were grown in

RPMI-1640 containing 10% foetal calf serum. Prior to labelling with sodium [ $^3\text{H}$ ]acetate, the cells were washed extensively with RPMI-1640. The cells were then cultured for 30 h in the presence of 5.55 MBq of sodium [ $^3\text{H}$ ]acetate per millilitre of serum-free RPMI-1640, before the culture medium was harvested and the radiolabelled macromolecules isolated as described below.

Conditioned medium containing  $^3\text{H}$ -labelled macromolecules was subjected to Q-Sepharose anion-exchange chromatography. The column was equilibrated with a buffer containing  $8\text{ mol l}^{-1}$  urea,  $0.2\text{ mol l}^{-1}$  NaCl,  $0.05\text{ mol l}^{-1}$  sodium acetate (pH 6.0), 0.5% Triton X-100 and protease inhibitors ( $20\text{ mmol l}^{-1}$  EDTA,  $2\text{ mmol l}^{-1}$  *N*-ethylmaleimide (NEM),  $2\text{ mmol l}^{-1}$  Pefabloc SC,  $1.5\text{ }\mu\text{mol l}^{-1}$  pepstatin A) and washed extensively with the same solution after the sample had been applied. Bound material was eluted with a gradient of 0.2 to  $1.2\text{ mol l}^{-1}$  NaCl in urea buffer and collected in fractions of 1.5 ml per 6 min. Samples were analyzed for radioactivity and conductivity, and the fractions containing the radiolabelled macromolecules were pooled. To reduce the volume of the material, the sample was diluted and re-applied to a column of Q-Sepharose equilibrated in the urea buffer described above. The bound macromolecules were eluted with a  $4\text{ mol l}^{-1}$  guanidine HCl solution containing  $0.05\text{ mol l}^{-1}$  sodium acetate (pH 6.0), 0.5% Triton X-100 and protease inhibitors. The radioactive eluate was passed through a Superose 6 HR 10/30 column in  $4\text{ mol l}^{-1}$  guanidine HCl,  $0.05\text{ mol l}^{-1}$  sodium acetate, 0.5% Chaps and protease inhibitors at flow rate of  $0.4\text{ ml min}^{-1}$ . Fractions of 0.4 ml were collected, and samples of these were analyzed for radioactivity. Selected fractions were pooled, dialyzed against distilled water and subjected to vacuum concentration.

To confirm that the radioactivity was incorporated in the chondroitin sulphate chains of the isolated material, a sample was treated with chondroitinase ABC, an enzyme that specifically depolymerizes chondroitin sulphate chains under the conditions used. The sample was incubated at  $37^\circ\text{C}$  for a minimum of 2 h with 0.05 units of chondroitinase ABC per millilitre of a buffer containing  $5\text{ mmol l}^{-1}$  Tris-HCl,  $5\text{ mmol l}^{-1}$  sodium acetate (pH 8.0), protease inhibitors and 0.5% Triton X-100. The chondroitinase-ABC-treated sample and an untreated sample were analyzed by Superose 6 HR 10/30 gel chromatography. More than 90% of the  $^3\text{H}$ -labelled macromolecules were depolymerized by the enzyme, confirming that they originated from chondroitin sulphate.

#### *Preparation of FITC-labelled hyaluronan*

FITC-labelled hyaluronan was prepared according to the method of Sørensen et al. (Sørensen et al., 1997).

#### *Anatomical distribution*

Five cod were anaesthetized by immersion in 0.004% (w/v) benzocaine solution and injected intravenously through the caudal vein with trace amounts of [ $^{125}\text{I}$ ]CSPG ( $0.5\text{--}1\text{ }\mu\text{g kg}^{-1}$  body mass) in a total injection volume of  $500\text{ }\mu\text{l}$  of PBS per fish. After 1 h, blood samples were collected from

the caudal vein approximately 2 cm caudal to the injection site. The fish were killed by a blow to the head, without prior anaesthesia, immediately after blood sampling. Heart, anterior kidney, liver and blood samples were removed and analysed for radioactivity in a Packard gamma counter.

#### *Preparation of EECs from cod atrium*

Functionally intact aEECs from cod were purified (according to the method of Koren et al., 1997). Briefly, the heart was dissected out and perfused with L-15 medium containing heparin ( $10\text{ i.u. ml}^{-1}$ ). The atria were dissected free and cut open. Ostial tissue rich in fibrocytes and macrophages was discarded before transfer to a 50 ml sterile plastic tube with 25 ml of calcium-free buffer (Pertoft and Smedsrød, 1987). After a 30 min incubation with horizontal shaking ( $250\text{ cycles min}^{-1}$ ), the buffer was changed and the atria were incubated with the following solutions: (i) trypsin ( $0.5\text{ mg ml}^{-1}$ ) and EDTA ( $0.1\text{ mg ml}^{-1}$ ) in PBS for 5 min and (ii) collagenase ( $0.5\text{ mg ml}^{-1}$  in L-15 medium supplemented with  $0.7\text{ mg ml}^{-1}$   $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ ) for 30 min. The contents of the tube were transferred to a sterile Petri dish, and the atrium was flushed several times with a jet from a 10 ml plastic syringe, re-using the cell suspension obtained. Remaining tissue was discarded, and the cell suspension was centrifuged for 5 min at  $400\text{ g}$ , after which the supernatant was removed and the pellet was resuspended in 10 ml of L-15 medium. Contaminating macrophages and other adherent cells were removed according to the method of Sørensen et al. (Sørensen et al., 1998). Non-adherent cells were seeded on tissue culture plastic (Falcon, Becton Dickinson & Company, NJ, USA) or glass slides, both precoated with fibronectin ( $0.5\text{ mg ml}^{-1}$ ) to enable attachment and spreading of aEECs. The incubation medium was L-15 supplemented with 10% foetal calf serum. The isolation procedure and incubations were carried out at  $12\text{--}14^\circ\text{C}$ , and the cells were washed with L-15 medium after 24 h and used in experiments the same day or the following day. The number of cells seeded per  $2\text{ cm}^2$  was approximately  $10^6$ . On average, more than 90% of the cells were aEECs, as evaluated by phase contrast microscopy. Contaminating cells were mostly cardiomyocytes and a few macrophages.

#### *Endocytosis of labelled ligands by cultured cod aEECs*

Cod aEEC cultures established in  $2\text{ cm}^2$  wells (approximately  $3\times 10^5\text{ cells cm}^{-2}$  attached and spread) were washed three times with L-15 medium and supplied with fresh L-15 medium with 1% HSA and trace amounts of radiolabelled hyaluronan ( $20\times 10^3\text{ cts min}^{-1}$ ; approximately 10–20 ng) or CSPG ( $20\times 10^3\text{ cts min}^{-1}$ ; approximately 10–20 ng) in a total incubation volume of  $200\text{ }\mu\text{l}$  per well. Incubations of radiolabelled hyaluronan at  $12^\circ\text{C}$  were terminated after various times by removing the incubation medium together with one washing volume of  $500\text{ }\mu\text{l}$  of PBS. The incubation medium was then transferred to a PD-10 column and eluted with PBS. Fractions of  $0.5\text{ ml}$  were collected. Intact ligand eluted in the void volume, and degradation products eluted in the total volume. Incubations of

radiolabelled CSPG were terminated by transferring the incubation medium together with one washing volume of 500 µl of PBS to tubes containing 800 µl of 20% trichloroacetic acid. This procedure precipitates only undegraded protein or intermediate degradation products of high molecular mass. The extent of degradation was determined by measuring the radioactivity in the pellet and the supernatant after centrifugation. Cell-associated hyaluronan and CSPG were quantified by solubilizing the cell layer with 1% SDS, followed by counting in a gamma counter.

The specificity of uptake of [<sup>125</sup>I]hyaluronan and [<sup>125</sup>I]CSPG was examined using ligand competition studies. Monolayer cultures were incubated for 2 h with trace amounts (20 × 10<sup>3</sup> cts min<sup>-1</sup>; approximately 10–20 ng) of labelled ligand alone (control) or together with excess amounts of non-labelled macromolecules (100 µg ml<sup>-1</sup>) or mannose (50 mmol l<sup>-1</sup>). Endocytosis experiments were terminated after 2 h at 12–14 °C by removing the incubation medium together with one washing volume of 500 µl of PBS, which were transferred to a PD-10 column and eluted with PBS. Intact ligand eluted in the void volume and degraded ligand in the total volume. Cell-associated ligand was quantified by solubilizing the cell layer with 1% SDS, followed by counting in a gamma counter.

#### *Pulse-chase studies on the intracellular degradation of [<sup>125</sup>I]hyaluronan and [<sup>125</sup>I]CSPG*

Monolayer cultures of aEECs (pooled cells from five cod, approximately 3 × 10<sup>5</sup> cells cm<sup>-2</sup>) were established in 9.6 cm<sup>2</sup> dishes. Incubation of cells with [<sup>125</sup>I]hyaluronan in 600 ml of L-15 medium supplemented with 1% HSA on ice for 1 h resulted in an average binding of 4.1% of the total added [<sup>125</sup>I]hyaluronan. Incubation of cells with [<sup>125</sup>I]CSPG in 600 ml of L-15 medium supplemented with 1% HSA on ice for 1 h resulted in the binding of approximately 7.4% of the total added ligand. Special care was taken to keep binding ('pulse') temperature constant below 1.5 °C. Following extensive washing, the cultures were transferred to 12 °C and incubated ('chased') for various periods. Transport and degradation were terminated by solubilizing both the cell layer and the incubation medium by adding 10% SDS in PBS to give a final concentration of 2% SDS. The protease inhibitors Pefabloc (2 mmol l<sup>-1</sup>), NEM (2 mmol l<sup>-1</sup>), EDTA (20 mmol l<sup>-1</sup>) and pepstatin A (1 µg ml<sup>-1</sup>) were added. Samples were stored at 4 °C until separation by gel filtration on a Sephacryl S-300 HR column (1.6 cm × 90 cm). The column was eluted with PBS containing 0.1% SDS and 0.02% NaN<sub>3</sub>. Flow rate was maintained at 42 ml h<sup>-1</sup>, and fractions of 3.5 ml were collected. The void volume and the total volume were determined by elution of Blue dextran and *N*-2,4-DNP-L-alanine, respectively. Eluted fractions were analyzed in a gamma counter.

#### *HPLC analysis of the catabolic products of [<sup>3</sup>H]hyaluronan and [<sup>3</sup>H]CSPG serglycin*

Monolayer cultures of aEECs (approximately 3 × 10<sup>5</sup> cells cm<sup>-2</sup>) were established in 9.6 cm<sup>2</sup> dishes. The cells were incubated at 12 °C with approximately 3 × 10<sup>5</sup> cts min<sup>-1</sup>

[<sup>3</sup>H]hyaluronan or [<sup>3</sup>H]CSPG serglycin in 600 ml of L-15 medium with 1% HSA. After 48 h, the medium was collected and analyzed by high-performance liquid chromatography (HPLC) on an Aminex HPX-87H cation-exchange column (300 mm × 7.8 mm, Bio-Rad) eluted isocratically with 0.004 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml min<sup>-1</sup>. Fractions (0.3 ml) were collected every 30 s on a fraction collector over 30 min. The amount of radioactivity in the eluted fractions was analyzed by adding liquid scintillation fluid (Ultima Gold™, 2 ml) before counting in a Packard Tri-Carb liquid scintillation analyzer. Samples of 50 µl of the collected medium were transferred to a PD-10 column and eluted with PBS to determine the quantitative conversion of <sup>3</sup>H-labelled ligands into low-molecular-mass products.

#### *Fluorescence microscopy of aEECs following administration of FITC-hyaluronan*

Cultures of aEECs were established on glass coverslips as described below. FITC-hyaluronan (10–50 µg ml<sup>-1</sup>) was incubated with the cultures for 2 h at 12 °C. The cultures were then fixed in 2.5% glutaraldehyde and embedded in anti-fading medium (Dako Fluorescent mounting medium, Glostrup, Denmark). Specific fluorescence due to FITC-hyaluronan was observed as a bright green to yellow colour, using a Zeiss Axiophot photomicroscope equipped with incident-light fluorescence optics (Carl Zeiss, Oberkochen, Germany). Photographs were taken on Kodak 64T or Ektachrome EPL 800 film (Kodak, Tokyo, Japan).

## Results

#### *Anatomical distribution of intravenously administered CSPG*

One hour after intravenous injection of trace amounts of [<sup>125</sup>I]CSPG, the content of radioactivity was highest in the atrium and ventricle. The high atrium: blood and ventricle: blood ratios compared with the much lower ratios in liver and kidney (Table 1) indicate a very effective uptake mechanism for [<sup>125</sup>I]CSPG in the cod heart.

#### *Endocytosis of FITC-hyaluronan in cod aEECs in vitro*

FITC-hyaluronan was incubated for 1 h with cultured aEECs. Subsequent fixation of the cultures and examination in the fluorescence microscope revealed that all cells accumulated

Table 1. *The ratio of tissue to blood specific radioactivity 1 h after intravenous injection of approximately 1 µg of [<sup>125</sup>I]chondroitin sulphate*

Atrium	36.4 ± 5.72
Ventricle	85.9 ± 11.4
Liver	0.17 ± 0.01
Kidney	3.06 ± 1.75
Blood	1 ± 0

Values represent means ± S.E.M. (N=3).

The specific radioactivity in blood 1 h after intravenous injection of [<sup>125</sup>I]chondroitin sulphate was 4.7 × 10<sup>3</sup> ± 2.9 × 10<sup>3</sup> cts min<sup>-1</sup> g<sup>-1</sup> tissue.

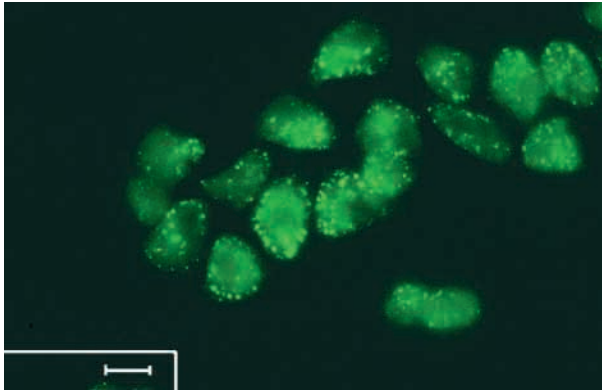


Fig. 1. Fluorescence micrograph of cod atrial endothelial cells cultured on a glass coverslip (pooled cells from three fish) and incubated with FITC-hyaluronan for 1 h at 12 °C. All endothelial cells in the monolayer cultures accumulated the probe. Note that fluorescence is confined to discrete vesicles, probably representing endocytic vesicles. Scale bar, 10  $\mu\text{m}$ .

large amounts of fluorescent material in distinct vesicles (Fig. 1).

#### Specificity of endocytosis of hyaluronan and CSPG

The specificity of endocytosis of [ $^{125}\text{I}$ ]hyaluronan and [ $^{125}\text{I}$ ]CSPG was studied by attempting to inhibit the uptake of radiolabelled ligands by cultured aEECs using excess amounts of unlabelled macromolecules ( $100\ \mu\text{g ml}^{-1}$ ) or mannose ( $50\ \text{mmol l}^{-1}$ ). Incubation of aEECs with [ $^{125}\text{I}$ ]hyaluronan in the presence of excess amounts of hyaluronan or CSPG resulted in a mean inhibition of uptake of approximately 46 % and 84 %, respectively (Fig. 2A). Neither FSA nor mannose inhibited endocytosis of [ $^{125}\text{I}$ ]hyaluronan. Incubation of aEECs with [ $^{125}\text{I}$ ]CSPG in the presence of excess amounts of CSPG, hyaluronan or FSA resulted in a mean inhibition of uptake of approximately 90 %, 42 % and 65 %, respectively

(Fig. 2B). Mannose did not inhibit endocytosis of [ $^{125}\text{I}$ ]CSPG. Unlabelled CSPG inhibited uptake of [ $^{125}\text{I}$ ]FSA by approximately 80 %. In the presence of  $5\ \text{mmol l}^{-1}$  EGTA, a  $\text{Ca}^{2+}$ -specific chelator, the ligands inhibited endocytosis of [ $^{125}\text{I}$ ]hyaluronan and [ $^{125}\text{I}$ ]CSPG by 40 % and 30 %, respectively.

#### Kinetics of endocytosis of hyaluronan and CSPG in vitro

To study the kinetics of endocytosis, cultured aEECs were incubated with trace amounts of [ $^{125}\text{I}$ ]hyaluronan and [ $^{125}\text{I}$ ]CSPG for 1–24 h. Of the [ $^{125}\text{I}$ ]hyaluronan added, 17 % was endocytosed after 1 h and 57 % after 24 h (Fig. 3A). The uptake of [ $^{125}\text{I}$ ]CSPG was 15 % of added ligand after 1 h and 65 % after 24 h (Fig. 3B).

#### Pulse–chase studies in vitro on the degradation of hyaluronan and CSPG

To study quantitatively the kinetics of degradation, cultures of aEECs were pulsed-labelled on ice ( $<1.5\ ^\circ\text{C}$ ) with [ $^{125}\text{I}$ ]hyaluronan or [ $^{125}\text{I}$ ]CSPG for 1 h. The cells were then washed and chased at 12 °C for increasing times. At the start of the chase period, 4.1 % of the [ $^{125}\text{I}$ ]hyaluronan added and 7.4 % of the [ $^{125}\text{I}$ ]CSPG added were cell-bound. The generation of  $^{125}\text{I}$ -labelled degradation products was analyzed by gel filtration on a Sepharose S-300 column.

[ $^{125}\text{I}$ ]Hyaluronan and [ $^{125}\text{I}$ ]CSPG that had been incubated in cell-free control wells and labelled material that bound to the cells at the start of the chase period eluted at  $K_{\text{av}}=0.1$ , where  $K_{\text{av}}$  is relative elution position, on gel filtration (Figs 4, 5). To estimate the speed of degradation, the undegraded proportion ( $K_{\text{av}}<0.5$ ) was calculated for each time point. Degradation of [ $^{125}\text{I}$ ]hyaluronan was detectable after a 30 min chase, as shown by a decrease in the amount of intact ligand from 72 % at the start of the period to 61 % at 30 min, and by the appearance of degradation products eluting at  $K_{\text{av}}>0.5$ . After 1 h and 2 h, 45 % and 30 %, respectively, of the endocytosed hyaluronan was left

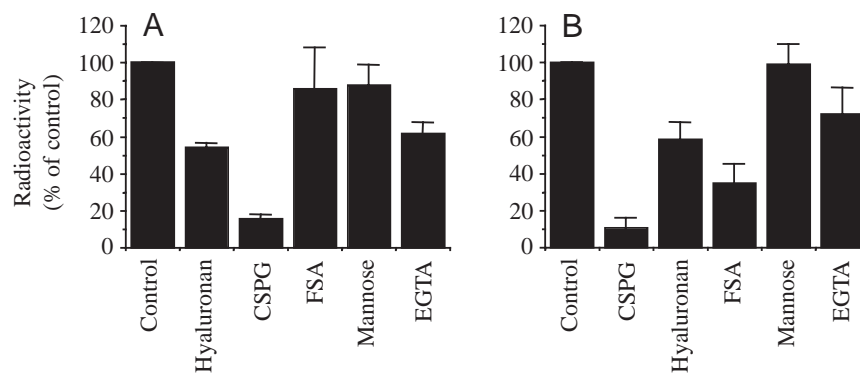


Fig. 2. Specificity of endocytosis of [ $^{125}\text{I}$ ]hyaluronan (A) and [ $^{125}\text{I}$ ]chondroitin sulphate proteoglycan (CSPG) (B) in cultured cod atrial endothelial cells (aEECs). Monolayer cultures of aEECs were incubated for 2 h at 12 °C with trace amounts of labelled ligand alone (Control) or together with excess unlabelled macromolecules ( $100\ \mu\text{g ml}^{-1}$ ) or mannose ( $50\ \text{mmol l}^{-1}$ ). The results are presented as a percentage of the radioactive content of the control samples and are means  $\pm$  S.E.M. of three independent experiments. The amounts of [ $^{125}\text{I}$ ]hyaluronan and [ $^{125}\text{I}$ ]CSPG bound in the control experiments were  $9.0\pm 1.63\%$  and  $7.67\pm 0.31\%$ , respectively, of the total radioactivity added. FSA, formaldehyde-treated serum albumin.

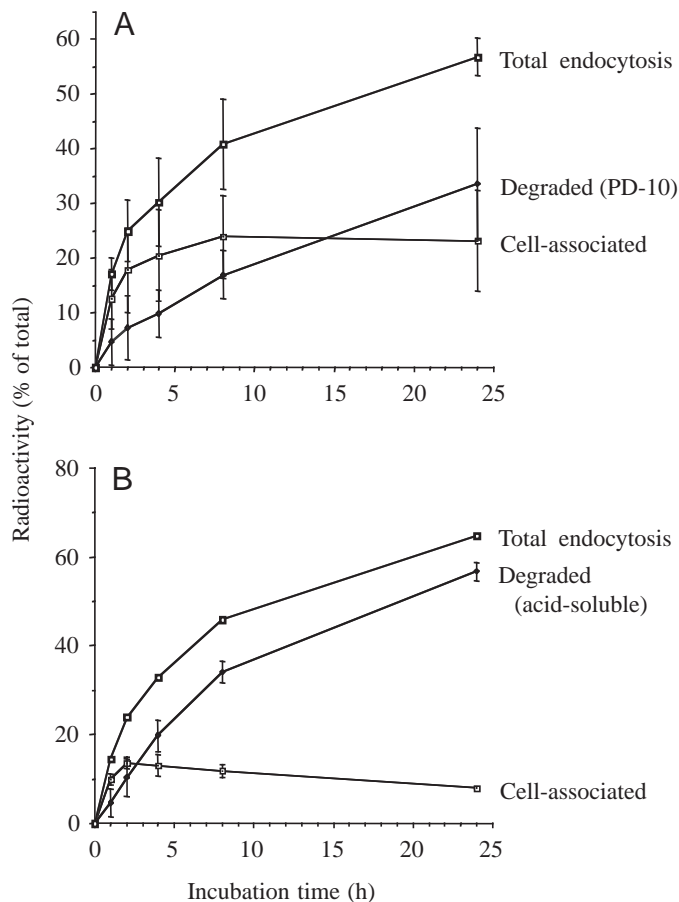


Fig. 3. Kinetics of endocytosis of [ $^{125}\text{I}$ ]hyaluronan (A) and  $^{125}\text{I}$ -labelled chondroitin sulphate proteoglycan (CSPG) (B) in cultured cod atrial endothelial cells (aEECs). Monolayer cultures of aEECs in  $2\text{ cm}^2$  wells were incubated with [ $^{125}\text{I}$ ]hyaluronan (approximately  $2 \times 10^4$  cts  $\text{min}^{-1}$ ; 3 ng) or [ $^{125}\text{I}$ ]CSPG (approximately  $2 \times 10^4$  cts  $\text{min}^{-1}$ ; 20 ng). Intact and low-molecular-mass radiolabelled ligands were measured after various periods of incubation at  $12^\circ\text{C}$ , as described in the Materials and methods section. Total endocytosis represents the sum of cell-associated and low-molecular-mass radioactivity in the supernatant. The results presented are means  $\pm$  S.E.M. of three independent experiments. PD-10, separation on a Sephadex G-25 column.

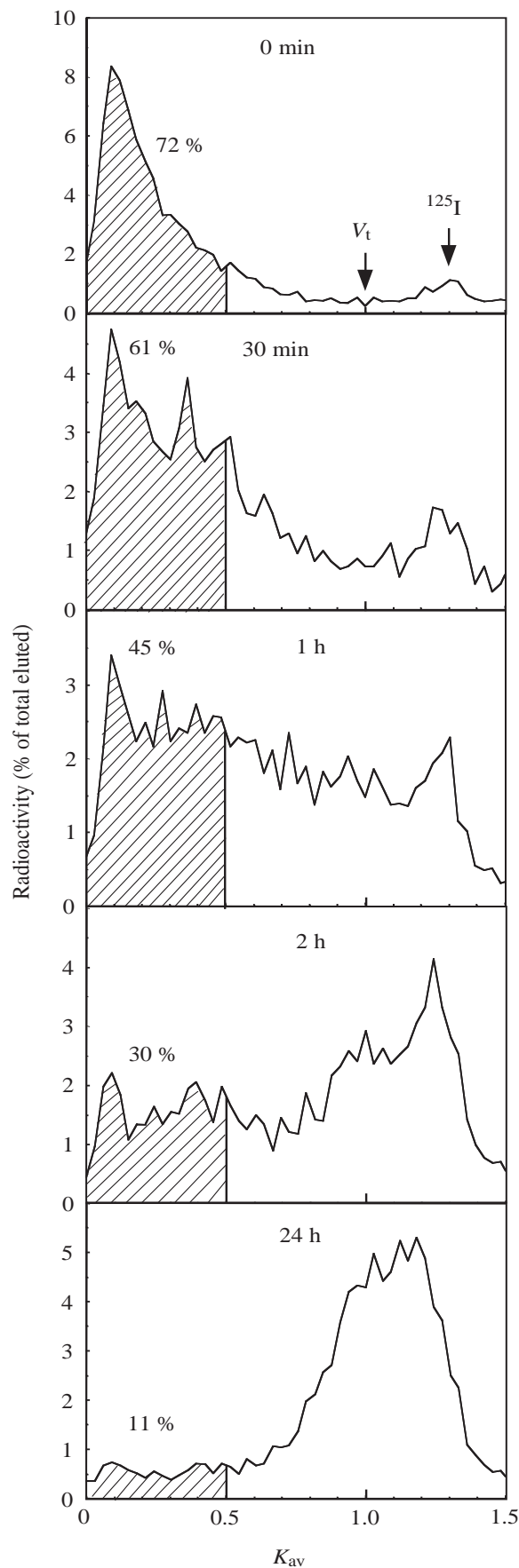
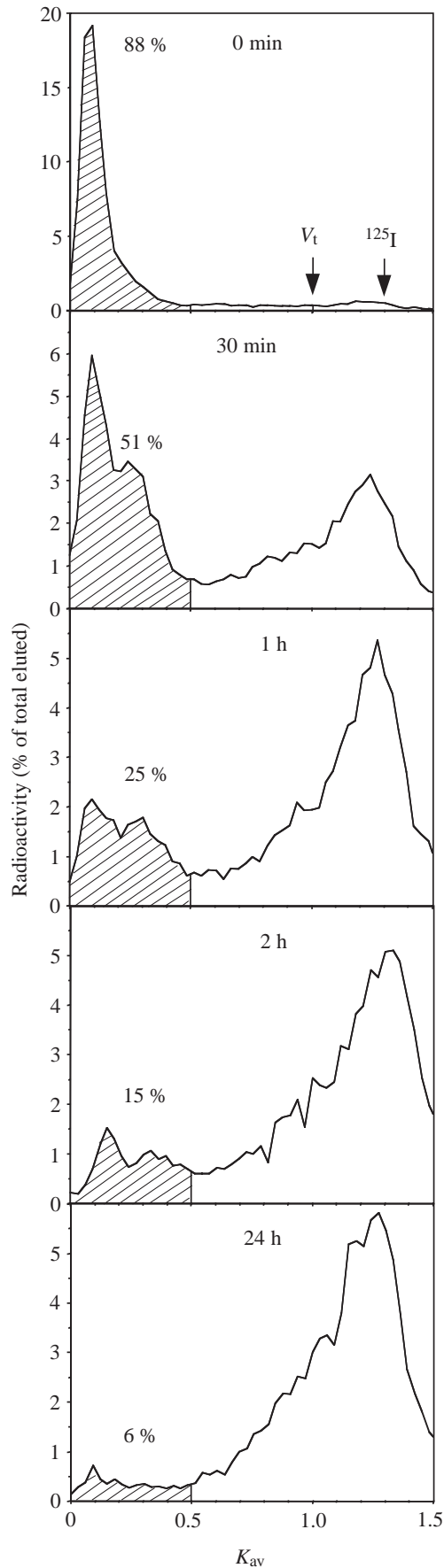


Fig. 4. Pulse-chase studies on the degradation of endocytosed [ $^{125}\text{I}$ ]hyaluronan. Cultured atrial endothelial cells were pulsed on ice with [ $^{125}\text{I}$ ]hyaluronan ( $3 \times 10^5$  cts  $\text{min}^{-1}$ ; 45 ng per  $600\ \mu\text{l}$ ) and chased at  $12^\circ\text{C}$  for varying periods. Approximately 4.1% of the added radioactivity remained cell-bound after washing. Solubilized cultures were analyzed by gel filtration on a Sepharose S-300 column. Elution profiles are given for five chasing periods: 0 min, 30 min, 1 h, 2 h and 24 h. Ordinates indicate the percentage of total radioactivity eluted. Shaded areas indicate the remaining percentage of intact molecules, calculated as the fraction of radioactivity eluting with  $K_{av} < 0.5$ , where  $K_{av}$  is relative elution position. Note that the final degradation products eluted at the same position as free  $^{125}\text{I}$  ( $K_{av} \approx 1.2$ ), relative elution position.  $V_t$ , total volume.



as intact material. After 24 h, only 11 % was undegraded (Fig. 4). The degradation of [ $^{125}\text{I}$ ]CSPG occurred more rapidly; after 30 min, the amount of intact ligand was 51 % compared with 88 % at the start of the period, whereas 25 % and 15 % remained undegraded after 1 h and 2 h, respectively. After 24 h, only 6 % of the [ $^{125}\text{I}$ ]CSPG was left as intact material (Fig. 5).

#### Identification of the degradation products of *in vitro* catabolism of hyaluronan and CSPG

The availability of biosynthetically labelled [ $^3\text{H}$ ]hyaluronan and [ $^3\text{H}$ ]CSPG serglycin facilitated detailed studies on the metabolic fate of hyaluronan and CSPG following endocytosis in aEECs. Fig. 6 shows a chromatographic profile of radioactivity accumulated in conditioned medium following a 48 h incubation of *N*-[ $^3\text{H}$ ]acetyl-labelled CSPG serglycin in monolayer cultures of aEECs. The labelled degradation products derived from [ $^3\text{H}$ ]CSPG serglycin (Fig. 6) and [ $^3\text{H}$ ]hyaluronan (not shown) eluted as standard [ $^3\text{H}$ ]acetate. At least 80 % of the [ $^3\text{H}$ ]CSPG serglycin and [ $^3\text{H}$ ]hyaluronan added was identified as low-molecular-mass degradation products after a 48 h incubation.

#### Discussion

The aim of the present study was to determine the catabolic fate of CSPG and hyaluronan following their endocytosis by EECs of cod. Recent reports indicate that EECs express endocytic receptors that enable a number of physiological and foreign waste molecules to be scavenged from the circulation (Koren et al., 1997; Smedsrød et al., 1995; Sørensen et al., 1997; Sørensen et al., 1998). We show here for the first time that circulating CSPG is taken up very efficiently by cod heart: only 1 h after intravenous injection of [ $^{125}\text{I}$ ]CSPG, the specific radioactivity of the heart was 122 times higher than that of the blood. In contrast, the specific radioactivities of the liver and kidney measured at the same time were only 0.2 and three times that of the blood.

Hyaluronan and CSPG represent major connective tissue macromolecules in vertebrates. It has been estimated that an adult human has hyaluronan stores of approximately 15 g, of which approximately one-third is turned over daily (Laurent and Fraser, 1992). Although such data are not available for

Fig. 5. Pulse-chase studies on the degradation of endocytosed [ $^{125}\text{I}$ ]-labelled chondroitin sulphate proteoglycan (CSPG). Cultured atrial endocardial endothelial cells were pulsed on ice with [ $^{125}\text{I}$ ]CSPG ( $3 \times 10^5$  cts  $\text{min}^{-1}$ ;  $0.3 \mu\text{g}$  per  $600 \mu\text{l}$ ) and chased at  $12^\circ\text{C}$  for varying periods. Approximately 7.4 % of the added radioactivity remained cell-bound after washing. Solubilized cultures were analyzed by gel filtration on a Sepharose S-300 column. Elution profiles are given for five chasing periods: 0 min, 30 min, 1 h, 2 h and 24 h. Ordinates indicate the percentage of total radioactivity eluted. Shaded areas indicate the remaining percentage of intact molecules, calculated as the fraction of radioactivity eluting with  $K_{\text{av}} < 0.5$ , where  $K_{\text{av}}$  is relative elution position. Note that the final degradation products eluted at the same position as free [ $^{125}\text{I}$ ] ( $K_{\text{av}} \approx 1.2$ ), relative elution position.  $V_t$ , total volume.

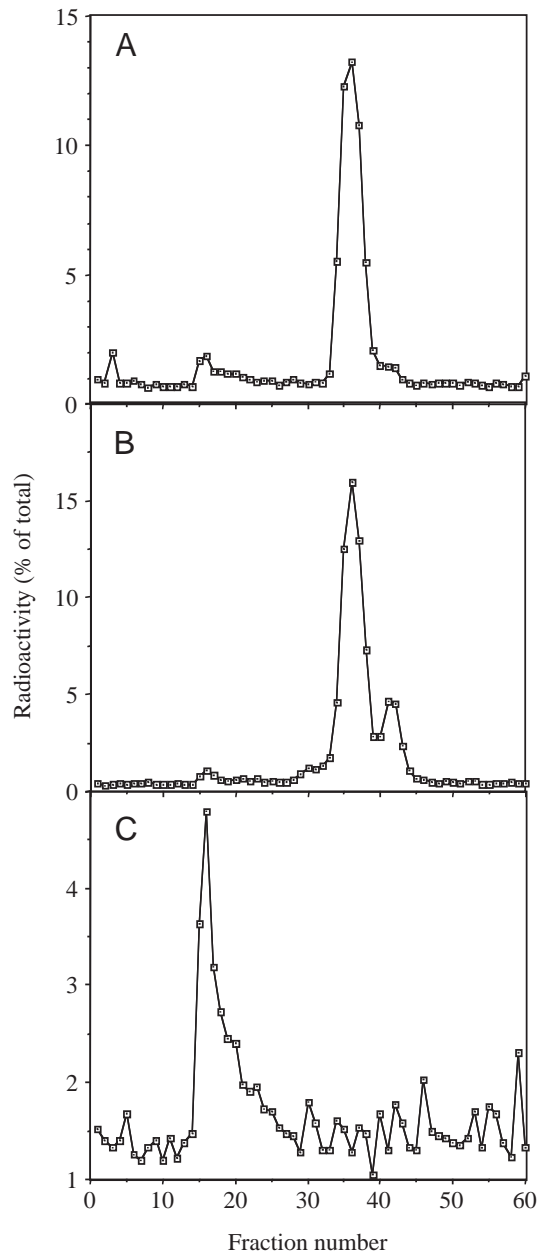


Fig. 6. HPLC analyses of the catabolic products after endocytosis of  $^3\text{H}$ -labelled chondroitin sulphate proteoglycan (CSPG) serglycin in atrial endocardial endothelial cells (aEECs). Monolayer cultures of aEECs were incubated with approximately  $3 \times 10^5$  cts  $\text{min}^{-1}$  of [ $^3\text{H}$ ]CSPG serglycin (A) for 48 h at 12 °C. The low-molecular-mass degradation products of the harvested culture medium were analyzed by HPLC on an Aminex HPX-87H column. Fractions were collected every 30 s. [ $^3\text{H}$ ]Acetate (B) was used as a standard. The elution profile of [ $^3\text{H}$ ]hyaluronan incubated in a cell-free culture (control) is shown in C.

CSPG, this proteoglycan is likely to turn over similarly rapidly since it forms complexes with hyaluronan in the tissues. Studies using isolated rat LECs have shown that hyaluronan and CSPG compete for the same receptor. This receptor, called the hyaluronan receptor, has been purified from rat LECs and partially characterized (McCourt et al., 1999).

To study whether cod EECs process hyaluronan and CSPG in the same manner as has been reported for rat LECs, we established purified primary monolayer cultures of cod aEECs, and measured variables such as the specificity and kinetics of endocytosis and the catabolic fate of endocytosed ligands. The observation that hyaluronan and CSPG competed reciprocally for endocytosis suggests that these two ligands are recognized by the same binding site on aEECs. This is compatible with the hyaluronan receptor specificity reported for rat LECs (Smedsrød et al., 1984). The findings in the present study, namely that FSA, a ligand for the scavenger receptor, and mannose, a ligand for the mannose receptor, have no inhibitory effect on the endocytosis of [ $^{125}\text{I}$ ]hyaluronan, suggest that neither of these two receptors is involved in the binding of hyaluronan to aEECs.

However, FSA inhibited endocytosis of [ $^{125}\text{I}$ ]CSPG by 60% and CSPG inhibited endocytosis of [ $^{125}\text{I}$ ]FSA by 80%, suggesting that CSPG is also recognized by the aEEC scavenger receptor. A similar phenomenon was observed in rat LECs (Eskild et al., 1986). These authors reported that FSA inhibited endocytosis of  $^3\text{H}$ -labelled chondroitin sulphate in a non-reciprocal manner. Moreover, McCourt et al. (McCourt et al., 1999) reported that antibodies against the hyaluronan receptor purified from rat LECs inhibited endocytosis of both hyaluronan and FSA.

The relationship between the hyaluronan and scavenger receptors is not clear. Mannose did not inhibit the endocytosis of [ $^{125}\text{I}$ ]CSPG, suggesting that the mannose receptor was not involved in the endocytosis of this proteoglycan in aEECs. EGTA, a  $\text{Ca}^{2+}$ -specific chelator, partially inhibited the endocytosis of hyaluronan and CSPG, suggesting that at least a subpopulation of the hyaluronan binding sites on aEECs depends on  $\text{Ca}^{2+}$  for binding. Hyaluronan-receptor-mediated endocytosis in rat LECs is known to be totally independent of  $\text{Ca}^{2+}$  (Smedsrød et al., 1984). A similar difference in  $\text{Ca}^{2+}$ -dependency between the rat and the cod scavenger receptor has been reported previously. Sørensen et al. (Sørensen et al., 1998) found that EGTA inhibited endocytosis of [ $^{125}\text{I}$ ]FSA by up to 30% in cod aEECs. This result is in contrast to a previous report demonstrating that the rat LEC scavenger receptor acts independently of  $\text{Ca}^{2+}$  (Eskild et al., 1986). Together, these results suggest that cod aEECs, by analogy with previous reports on rat LECs, endocytose hyaluronan, CSPG and FSA by a common binding site, supporting the idea that at least one category of vertebrate scavenger receptors also recognizes negatively charged connective tissue polysaccharides. The finding that a subpopulation of these binding sites in cod aEECs depends on  $\text{Ca}^{2+}$  for ligand binding represents a clear difference from the corresponding endocytosis receptor in rat LECs, which has no requirement for  $\text{Ca}^{2+}$ .

The uptake of hyaluronan and CSPG by aEECs *in vitro* was rapid, with approximately 17% and 57% of added [ $^{125}\text{I}$ ]hyaluronan being taken up after 1 and 24 h, respectively. The corresponding figures for uptake of [ $^{125}\text{I}$ ]CSPG were 15% and 65%. This rate of uptake is similar to the endocytosis of  $^{125}\text{I}$ -labelled N-terminal propeptide of type I procollagen



( $[^{125}\text{I}]\text{PINP}$ ) via the cod aEEC scavenger receptor, but slower than endocytosis of  $[^{125}\text{I}]\text{FSA}$  by the same receptor (Sørensen et al., 1998). Incubation at 1.5 °C to prevent intracellular degradation resulted in binding of 25 % of added  $[^{125}\text{I}]\text{FSA}$  and 4 % of added  $[^{125}\text{I}]\text{PINP}$  (Sørensen et al., 1998) compared with 4.1 % of added  $[^{125}\text{I}]\text{hyaluronan}$  and 7.4 % of added  $[^{125}\text{I}]\text{CSPG}$  in the present report. The difference in endocytosis between different ligands may reflect a difference in the ligand affinity of the binding site or a difference in the number of binding sites present on the surface of the aEEC. Pulse-chase studies revealed that the degradation of endocytosed  $[^{125}\text{I}]\text{hyaluronan}$  and  $[^{125}\text{I}]\text{CSPG}$  in cod aEECs at 12 °C occurred at a slower rate than the degradation of the  $[^{125}\text{I}]\text{FSA}$  and  $[^{125}\text{I}]\text{PINP}$  internalized by the cod scavenger receptor (Sørensen et al., 1998). These results indicate that cod aEECs degrade endocytosed proteins more effectively than they degrade hyaluronan and CSPG. The pulse-chase studies demonstrated further that the degradation of  $[^{125}\text{I}]\text{hyaluronan}$  and  $[^{125}\text{I}]\text{CSPG}$  in aEECs yields free iodine, suggesting the presence of dehalogenase activity in aEECs, as has been described previously in cod aEECs (Sørensen et al., 1998) and rat LECs (Hellevik et al., 1996).

To identify the degradation products, endocytosis studies were carried out with CSPG and hyaluronan that had been biosynthetically labelled with  $^3\text{H}$  in the acetyl groups of the aminosugars of the polysaccharides. HPLC analyses of the spent medium demonstrated that virtually all  $^3\text{H}$ -labelled low-molecular-mass degradation products were  $[^3\text{H}]\text{acetate}$ , indicating that these cells operate anaerobically. Thus, not only are the specificity and kinetics of endocytosis of hyaluronan and CSPG alike in rat LECs and cod aEECs, the anaerobic catabolism of endocytosed polysaccharides is also similar in the two cell types.

Although rat LECs and cod EECs are located in different anatomical sites and occur in species that are phylogenetically distant from each other, the cells show some striking similarities in structure and function: (i) they line sinusoids or sinusoid-like blood spaces that carry blood of very low oxygen tension; (ii) they represent major sites of uptake of an array of soluble and colloidal waste macromolecules; (iii) they express endocytosis receptors that recognize ligands with strikingly similar specificities; (iv) they contain few mitochondria (Blouin et al., 1977); (v) the degradation products they secrete reflect an anaerobic mode of metabolism; (vi) they are very closely apposed on the abluminal side to epithelial cells that contain large numbers of mitochondria and consume large amounts of energy.

The present study of the catabolism of hyaluronan and CSPG serglycin in cod aEECs employed ligands biosynthetically labelled with  $^3\text{H}$  in only the acetyl groups, restricting our assay systems to detect only degradation products containing  $^3\text{H}$ . Studies on the catabolism of CSPG and hyaluronan labelled with  $^{14}\text{C}$  in the ring structures of the pyranose units or  $^3\text{H}$  in the acetyl residues have shown that endocytosis of these macromolecules in rat LECs leads to the generation of both  $[^3\text{H}]\text{acetate}$  and  $[^{14}\text{C}]\text{lactate}$  (Smedsrød et

al., 1984). This fact, together with the anaerobic metabolism of these cell types, suggests that lactate would also represent a major degradation product in cod aEECs.

The major fuels for the vertebrate heart are generally considered to be glucose, lipid in the form of free fatty acids, lactate and ketone bodies (Driedzic, 1992). Although carbohydrates and fatty acids are available as energy sources for the teleost heart, lactate appears to be preferentially utilized even when glucose is available (Lanctin et al., 1980).

By producing acetate, and probably also lactate, anaerobically from normal connective tissue turnover, the aEECs of cod heart provide high-energy-yielding metabolites that may support the energy-consuming muscle cells of the cod heart. This, together with the observation that intact fish hearts metabolize acetate (Driedzic and Hart, 1984), indicates that the cardiomyocytes do in fact utilize acetate for mitochondrial energy production.

In conclusion, our results indicate that the population of EECs represents the major site of uptake of hyaluronan and CSPG from the circulation in the Atlantic cod. Studies on purified cultures of aEECs suggest that these cells express a specific binding site(s) that mediates endocytosis of hyaluronan, CSPG and FSA. The labelled degradation product derived from hyaluronan and CSPG biosynthetically labelled with  $^3\text{H}$  in the acetyl groups was identified as mainly acetate, indicating an anaerobic mode of metabolism. Thus, cod EECs are strikingly similar to rat LECs in most functional aspects studied. The observation that these cells produce acetate (and possibly other metabolites such as lactate that readily release energy in the form of ATP upon mitochondrial oxidation), raises the possibility that rat LECs and cod EECs may simultaneously serve two important functions, namely (i) a scavenger function for the clearance of waste macromolecules from the blood circulation, and (ii) a supply function delivering energy substrates to their neighbouring energy-consuming epithelial cells. According to this hypothesis, substrates for energy-consuming reactions in epithelial cells (hepatocytes in mammals; cardiomyocytes in cod) are produced locally by the scavenger endothelial cells. Studies are under way in our laboratory to determine whether this metabolic principle is in operation in all organs that carry specialized scavenger endothelial cells.

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