SCAVENGER-RECEPTOR-MEDIATED ENDOCYTOSIS IN ENDOCARDIAL ENDOTHELIAL CELLS OF ATLANTIC COD GADUS MORHUA

KAREN K. SØRENSEN1,*, JUKKA MELKKO2 AND BÅRD SMEDSRØD2

1Department of Arctic Veterinary Medicine, Norwegian College of Veterinary Medicine, N-9005 Tromsø, Norway and
2Department of Experimental Pathology, University of Tromsø, Norway

*e-mail: Karen.Sorensen@vetinst.no

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Summary

Scavenger receptors are multifunctional integral membrane proteins that mediate the endocytosis of many different macromolecular polyanions and also participate in host defence reactions and cell adherence. In Atlantic cod (Gadus morhua L.), two intravenously injected scavenger receptor ligands, [125I]tyramine-cellobiose-labelled formaldehyde-treated serum albumin ([125I]-TC-FSA) and [125I]-labelled N-terminal propeptide of type I procollagen ([125I]-PINP), distributed mainly to the heart. Cellular uptake was visualized by injections of fluorescently labelled FSA (FITC-FSA), which was recovered in discrete vesicles in endocardial endothelial cells of both heart chambers. Studies in vitro showed that radioiodinated FSA and PINP were endocytosed and degraded very efficiently by cultured atrial endocardial endothelial cells. Moreover, uptake of [125I]-FSA was Ca2+-independent. Out of a range of unlabelled ligands, only the scavenger receptor ligands FITC-FSA, polyinosinic acid and, to a varying extent, FSA, acetylated low-density lipoprotein (AcLDL) and PINP, were able to compete with radioiodinated FSA, PINP or AcLDL for uptake in isolated endocardial cells. From our findings, we conclude that the endocardial endothelial cells are major carriers of scavenger receptors in cod. In addition, our results strengthen the hypothesis that these cells in cod play the same important function as that established for the scavenger endothelial cells of the mammalian liver.

Key words: endocytosis, endothelium, endocardium, scavenger receptor, formaldehyde-treated albumin, procollagen propeptide, acetylated low-density lipoprotein, teleost fish, Atlantic cod, Gadus morhua.

Introduction

Scavenger receptors are a broad family of integral membrane proteins that mediate the cellular binding and internalization of an extraordinarily wide range of negatively charged macromolecules. Scavenger receptor ligands include chemically modified proteins and lipoproteins (Goldstein et al. 1979; Henriksen et al. 1981; Steinbrecher et al. 1984; Blomhoff et al. 1984b), polyribonucleotides such as poly(I) and poly(G) (Brown et al. 1980), advanced glycation end-products (AGEs) (Takata et al. 1988; Araki et al. 1995), endogenous products of connective tissue turnover (Melkko et al. 1994), certain lipopolysaccharides of Gram-negative bacteria (Hampton et al. 1991; Schnyra and Lindberg, 1995) and lipoteichoic acid of Gram-positive bacteria (Dunne et al. 1994).

The first scavenger receptor to be discovered was a receptor mediating the uptake of modified lipoproteins in macrophages. Following uptake, the macrophages were converted into cholesterol-loaded foam cells and deposited in developing atherosclerotic plaque (Goldstein et al. 1979; Brown et al. 1980; Brown and Goldstein, 1983). To date, three classes of functionally related scavenger receptor proteins have been characterized (for a review, see Freeman, 1997) and, in addition to macrophages, many cell types, including mammalian liver sinusoidal endothelial cells (LECs), hepatocytes, fibroblasts and smooth muscle cells, have been reported to express scavenger receptors (Nagelkerke et al. 1983; Pitas, 1990; van Berkel et al. 1991; Kamps et al. 1992). Studies in rats have shown that the LECs are indeed the main site of uptake of several circulating artificial and physiological scavenger receptor ligands (Nagelkerke et al. 1983; Blomhoff et al. 1984a,b; Melkko et al. 1994; Smedsrød et al. 1997).

Scavenger receptors are multifunctional proteins that also participate in physiological processes such as cell adherence (Fraser et al. 1993; Suzuki et al. 1997) and host defence reactions. Surface constituents of both Gram-positive and Gram-negative bacteria, as well as the intact bacteria, are known ligands (Hampton et al. 1991; Dunne et al. 1994; Schnyra and Lindberg, 1995), and it has recently been reported that mice deficient in type I and type II class A macrophage scavenger receptors showed an increased susceptibility to bacterial and viral infections (Suzuki et al. 1997).

Information about scavenger receptors in non-mammalian species is scarce. Nevertheless, scavenger receptor function seems to be well preserved during evolution. The finding that
macrophages from Drosophila melanogaster specifically endocytosed acetylated low-density lipoprotein (AcLDL) (Abrams et al. 1992) led to the cloning of a class C scavenger receptor with no known vertebrate homologue (Pearson et al. 1995).

In fish, scavenger-receptor-mediated endocytosis has been studied in salmonids. In these animals, circulating AcLDL was found to be cleared by endothelial cells lining the venous sinusoids of the kidney (Gjøen and Berg, 1992). Also, soluble chemically modified albumin was taken up mainly by a non-phagocytic population of kidney cells, most probably the sinusoidal endothelial cells (Dannevig and Berg, 1986; Dannevig et al. 1990, 1994).

Fish represent a large and extremely diverse group of animals. The Atlantic cod Gadus morhua, a marine fish of great importance in commercial fisheries and with an interesting future in aquaculture, is phylogenetically distant from the salmonids. In the present study on cod, we first examined the organ and cell distribution of intravenously injected scavenger receptor ligands. Unlike the results reported for salmonid fish or mammalian species, the endocardium was the main site of uptake. To study scavenger-mediated endocytosis in detail, a newly described cell isolation procedure (Koren et al. 1997) was used to establish primary cultures of cod endocardial endothelial cells.

Materials and methods

Medium and chemicals

Leibowitz 15 (L-15) culture medium was obtained from Gibco (Grand Island, NY, USA), adjusted to 360mosmol l\(^{-1}\) tonicity using 0.5mol l\(^{-1}\) NaCl and supplemented with 0.33g l\(^{-1}\) glucose and 0.05g l\(^{-1}\) gentamycin. Foetal calf serum and heparin were purchased from Hyclone (Logan, UT, USA) and Novo Nordisk (Copenhagen, Denmark). Human serum albumin (HSA) and trypsin (1:250) were purchased from Gibco (Grand Island, NY, USA), adjusted to 360 mosmol l\(^{-1}\) GIBCo (Grand Island, NY, USA), and purified of unlabelled 125I on a PD-10 column (prepacked Sephadex G-25, Pharmacia, Uppsala, Sweden) eluted with phosphate-buffered saline (PBS). The resulting specific radioactivities were approximately 10⁶ to 2×10⁶ cts min\(^{-1}\) µg\(^{-1}\) for FSA and 10⁶ cts min\(^{-1}\) µg\(^{-1}\) for PINP. Acetylated low-density lipoprotein (AcLDL) was radioiodinated using the procedure described by McFarlane (1958), with a resulting specific radioactivity of approximately 2×10⁶ cts min\(^{-1}\) µg\(^{-1}\).

To prevent escape of marker isotope from the site of uptake, FSA was labelled with 125I-tyramine cellulose (TC), as described by Pittman et al. (1983), with modifications according to Smedsrød (1988). This adduct is non-biodegradable and is trapped intralysosomally after endocytosis. The resulting specific radioactivity was approximately 10⁶ cts min\(^{-1}\) µg\(^{-1}\). Radioactivity was measured using a Packard gamma counter (Packard Instrument Company, IL, USA).

FITC-labelled FSA was prepared by incubating ligand (5 mg ml\(^{-1}\)) with FITC (5 mg ml\(^{-1}\)) in 0.2 mol l\(^{-1}\) sodium bicarbonate buffer (pH 9.5) for 20h at 4°C. Unreacted dye was removed by gel filtration on a PD-10 column.

Distribution studies

To study the organ distribution of scavenger receptor ligands in cod, 125I-PINP and 125I-TC-FSA were injected into the caudal vein. For inhibition studies, 125I-TC-FSA was co-injected with unlabelled FSA or FITC-FSA. Parallel series of 3–5 fish were used at each time point. At specified time intervals, 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 immediately after blood sampling, and the heart, anterior and posterior kidney, liver, gall bladder, gills, spleen, stomach, intestine and blood were removed and analyzed for radioactivity. As a control for accurate intravenous injection, the tissue at the injection site was
excised and radioactivity was quantified in all fish. Total blood mass was set to 5% of body mass.

Prior to injections and blood sampling, the fish were anaesthetized by immersion in 0.004 % (w/v ratio) benzocaine solution.

Histological preparations

FITC-FSA was injected intravenously into four fish. The fish were killed after 1 or 4 h, and the organs were dissected out, fixed in 4% formaldehyde, prepared and sectioned for fluorescence microscopy.

Purified cultures of atrial endothelial cells were established on glass coverslips as described below. FITC-FSA (100 μg ml⁻¹) was incubated together with the cultures for 1 h at 12 °C. Control cultures were incubated with FITC only. The cultures were fixed in 4% formaldehyde and embedded in an antifade medium consisting of 1 g of DABCO (Sigma) in a solution of 5 ml of PBS and 5 ml of glycerol.

Sections and fixed cultures were examined using a Leica confocal laser scanning inverted microscope (CLSM-Fuvelt FT) equipped with an argon/krypton mixed-gas laser or in a Zeiss Axiopt photomicroscope equipped with incident-light fluorescence optics (Carl Zeiss, Obercochen, Germany). Pictures were recorded on Kodak 64T or Ectachrome EPL 800 films (Kodak, Tokyo, Japan).

For scanning electron microscopy, heart tissue was fixed in McDowell’s fixative (McDowell and Triumph 1976), postfixed in 1% aqueous OsO₄, dehydrated in a graded ethanol series and critical-point-dried (Hitachi CPI, Tokyo, Japan) using carbon dioxide as the transitional fluid. Preparations were covered with gold (Polaron Sem Coating Unit E 500) and analyzed in a JEOL JSM-5300 scanning electron microscope (JEOL, Tokyo, Japan) at 15 kV and a tilt angle of 21°. Pictures were recorded on Kodak TMX-120 films.

Isolation and cultivation of endocardial endothelial cells from cod atrium

The preparation of highly purified cultures of functionally intact cod atrial endocardial cells has been detailed by Koren et al. (1997). Briefly, the heart was dissected out and perfused free of blood with L-15 medium supplemented with heparin (10 U ml⁻¹). The atria were dissected free, cut open, transferred to 50 ml sterile plastic centrifuge tubes and incubated in a horizontal shaker set to 250 cycles min⁻¹. Consecutive incubations were performed with 20 ml of the following solutions: (i) calcium-free buffer (Pertoft and Smedsrød, 1987) for 30 min; (ii) trypsin (0.5 mg ml⁻¹) and EDTA (0.1 mg ml⁻¹) in PBS for 5 min; (iii) collagenase (0.5 mg ml⁻¹ in L-15 medium supplemented with 0.7 mg ml⁻¹ CaCl₂·2H₂O) for 30 min. The contents of the tube were transferred to a sterile Petri dish, and the endocardium was flushed several times with the jet from a 10 ml plastic syringe, re-using the resulting cell suspension. The remaining tissue was discarded. The resulting cell suspension was centrifuged for 5 min at 400 g, the supernatant was removed and the pellet was resuspended in L-15.

To remove contaminating macrophages, we introduced a panning step of 45 min, making use of the fact that only macrophages attach and spread on 1% human serum albumin (HSA)-coated plastic during this short period. The non-adherent cells were seeded on plastic (Falcon, Becton Dickinson & Company, NJ, USA) or glass tissue culture slides. Plastic and glass were precoated with gelatin (0.1%) for a minimum of 5 min. Cells adhered best to plastic. The incubation medium was L-15 supplemented with 10% foetal calf serum. The isolation procedure and incubations were carried out at 12 °C.

The cells were washed after 24 h and used in experiments the same day or the next day. When cells were seeded at approximately 0.75×10⁶ to 10⁶ cells cm⁻², they produced monolayer cultures of approximately 3×10⁵ cells cm⁻². Cell numbers were assessed by visual counting of glutaraldehyde-fixed cultures using a phase-contrast microscope. On average, more than 90% of the cells were atrial endothelial cells (AECs); contaminating cells were predominantly cardiomyocytes and a few macrophages. Pooled endothelial cells from 2–4 atria were used in the experiments.

Endocytosis of labelled ligands by cultured endocardial cells

AEC cultures established in 2 cm² wells (approximately 3×10⁵ cells attached and spread per cm²) were washed several times with L-15 medium and supplied with fresh medium containing 1% HSA and trace amounts of radiolabelled protein (approximately 2×10⁶ cts min⁻¹) in a total incubation volume of 200 μl per well. Incubations carried out at 12 °C for various times were terminated by transferring the incubation medium along with the first of three washes of 500 μl of PBS to tubes containing 800 μl of 20% trichloroacetic acid (TCA). 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 supernatant after centrifugation. Cell-associated ligand was quantified by counting the radioactivity in washed cells following solubilization in 1% sodium dodecyl sulphate (SDS). Total endocytosis was determined by adding the cell-associated and acid-soluble radioactivities in the medium.

Pulse-chase studies on intracellular degradation of ¹²⁵I-FSA and ¹²⁵I-PINP

Monolayer cultures of AECs (approximately 4×10⁵ cells adhered per cm² in the FSA studies and approximately 2×10⁵ cells adhered per cm² in the PINP studies) were established in 9.6 cm² dishes. Incubation of cells with ¹²⁵I-FSA (3×10⁵ cts min⁻¹; 0.25 μg) in 600 μl of L-15 supplemented with 1% HSA on ice for 1 h resulted in the binding of approximately 25% of the total added ligand. Incubation of cells with ¹²⁵I-PINP (2×10⁵ cts min⁻¹; 0.25 μg) in 600 μl of L-15/1% HSA on ice for 1 h resulted in the binding of approximately 4% of the total added ligand. Special care was taken to keep binding (‘pulse’) temperature constantly below 1.5 °C. Following extensive washing, cultures were transferred to 12 or 4 °C and incubated (‘chased’) for various periods. Transport and
degradation were terminated by solubilizing both the cell layer and the incubation medium by the addition of 10% SDS in PBS to give a final concentration of 2% SDS. Proteinase inhibitors, Pefabloc (2 mmol l\(^{-1}\)), NEM (2 mmol l\(^{-1}\)), EDTA (20 mmol l\(^{-1}\)) and pepstatin A (1 μg ml\(^{-1}\)) were added. At the start of the chase, the cell layer and supernatant were solubilized separately; for the other chase periods, the cell layer and supernatant were solubilized together. Samples were stored at 4 °C until separation, in random order, by gel filtration on a Sephacryl S-300 HR column (1.6 × 90 cm; Pharmacia, Uppsala, Sweden). The column was eluted with PBS containing 0.1% SDS and 0.02% NaN\(_3\). Flow rate was maintained at 42 ml h\(^{-1}\), and fractions of 3.5 ml were collected. The void volume (\(V_0\)) and total volume (\(V_t\)) were determined by elution of Blue Dextran and N-2,4-DNP-L-alanine, respectively. Eluted fractions were analyzed in a gamma-counter, and chromatograms were constructed by calculating the relative elution volume \(K_{av}\) (abscissa) and the percentage of radioactivity eluted per fraction (ordinate).

In the chromatograms, a ‘late peak’ was observed to elute after \(V_t\) with a \(K_{av}\) of approximately 1.2. The proportion eluting at this position increased significantly with prolonged incubation, indicating hydrophobic interactions of degradation products with the column. Unconjugated Na\(^{125}\)I eluted at this position in cell-free controls. After 15 min, 1 h and 24 h, radioactivity eluted at this position increased significantly with prolonged incubation, indicating hydrophobic interactions of degradation products with the column. Unconjugated Na\(^{125}\)I eluted at the same position, and the peak judged to represent free \(^{125}\)I also eluted at this position in cell-free controls.

### Results

#### Distribution studies

The organ distribution of radiolabelled FSA was studied following intravenous injections of FSA labelled with \(^{125}\)I-tyramine cellobiose (\(^{125}\)I-TC). After 15 min, 1 h and 24 h, 14.5%, 2.9% and 0.9%, respectively, of the recovered label was still present in the blood, whereas the uptake in the heart increased from 58.6 to 68.1% during the same period (Table 1). Except for approximately 20% uptake in liver, radioactivity in the other organs was low at all times. The recovered radioactivity in the organs and tissues represented approximately 85% of the injected radioactivity (tested by quantifying the radioactivity in total body tissue of three fish at 1 h). The remaining 15% of label was evenly distributed throughout the fish.

When expressed with respect to organ mass (cts min\(^{-1}\) g\(^{-1}\)), the mean atrium/ventricle ratio of radioactivity was 0.8±0.1 (mean ± s.d., \(N=5\)) after 15 min, 0.7±0.2 after 1 h and 0.8±0.3 after 24 h. The heart (atrium and ventricle) constituted only 0.16±0.03% (mean ± s.d., \(N=15\)) of the body mass, and the high heart/blood ratio of specific radioactivity indicates a very effective uptake mechanism for radioiodinated FSA in this organ (Table 2).

Co-injections of \(^{125}\)I-TFSA (approximately 1 μg) with unlabelled FSA (500 μg) or FITC-FSA (300 μg, the dose used in the morphological study) effectively inhibited endocytosis of radiolabelled ligand in the heart, whereas the amount of liver-associated radioactivity increased by a factor of approximately 3 and 4, respectively (Fig. 1), indicating that an alternative route of uptake of FSA exists in cod.

Procollagen propeptides are produced in large amounts when procollagen is secreted from connective tissue cells. The

### Table 2. The ratio of tissue to blood specific radioactivity following intravenous injection of approximately 1 μg of \(^{125}\)I-TC-FSA

<table>
<thead>
<tr>
<th>Time</th>
<th>Atrium</th>
<th>1 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>147±83</td>
<td>533±128</td>
<td>2184±1390</td>
</tr>
<tr>
<td>Vent</td>
<td>177±115</td>
<td>799±278</td>
<td>2684±710</td>
</tr>
</tbody>
</table>

Specific activity was calculated as \((\text{cts min}^{-1} \text{ g}^{-1} \text{ tissue})/\ (\text{cts min}^{-1} \text{ g}^{-1} \text{ blood})\).

Values at each time point represent means ± s.d. of five experiments.

### Table 1. Recovery of radioactivity in heart (atrium and ventricle), liver and blood, presented as percentage of the total recovered radioactivity, following intravenous injection of approximately 1 μg of \(^{125}\)I-TC-FSA

<table>
<thead>
<tr>
<th>Time</th>
<th>15 min</th>
<th>1 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>58.6±3.4</td>
<td>66.1±8.7</td>
<td>68.1±4.1</td>
</tr>
<tr>
<td>Liver</td>
<td>14.8±2.4</td>
<td>19.7±6.3</td>
<td>20.1±3.0</td>
</tr>
<tr>
<td>Blood</td>
<td>14.5±5.2</td>
<td>2.9±0.8</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>Other organs*</td>
<td>12.1±1.2</td>
<td>11.3±2.9</td>
<td>10.9±1.9</td>
</tr>
</tbody>
</table>

Values at each time point represent means ± s.d. of five experiments.

*Bulbus arteriosus, anterior and posterior kidney, gall bladder, gills, spleen, stomach and intestine.

![Fig. 1. Organ distribution of \(^{125}\)I-TFSA 1 h after intravenous injection. Cod were injected with approximately 1 μg of \(^{125}\)I-TFSA either alone (filled columns, \(N=5\)) or along with 500 μg of unlabelled FSA (shaded columns, \(N=3\)) or 300 μg of FITC-FSA (open columns, \(N=3\)). The results are presented as a percentage of the total recovered radioactivity. Values represent means ± s.d. AK, anterior kidney; PK, posterior kidney; Spl, spleen; St, stomach; Int, intestine.](image-url)
Scavenger receptors in Atlantic cod 1711

N-terminal propeptide of type I procollagen (PINP) is a known physiological ligand for the scavenger receptor in rat liver sinusoidal endothelial cells (Mellko et al. 1994). Radiolabelled PINP was administered intravenously to three cod, and the radioactivity in tissues and organs was measured after 30 min (Fig. 2). Except for a slower rate of blood clearance, the distribution pattern was the same as for FSA, the heart being the main site of uptake. The atrium/blood ratio of specific radioactivity following the 125I-PINP injections, was 26.1±8.9 (mean ± s.d., N=3), the ventricle/blood ratio was 13.1±4.8 and the atrium/ventricle ratio was 2.0±0.1.

Morphology

Morphological distribution studies showed that, 1 h after intravenous injection of FITC-FSA, the fluorescence was located along the periphery of circular vesicles or in discrete filled vesicles distributed throughout the cytoplasm of the endocardial endothelial cells in both the atrium and ventricle (Fig. 3A,B). Specific fluorescence was also seen in cells evenly distributed throughout the liver parenchyme (Fig. 3C), in large macrophage-like cells in kidney blood sinusoids and interstitium (Fig. 3E) and in scattered cells and ellipsoid structures in the spleen (data not shown).

Heart tissue was also prepared for scanning electron microscopy (Fig. 4A,B). A special type of tall endothelial cell covered the muscular trabeculae of the atrium. The ventricular endocardial endothelial cells were flat, with taller cells protruding at the trabecular edges. Furthermore, the intertrabecular spaces were narrower in the ventricle than in the atrium.

Atrial endocardial endothelial cells (AECs) were isolated for in vitro studies (Fig. 5A). Cells incubated at a standardized temperature of 12°C were used in the in vitro experiments regardless of sea temperature, which could vary from 3 to 10°C during the experimental period. To visualize the uptake via the scavenger receptor, FITC-FSA (100 μg ml⁻¹) was given to cultured AECs (24 h cultures of pooled cells from three fish). The cells were fixed after 1 h and examined in the confocal laser scanning microscope (Fig. 5B). Fluorescence was seen in discrete vesicles in all AECs in the preparations. Administration of unconjugated FITC caused no staining (data not shown).

Kinetics of uptake of FSA and PINP in cultured AECs

To study the kinetics of endocytosis, cultured AECs were incubated with trace amounts of 125I-FSA for 1–6 h (Fig. 6). Approximately 25% of added ligand was endocytosed after 1 h and 75% after 6 h, indicating a very effective uptake mechanism in these cells. The uptake of 125I-PINP (Fig. 7) was slower, with 4% and 55% endocytosed after 1 h and 24 h, respectively.

Scavenger-receptor-mediated endocytosis is known to be Ca²⁺-independent (Eskild et al. 1986). When 5 mmol l⁻¹ EGTA was added to the culture medium, the uptake of 125I-FSA after 1 h was approximately 70–90% of that of control cultures without EGTA (means of triplicate cultures in two independent experiments; results not shown).

Pulse-chase studies in vitro on the degradation of endocytosed FSA and PINP

To study quantitatively the kinetics of degradation of ligands taken up via the scavenger receptor, cultures of AECs were pulsed on ice (<1.5°C) with 125I-FSA or 125I-PINP for 1 h, washed and chased at 12°C (FSA and PINP) or 4°C (PINP) for increasing periods. Approximately 25% of the added 125I-FSA and 4% of the added 125I-PINP were cell-bound at the start of the chase period. The presence of 125I-labelled degradation products was analyzed by gel filtration.

Ligands in cell-free controls and intact ligands that associated with cultures at the start of the chase period both eluted in the void volume with Kav=0.25 for FSA (Fig. 8A,B) and Kav=0.45 for PINP (data not shown). To illustrate the extent of degradation, the proportion remaining as undegraded material eluting before Kav=0.5 (FSA) or Kav=0.6 (PINP) was calculated for each time point.

Degradation of FSA was detectable after a 30 min chase, as shown by a decrease in the amount of intact ligand from 87% at the start of the chase period both eluted in the void volume with Kav>0.5 (Fig. 8C). After 1 h and 2 h, 44% and 21%, respectively, of endocytosed FSA was left as intact material (Fig. 8D,E), and after 15 h only 4% was undegraded (Fig. 8F). After a 1 h chase on ice, degradation of FSA was negligible compared with that at the start of the chase period (results not shown).

The degradation of PINP occurred rapidly at both 12 and 4°C, as measured in pooled cells from four cod captured at a sea temperature of 4°C. At 12°C, 52% of the endocytosed material eluted at Kav<0.6 after a 30 min chase, compared with 98% at the start of the chase period, whereas 24% and 16% were undegraded after 1 h and 2 h, respectively. When chased at 4°C, 28% of PINP was left as intact material after 2 h,
Fig. 3. Fluorescence micrographs of (A) a section of atrium and (B) a section of ventricle 1 h after intravenous injection of approximately 300μg of FITC-FSA. Specific fluorescence (arrows) is seen in the endocardial endothelial cells (e). m, muscular trabeculae; b, blood space. (C) Uptake of FITC-FSA (arrows) in cod liver 1 h after injection. Fluorescence was observed in cells evenly distributed throughout the liver. sv, central vein. (D) Liver tissue stained with haematoxylin and eosin. sv, central vein; h, hepatocyte; arrows, sinusoids. (E) The uptake of FITC-FSA (arrow) in large sinusoidal cells in kidney 4 h after injection of ligand. t, renal tubuli. Scale bars, 25μm.

Fig. 4. Scanning electron micrographs of cod atrial (A) and ventricular (B) endocardium. The atrial muscular trabeculae are covered with tall endothelial cells, which are interconnected at the base of the cell. The ventricular endocardial endothelium is flatter, with taller cells protruding at the trabecular edges. Scale bars, 10μm.
Scavenger receptors in Atlantic cod decreasing to 12% after 24 h (data not shown). The final degradation products eluted in the same position as free 125I ($K_{av} \approx 1.2$).

Specificity of endocytosis in cultured AECs

The specificity of endocytosis of 125I-FSA, 125I-PINP and 125I-AcLDL was studied by attempting to inhibit the uptake of radiolabelled ligands in cultured AECs using excess amounts of unlabelled macromolecules (100 µg ml$^{-1}$) or mannose (50 mmol l$^{-1}$).

Incubation of AECs with 125I-FSA in the presence of excess amounts of FSA, PINP, poly(I) and AcLDL resulted in a mean inhibition of uptake of approximately 80%, 30%, 95% and 55%, respectively, compared with control values (Fig. 9A). Hyaluronan, LDL and mannose did not inhibit endocytosis of the labelled ligand.

Excess amounts of FSA inhibited the uptake of 125I-PINP by approximately 30%, whereas inhibition by AcLDL and poly(I) was approximately 30 and 70%, respectively (Fig. 9B, C). Co-incubation with hyaluronan, LDL, mannose or denatured cod skin collagen had little or no inhibitory effect.

In a control study on the endocytosis of 125I-AcLDL in AECs, co-incubation with FSA and poly(I) resulted in approximately 20% and 75% inhibition, respectively, whereas LDL, hyaluronan and mannose had negligible effects (Fig. 9D).

Conjugating proteins with FITC cause the net negative charge on the molecule to increase because positively charged primary amino groups are neutralized. In two independent experiments, FITC-FSA inhibited the uptake of 125I-PINP by approximately 70% (Fig. 9B), whereas the uptake of 125I-FSA was completely blocked (data not shown). Mannose, hyaluronan and cod skin collagen did not inhibit the uptake of labelled ligands in these experiments.

**Discussion**

In mammals, scavenger receptors are expressed on several
different cell types, including macrophages, hepatocytes and LECs (Goldstein et al. 1979; Blomhoff et al. 1984a,b; Kamps et al. 1992). Studies in rats have shown that an array of circulating artificial and physiological scavenger receptor ligands, such as formaldehyde-treated albumin (Blomhoff et al. 1984b), acetylated LDL (Nagelkerke et al. 1983; Blomhoff

1984a), some procollagen propeptides (Melkko et al. 1994) and AGE proteins (Smedsrød et al. 1997), are taken up mainly by LECs. These specialized scavenger endothelial cells play an important part in the reticuloendothelial system by clearing the macromolecular waste products of both physiological and pathological processes from the blood via several distinct receptors (for a review, see Smedsrød et al. 1990).

As in mammals, specialized endothelial cells are also responsible for the main blood clearance of circulating soluble scavenger receptor ligands in salmonid fish (Dannevigh et al. 1994; Gjøen and Berg, 1992). In the salmonids, however, these cells are located in the blood sinusoids of the kidney and not in the liver.

The present results reveal that the anatomical location of scavenger-receptor-mediated clearance in cod is quite different from that in the salmonid fish and mammalian species investigated. 125I-TC-FSA administered intravenously to cod distributed mainly to the heart. The heart was also the main uptake organ for intravenously injected 125I-PINP, although the clearance of this ligand from the blood was slower.

When a trace amount of 125I-FSA was co-injected with excess amounts of unlabelled FSA or FITC-FSA, liver-associated radioactivity after 1 h increased from approximately 20% to 57% and 77%, respectively, of recovered radioactivity, indicating that there is an alternative route of uptake of FSA in cod. The liver receptor may have a lower affinity, but a larger capacity, for the ligand than the heart receptor. To our knowledge, this is the first report of FSA being cleared in two different organs.

The cells responsible for the uptake of scavenger receptor ligands were visualized by intravenous injections of FITC-FSA and subsequent examination of tissue sections in the fluorescence microscope. Specific fluorescence could be seen in endocardial endothelial cells of both the atrium and ventricle, and in cells evenly distributed throughout the liver. Fluorescence was also detected in macrophage-like cells in the kidney and in scattered cells and ellipsoid structures in the spleen. The cod liver contains large amounts of fat, which

Fig. 8. (A–F) Pulse-chase studies on the degradation of endocytosed 125I-FSA. Cultured cod atrial endocardial endothelial cells (AECs) (approximately 4x10^6) were pulsed on ice with 125I-FSA (300x10^6 cts min^-1; 0.25 ug per 600 ml) and chased at 12°C for varying periods. Approximately 25% of the added radioactivity remained cell-bound after washing (see Materials and methods section for protocol). Elution profiles are given for cell-free controls (A) and for five chasing periods: (B) 0 min, (C) 30 min, (D) 60 min, (E) 2 h and (F) 15 h. Ordinates indicate the percentage of total eluted radioactivity. To illustrate in a more exact manner the extent of degradation, the proportion remaining as undegraded material eluting before Kav=0.5 (shaded portion of the curves) is given for each time point. Degradation was detectable after 30 min of incubation (C). Extensive degradation was evident after 2 h of chasing (E); after 15 h (F), complete degradation of internalized ligand had occurred. Note that the final degradation products eluted at the same position as free 125I (Kav=1.2). Kav, relative elution position.
makes it difficult to interpret the tissue structure in paraffin sections. Nevertheless, the distribution pattern of stained cells, taken together with an earlier observation that Kupffer cells are not found in cod liver (Morrison, 1987) and our own observation that intravenously injected 2 μm latex beads were not recovered in liver (K. K. Sørensen and B. Smedsrød, unpublished results), indicating that cod liver is devoid of macrophages, is evidence that the probe may accumulate in parenchymal cells.

Ultrastructural studies on cod endocardium have shown that the atrial and ventricular endocardial endothelial cells have a well-developed endocytotic apparatus (Smedsrød et al. 1995). The cells also contain numerous (1–3 μm) ‘moderately dense bodies’ of unknown function, with a fibrillar content (Sætersdal et al. 1974; Leknes, 1981; Smedsrød et al. 1995; Koren et al. 1997). Other species of Gadidae have been reported to have a type of endocardial cell similar to those of the Atlantic cod (Leknes, 1980). In the present study, the surface morphology of the cod endocardium was visualized in the scanning electron microscope. The highly developed network of muscular trabeculae, covered with tall, cylindric or club-shaped endothelial cells in the atrium, and both flat and protruding endothelial cells in the ventricle, provides a large surface area facing the blood. This, together with the centralized anatomical location of these cells, provides an excellent facility for effective blood surveillance and clearance of potentially harmful endogenous and foreign macromolecules. This hypothesis is supported by the recent finding that the cod endocardial endothelial cells were the most important scavenger cells for circulating collagen fragments (Smedsrød et al. 1995), hyaluronan (Sørensen et al. 1997), β-(1,3)-glucan laminaran (Dalmo et al. 1996) and Aeromonas salmonicida lipopolysaccharide (LPS) (Dalmo et al. 1998).

The receptors mediating the uptake of LPS in cod were not studied but, from the mammalian literature, it appears that the scavenger receptor is a possible candidate (Hampton et al. 1991; Schnyra and Dalberg, 1995).

A newly described method (Koren et al. 1997) allowed us to establish highly purified primary cultures of cod AECs. To obtain enough cells for the in vitro studies, we had to pool cells from different individuals. Fluorescence microscope studies of mixed cod AECs isolated from three fish and incubated with FITC-FSA for 1 h showed fluorescence in discrete vesicles in all AECs in the preparations, indicating good cell viability in the pooled cultures.

Endocytosis of FSA was rapid, with approximately 25% of

Fig. 9. Specificity of endocytosis of 125I-FSA (A), 125I-PINP (B,C) and 125I-AcLDL (D) in cultured cod atrial endocardial endothelial cells (AECs). Monolayer cultures were incubated for 60 min at 12°C with trace amounts of labelled ligand alone (control) or together with excess amounts of unlabelled macromolecules (100 μg ml⁻¹) or mannose (50 mmol l⁻¹). The mean values for three independent experiments are shown in A and B, whereas the mean value for two experiments or the value for one experiment only is shown in C and D. The results of each experiment, presented as a percentage of the control value, are means of triplicate measurements. (A) Endocytosis of 125I-FSA in control cultures, as a percentage of the total added ligand, was 26.0±1.09%, 30.4±0.40% and 20.6±1.21% in the three experiments (means ± S.D.). Mean values of inhibition, presented as a percentage of the control value, varied in the experiments as indicated: +FSA (4.9–27.7), +PINP (52.2–85.4), +poly(I) (1.9–11.1), +AcLDL (30–55.1), +LDL (96.3–109.4), +hyaluronan (92.5–102), +mannose (97.9–110.7). (B,C) Endocytosis of 125I-PINP in control cultures was 8.7±0.96%, 8.0±1.32% and 7.5±0.78% of total added ligand, was 26.0±1.09%, 30.0±0.40% and 20.6±1.21% in the three independent experiments (means ± S.D.). Mean values of inhibition, presented as a percentage of the control value, are means of results of each experiment, presented as a percentage of the control value, varied in the different experiments as indicated: (B) +FSA (55–68.5), +FITC-FSA (26.9–29.1), +hyaluronan (94–100), +mannose (109.2–115.5), +collagen (88.5–114.6); (C) +FSA (69.5–75), +poly(I) (27.3–30.4), +AcLDL (59.1–73.4), +LDL (102–104.3), +hyaluronan (89–95.6). An asterisk indicates that FITC-FSA was only used in two experiments. (D) Endocytosis of 125I-AcLDL in control cultures was 18.2±0.7% (mean ± S.D.) of total added ligand. S.D. varied from 3 to 13% of the mean value.
Scavenger-receptor-mediated endocytosis is known to be Ca\(^{2+}\)-independent (Eskild et al. 1986). When EGTA was added to the culture medium, the uptake of \(^{125}\)I-FSA after 1 h was approximately 70–90% compared with control cultures lacking EGTA. If scavenger-receptor-mediated endocytosis in cod AECs is truly Ca\(^{2+}\)-dependent, like endocytosis mediated by the mannose receptor in these cells (K. K. Sørensen, O. K. Tollersrud, G. Evjen and B. Smedsrød, unpublished results), one would expect no endocytosis of \(^{125}\)I-FSA after EGTA treatment. The 10–30% inhibition was therefore judged to be caused by other effects of Ca\(^{2+}\) depletion on the cells.

Pulse-chase studies showed that degradation of endocytosed FSA and PINP in cod AECs at 12 °C occurred at a rate comparable with the degradation of the same ligands in rat liver endothelial cells at 37 °C (Eskild et al. 1986; Melkko et al. 1994). Only approximately 20% of the internalized ligands was left as undegraded material after 2 h. The cells also showed extensive degradation of PINP at 4 °C, 28% being undegraded after 2 h and 12% after 24 h. These results indicate that the cod endocytotic apparatus is well-adapted to low temperatures.

The observation that uptake of \(^{125}\)I-FSA, \(^{125}\)I-PINP and \(^{125}\)I-AcLDL was effectively inhibited by excess poly(I) supports the idea that cod AECs express scavenger receptors. However, the degree of cross-competition was low. Excess amounts of unlabelled FSA inhibited approximately 20% and 30% of the uptake of \(^{125}\)I-AcLDL and \(^{125}\)I-PINP, respectively, whereas PINP reduced the uptake of FSA by approximately 30%. On average, AcLDL inhibited 55% of the uptake of \(^{125}\)I-FSA, but only approximately 30% of the uptake of \(^{125}\)I-PINP. These observations may be explained by the existence of different binding sites on the same receptor (Freeman et al. 1991) or by the existence of a family of different scavenger receptors (for a review, see Freeman, 1997). From the results presented here, it is possible that FSA and PINP interact with different receptors. In addition to the low level of cross-competition for uptake in vitro, it is worth noting that different heart chambers favoured the uptake of the two different ligands in vivo, the atrium/ventricle ratio of specific radioactivity being approximately 2.0 and 0.75 following distribution of radiolabelled PINP and FSA, respectively. This observation indicates that endothelial cells in the two chambers may express scavenger receptors of different types or binding affinities.

Although scavenger receptor ligands are all negatively charged macromolecules, not all polyamions are ligands for scavenger receptors. As also reported from studies on endocytosis by rat LECs (Melkko et al. 1994), the major tissue polysaccharide hyaluronan, a highly negatively charged glycosaminoglycan, had little or no inhibitory effect on endocytosis of radiolabelled FSA, AcLDL or PINP in cod AECs.

Taken together, the results presented here and earlier studies show that cod endocardial endothelial cells, although located in a different organ, show striking functional similarities with the specialized mammalian liver endothelial cells (Smedsrød et al. 1990, 1995; Sørensen et al. 1997). We suggest that, in order to eliminate circulating endogenous macromolecular waste products and harmful foreign molecules, all vertebrates carry a highly characteristic population of specialized scavenger endothelial cells. These cells, which must be regarded as an important arm of the reticuloendothelial system, are equipped with multiple endocytotic receptors and a well-developed endocytotic apparatus. Moreover, this population of scavenger endothelial cells is found at strategic locations in the blood circulatory system: in the liver of mammals, in the kidney of salmonids and in the heart of cod.

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