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High concentrations of low density lipoprotein decrease basement membrane-associated heparan sulfate proteoglycan in cultured endothelial cells*

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The effect of increasing low density lipoprotein (LDL) concentrations on the synthesis of basement membrane components was investigated in proliferating porcine aortic endothelial cells (PAEC) in culture. Basement membrane-associated heparan sulfate proteoglycan (HSPG) and fibronectin were determined by enzyme immunoassay. Low extracellular LDL-levels increase, high extracellular LDL-levels decrease the HSPG content of PAEC. Fibronectin synthesis was only slightly affected while proliferation and metabolic activity as assessed by lactate production were constant. Insulin or high extracellular glucose did not influence the effect of LDL on basement membrane components.

Endothelial cell; Porcine aorta; Basement membrane; Heparan sulfate; Hypercholesterolaemia

1. INTRODUCTION

Alterations of function or composition of the vascular basement membrane may be regarded as a primary event during atherogenesis [1]. They may lead to an increase in the permeability of the intimal layer and to loss of barrier functions [2,3]. Basement membranes are composed of collagens, mainly collagen type IV and of other noncollagenous constituents like laminin, entactin and HSPG [4,5]. Both cell surface- and basement membrane-associated HSPGs consist of linear sulfated polysaccharide chains, the heparan sulfate moieties, which are covalently attached to a core protein [6–8]. The possible functions of basement membrane components in cell attachment, proliferation, migration and in controlling vascular permeability, mainly attributed to HSPG, have been described [4,5,9].

We have recently isolated a HSPG from porcine glomerular basement membrane and have raised antibodies against it in rabbits [10,11]. These antibodies were shown to specifically recognize the core protein of basement membrane HSPG from porcine and human glomerular and other blood vessels. A quantitative enzyme immunoassay was developed for the determination of HSPG content of cultured cells [11]. Among the cells of the vascular wall, endothelial cells have been shown to be an important site of basement membrane HSPG synthesis [11,12].

Plasma lipoproteins, especially LDL, have been identified to be the most important risk factors for the development of arterial lesions and coronary heart disease [13]. The present study provides evidence that elevated LDL-levels may decrease subendothelial HSPG and may therefore cause nondenuding injury.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Endothelial cells were isolated from the thoracic aorta of 5–6-month-old pigs and grown to confluence as described [14]. Experiments were performed using secondary cultures on 24 mm 6-well plates (split ratio 1:2 after trypsinisation). Medium was exchanged every 12 h.

2.2. *Preparation of LDL and lipoprotein deficient serum (LPDS)*

Human LDL (d = 1.02–1.06 g/ml) was prepared from the plasma of fasting normal subjects by sequential ultracentrifugation [15]. To avoid lipid oxidation, LDL and LDL-containing culture media were handled and stored under a 5% CO_2/argon atmosphere [16]. LPDS was obtained by removing lipoproteins from human serum (adjusted to d = 1.25 g/ml) by ultracentrifugation at 120000 × g for 48 h followed by extensive dialysis against phosphate-buffered saline (PBS).

2.3. *Determination of basement membrane components*

Laminin, fibronectin and HSPG were determined by enzyme immunoassay as described recently [11]. Before harvesting, cells were washed 3 times with PBS. The cell layer was then incubated with 0.6 ml 4 mol/l guanidinium-HCl containing 0.5% Chaps and protease inhibitors (1 mM phenylmethylsulfonyl fluoride/10 mM *N*-ethylmaleimide/10 mM EDTA/10 mM 6-amino-hexanoic acid/

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5 mM benzamidine-HCl) for 1 h at room temperature. Solubilisation was examined by phase-contrast microscopy to ensure total cellular disruption. Samples were stored at −30°C until measurement. After 1 : 100 dilution with PBS an 100 μl aliquot was used to coat each well (multiwell chambers from Nunc Roskilde, DK). Then the appropriate antiserum was added. Fibronectin antiserum was purchased from Behringwerke (Marburg, FRG) and HSPG antiserum from rabbit was prepared and characterized as described [11]. For calibration, fibronectin prepared from porcine plasma and HSPG isolated from porcine glomerular basement membrane [10] were used. When the effect of elevated glucose was studied, media were corrected for constant osmolarity with NaCl.

2.4. *Substrate assays*

DNA content of the cell pellets was measured in parallel incubations [17]. Cellular cholesterol content was measured fluorimetrically [18]. Lactate was measured as described [14].

3. RESULTS AND DISCUSSION

Freshly isolated PAEC contain substantial amounts of HSPG (450 ng/μg DNA) due to basement membrane material associated with the cell colonies formed during seeding which is not removed by the collagenase digestion during the isolation procedure (immunofluorescence, not shown). Since this material does not reflect HSPG synthesis by the cultured cells, only secondary cultures were used for experiments.

Growing cultures were harvested after various periods of time and HSPG and fibronectin content were determined and compared to DNA content. HSPG and fibronectin were synthesized during logarithmic phase of growth reaching a plateau when cells became confluent as indicated by constant DNA content (Fig. 1). In order to measure synthesis of HSPG and fibronectin, incubations with LDL were therefore initiated 2 h after seeding when cells were firmly attached to the plates and terminated after 48 h. The effect of increasing concentrations of LDL in the incubation media is shown in Fig. 2. Low LDL concentrations increased the HSPG content of the cultures whereas further elevation of LDL-concentration to ranges known to be atherogenic diminished HSPG-synthesis in a concentration-dependent manner. The diminution of HSPG synthesis by LDL was selective since the level of fibronectin remained essentially unchanged when high LDL-concentrations were compared to controls. However, a small increase was found for low LDL-levels. No effect of LDL-concentration on cell proliferation and density, as assessed by DNA, was found (Fig. 2B). Furthermore, metabolic activity as measured by lactate release into the medium was unchanged (data not shown). As expected, an increase in cellular total cholesterol content with increasing LDL-content of media was observed in agreement with previous results by Reckless et al. [19] and Fielding et al. [20]. To exclude an influence of LDL-concentration in the incubation media on the analytical procedures, a control incubation was performed adding 1.5 mg/ml LDL 1 h before harvesting. No significant difference in

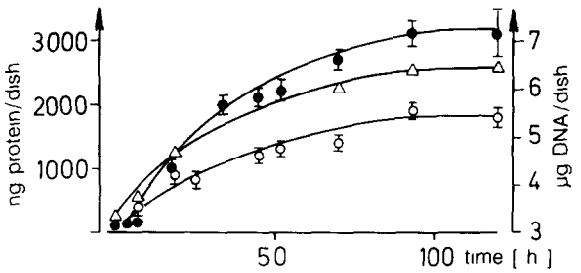

Fig. 1. Endothelial HSPG (•) and fibronectin (○) content during growth; secondary cultures were harvested after various times and analyzed for DNA (△) and content of basement membrane components. Data are expressed as mean ± SD of 4 dishes.

HSPG and fibronectin content was found (Table I). Neither elevation of 5 mmol/l to 40 mmol/l glucose nor addition of insulin to the media modified the effect of 1.5 mg/ml LDL-cholesterol (Table I) on HSPG synthesis.

Alterations of vascular basement membrane composition, especially a decrease in HSPG content, may have severe functional consequences. By virtue of heparan sulfate side chains, HSPG can complex different molecules and may modulate their biologic activity. For example, endothelial cell growth factor binding to heparan sulfate is well documented [21]. Furthermore, HSPG seems to play an important role in

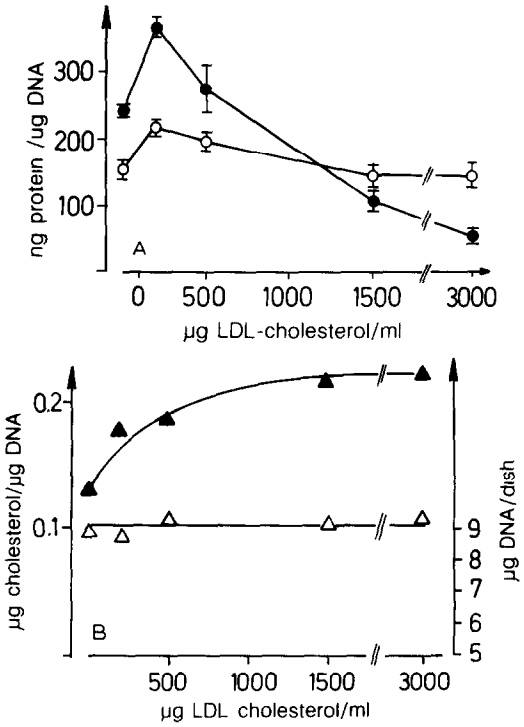

Fig. 2. (A) Influence of LDL in the growth medium (medium 199 + 10% LPDS) on HSPG (•) and fibronectin (○) content of PAEC. Secondary cultures obtained by trypsinisation of primary cultures were plated with a seeding density of about 0.6 μg/cm^2 and harvested at preconfluence after 48 h. Points represent mean ± SD of 4 dishes each. (B) Total cholesterol (▲) and DNA (△) content of PAEC under the same conditions as above. Points represent the mean of triplicate determinations.

Table I

Effect of different medium conditions on HSPG and fibronectin content of PAEC 48 h after passage (mean ± SD of 4 dishes each)

Incubation conditions	% of control	
	HSPG	Fibronectin
5 mmol/l glucose (control)	100 ± 6	100 ± 9
5 mmol/l glucose + LDL[a] 1 h before harvest	112 ± 7	87 ± 3
5 mmol/l glucose + LDL[a]	46 ± 21	82 ± 10
5 mmol/l glucose + LDL[a] + insulin[b]	66 ± 7	82 ± 9
40 mmol/l glucose + LDL[a]	67 ± 11	79 ± 5
40 mmol/l glucose + LDL[a] + insulin[b]	55 ± 25	82 ± 6

[a] 1.5 mg LDL-cholesterol/ml
[b] 2×10^{-8} mol/l

the attachment of cells to the basement membrane [4,5]. Due to its negative charge, HSPG is believed to be an important regulator of permeability and charge selectivity [9] of vascular basement membrane. For example, impairment of glomerular basement membrane in the genesis of glomerular sclerosis is well established [5,22].

Following the widely accepted response to injury thesis [1], alterations of vascular basement membrane composition may thus be regarded as a nondenuding form of endothelial or intimal injury. The results presented may therefore provide a possible link between high LDL-concentrations in plasma and the development of vascular lesions.

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