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Anti-atherosclerotic effect of microbial hyaluronate lyase from group B streptococci

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The effect of the microbial hyaluronic acid splitting enzyme hyaluronate lyase produced by *Streptococcus agalactiae* was investigated *in vitro* in human atherosclerotic plaque specimens and *in vivo* on Watanabe heritable hyperlipidaemic rabbits (WHHL) as an animal model for familiar hypercholesteraemia. The *in vitro* presence of the enzyme caused a partial destruction of the atherosclerotic plaque surfaces as well as releasing of glucuronic acid and solid calcium-containing materials from pieces of atherosclerotic plaques in human arteries. Accordingly hyaluronic acid seems to be the main component for anchoring of calcium deposits on the plaque surfaces. Repeated intravenous injections of hyaluronate lyase in WHHL rabbits resulted in a tendency of decreased formation of atherosclerotic plaques. The observed effects are discussed to be primary the result of the splitting of hyaluronic acid in the vessels.

1. Introduction

The mucopolysaccharide hyaluronic acid is an important part of connective tissue and mediator for a number of physiological functions (Lee and Spicer 2000). The splitting of hyaluronic acid by hyaluronidases as hydrolases or hyaluronate lyases as eliminases causes structural alterations in tissues, vessels and vessel fluids. Therefore hyaluronidase has been applied therapeutically (Kreil 1995; Menzel and Farr 1998). The enzyme considerably improves the permeability of tissues and enhances the absorption of drugs administered subcutaneously (Gross and Kietzmann 2006). It accelerates the healing of oedema (Modena et al. 1998), limits infarct size (Wetstein et al. 1982) with mutually contradictory results (Premaratne et al. 1995) and helps to overcome cytotoxic drug resistance (Spruss et al. 1995). In contrast to application of hyaluronidase in ophthalmology and oncology, anti-atherosclerotic administration of hyaluronidase has been neither described, nor have the small, but promising beginnings of the sixties been evaluated (Thurnherr and Koch 1962). Probably, at that time there was no chance that supply could be satisfied in a reasonable quantity and quality. Consequently, the amounts of enzyme activity administered could have been much to low to be successful. It was shown that a monthly administration of a very small hyaluronidase activity of 300 IU to hypercholesterinemic rabbits had no therapeutical influence on the atherosclerotic plaques. But after repeated administration of an equal dose over a time period of three months, cholesterol and fibrin levels in plasma were reduced and free heparin and vascular permeability of tissue were increased (Pertsovskii et al. 1973).

Because it is possible to produce hyaluronate lyase in *Streptococcus agalactiae* in sufficient amounts and with high purity and it has already been shown that microbial enzymes can possibly substitute the hyaluronidase in medical administrations (Oettl et al. 2003), effect of hyaluronate lyase on hyaluronic acid containing atherosclerotic plaques in arterial vessels was investigated. For the investigations *in vitro,* surgical isolated segments from arteries were used, and *in vivo* hyperlipedaemic effects were studied in rabbits with the disposition to atherosclerotic deposits (WHHL-rabbits). Since hyaluronic acid seems to be an outstanding constituent of the atherosclerotic plaques (Papakonstantinou et al. 1998; Wight and Merrilees 2004), it should be possible to destruct the hyaluronic acid present in atherosclerotic plaques using the microbial enzyme hyaluronate lyase. The results reported here show that *in vitro* microbial hyaluronate lyase is able to split the hyaluronic acid in atherosclerotic plaques under release of calcium deposits and reduces *in vivo* the development of atherosclerotic lesions in hyperlipidaemic rabbits.

2. Investigations and results

2.1. Effect of microbial hyaluronate lyase on isolated human atherosclerotic plaques tissue

The incubation of unfixed human atherosclerotic plaques tissue obtained through necropsy of specimens of *Arteria carotis communis* with hyaluronate lyase for about 96 h showed a decomposition of accumulated hyaluronic acid

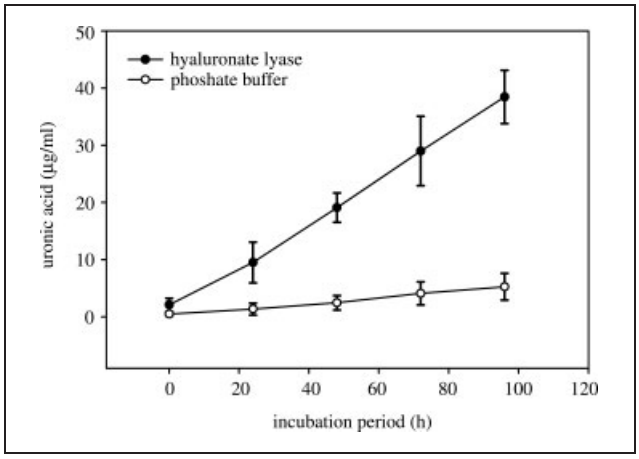

Fig. 1: Enzymatic release of uronic acid from human atherosclerotic plaques of *Arteria carota communis* with hyaluronate lyase (10,000 IU/ml) in 0.05 M phosphate buffer pH 7.0

in the plaque tissue estimated by the released uronic acid in the liquid phase (Fig. 1). The action of hyaluronate lyase caused a considerable increase of free uronic acid, which was explicitly higher than in the control. The low quantities of uronic acid released in the control showed the strong anchoring of hyaluronic acid in the plaque tissue. The destruction of hyaluronic acid simultaneously caused a release of insoluble material. After treatment of pieces with hyaluronate lyase, solid material released (11 mg, estimated as dry weight) probably consisting of calcium-phoshate. In contrast, 3 mg solid material were released from the incubation of equally long pieces without enzyme added.

The surgically isolated material of arteriosclerotic *Arteria femoralis superficialis* shows a clear intima fibrosis with developing fibrotic plaques correlated to extensive dystrophic areales of calcareous deposits as shown by haematoxylin-eosin staining (Fig. 2a). After 72 h of incubation with hyaluronate lyase the calcareous deposits were completely absent (Fig. 2b). The controls showed no changes in the superficial calcareous deposits.

The distribution of hyaluronic acid in atherosclerotic plaque sections is shown in Fig. 3a. Its favoured position in a marginal area of the atherosclerotic plaque is designated by the brown-coloured zones of HBP-marked hyaluronic acid (1). The special position implies a function in fixation and embedding of the violet stained zones of solid calcium deposits (2). The violet coloured calcium deposits envelope the plaque surfaces. Hyaluronic acid is supposedly the connective substance forming compact crystalline areas on the plaque surface. After treatment of atherosclerotic plaques with hyaluronate lyase the hyaluronic acid stained with HBP as well as the calcareous deposits disappeared completely (Fig. 3b). The breakdown of hyaluronic acid simultaneously caused a release of calcium deposits besides visible changes in the interior structure of plaques.

2.2. Administration of microbial hyaluronate lyase in WHHL rabbits

Repeated intravenous administrations of saline or saline plus hyaluronate lyase infusions had no influence on the general condition of rabbits. The spontaneous behaviour of all animals was without pathological findings. In the injection areas of marginal ear veins of control and hyaluronate lyase-treated rabbits no local reactions like oedema, inflammations or necrosis could be found. In the sera of treated animals anti-hyaluronate lyase was found in amounts between 4.8 and $12 \times 10^3 \pm 2.1$ U/ml.

During and after infusion no differences in general behaviour, body mass gain and body temperature could be found between the treated group and the control group. There was no indication of any infectious diseases.

After dissection of all the rabbits, no pathological changes were found in liver, stomach, small intestine, large intes-

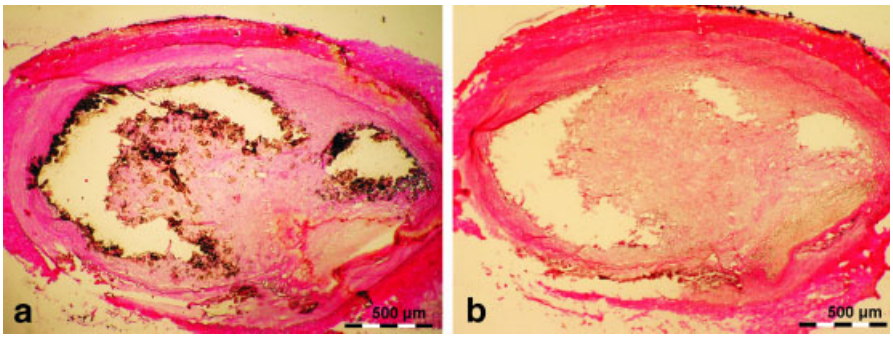

Fig. 2:
Decomposition of atherosclerotic plaques in neointima of *Arteria femoralis superficialis* with hyaluronate lyase (10,000 IU/ml) for 72 h at 37 °C. Haematoxylin-eosin staining of atherosclerotic plaques before (A) and after incubation (B) with hyaluronate lyase. The calcareous deposits are visible on the surface of atherosclerotic plaques as dark brown pigments, which are missing after the incubation

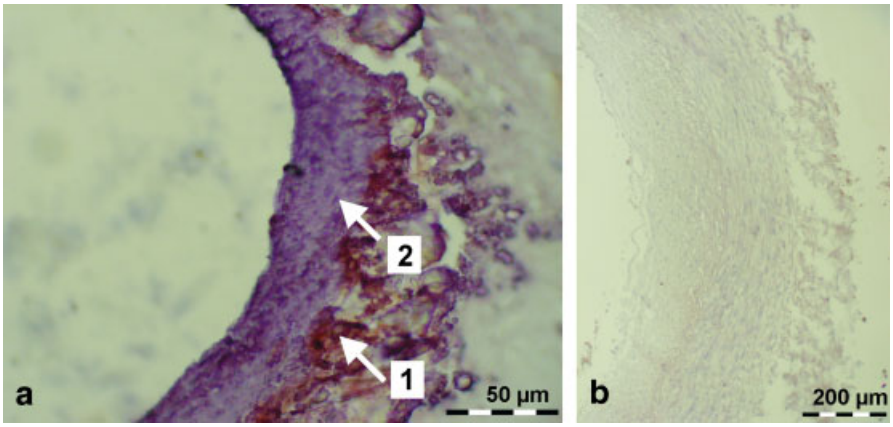

Fig. 3:
Hyaluronic acid distribution in the atherosclerotic plaques surface from arteriosclerotic *Arteria femoralis superficialis* before and after treatment with hyaluronate lyase (10,000 IU/ml). The hyaluronic acid is staining using hyaluronic acid-bonding biotinylated protein (HBP) and acidin-biotin-complex method. In frozen section (thickness 8 µm) of untreated atherosclerotic plaques surface (A) the hyaluronic acid containing area is coloured brown (1) whereas the calcium deposits was coloured violet (2). It is show that the calcium deposits are bounded directly on hyaluronic acid. After treatment with hyaluronate lyase neither atherosclerotic calcium deposits nor hyaluronic acid are detectable (B)

tine, kidneys, adrenals, brain and heart. Only in the lungs slight acute blood stasis and marginal emphysema with distension of different degrees were found.

After longitudinal opening of the aorta from the ascending aortic arch to the iliac bifurcation, atherosclerotic lesions with slightly to severely pronounced plaques were visible along the entire length. The areas of the aortic arch and thoracic segments showed more pronounced atherosclerotic plaques than the abdominal segments.

Slight to moderate atherosclerosis was found in the pulmonary arteries. The diameters of atherosclerotic plaques were 0.5 to 1.5 mm.

The hyaluronate lyase-treated rabbits, the intimal surfaces of the aortic arches were moderately to severely atherosclerotic, or the intimal surfaces were slightly to moderately atherosclerotic.

In the controls, the intimal surfaces of thoracic aorta segments were severely atherosclerotic or the intimal surfaces were moderately atherosclerotic (Fig. 4a).

In microbial hyaluronate lyase-treated animals the intimal surfaces of the thoracic aorta were scarcely or only slightly atherosclerotic (Fig. 4b).

Similar results were found in the controls where the intimal surfaces of the abdominal aorta were severely atherosclerotic or the abdominal aorta was slightly to moderately atherosclerotic (Fig. 4c) while in the microbial hyaluronate lyase-treated rabbits, the intimal surfaces of the abdominal aorta segment were moderately atherosclerotic or the abdominal aorta was slightly atherosclerotic (Fig. 4d).

The dissected preparations of hearts, adrenal glands, brains (cerebrum and cerebellum) as well as *Hypophysis cerebri* and their photo microscopic copies did not show any morphologically distinctive findings, like cellular infiltrates or focal degenerations. Cellular infiltrates nor cellular necrosis were not detectable in the lungs. There was acute blood congestion in the lung slits (or slices) and the animals atelectasic areas and spheres of emphysema were demarcated. The liver slices had predominantly no patho-

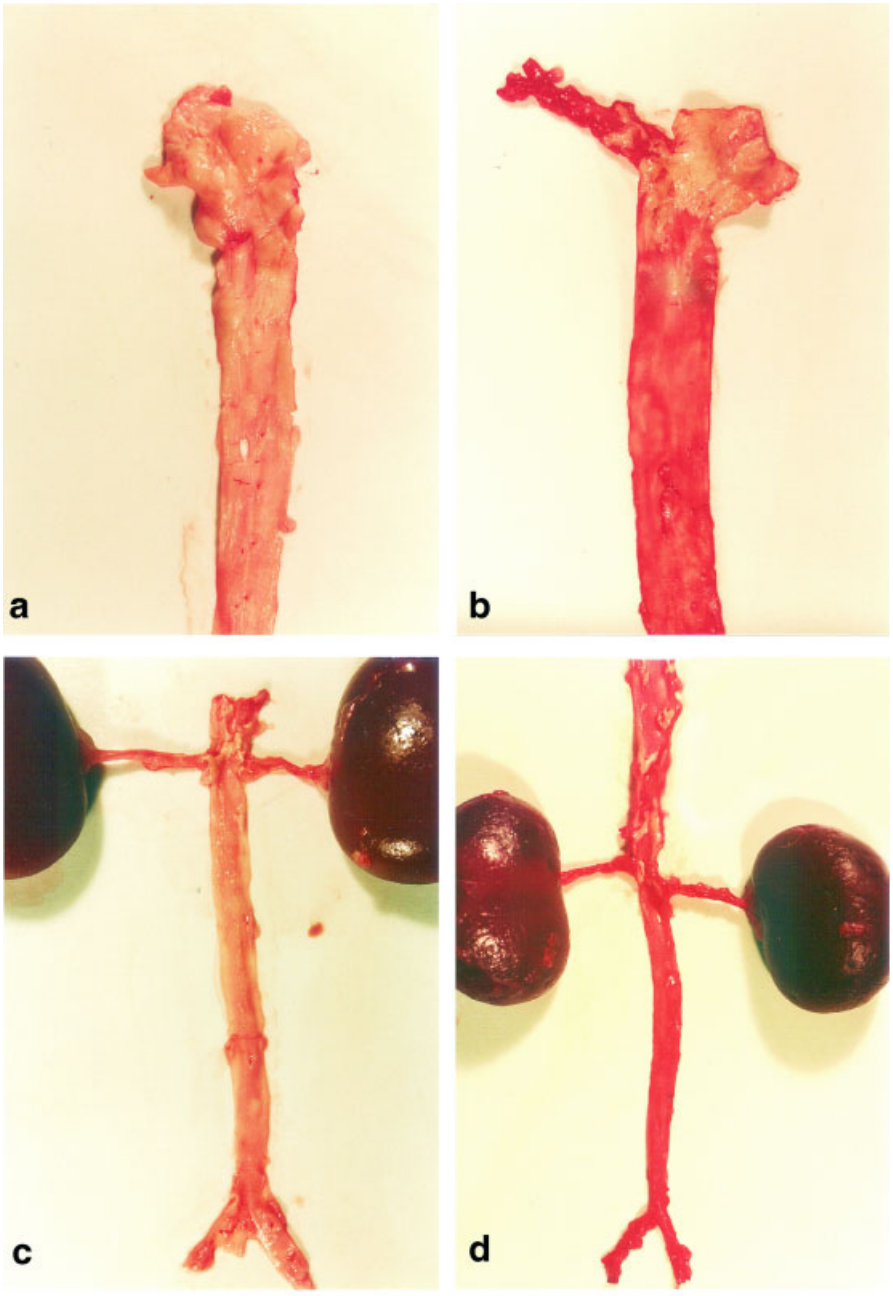

Fig. 4:
Macroscopic alterations in arterial unstained vessels of WHHL rabbits after intravenous administration of hyaluronate lyase and controls. a) Intimal surface of aortic arch and thoracic aorta segment of a untreated WHHL rabbit. The intimal surface is covered with severe atherosclerotic lesions. b) Intimal surface of aortic arch and thoracic aorta segment of a hyaluronate lyase treated WHHL rabbit. The intimal surface is only scarcely or slightly atherosclerotic. c) Intimal surface of abdominal aorta segment of a untreated WHHL rabbit. The intimal surface is covered with severe atherosclerotic lesions. d) Intimal surface of abdominal aorta segment of a hyaluronate lyase treated WHHL rabbit. The intimal surface is slightly atherosclerotic

logically distinguished variations like fatty streak generation, necrosis, and infiltrates after inflammation. The investigation of aorta segments in all rabbits demonstrated atherosclerotic plaques with submucous infiltrates of foam cells to different degrees. It was not possible to detect cellular infiltrations in the wall structures (*Aorta media* and *Aorta adventitia*). Neither in the control group nor in the attended group, symptoms for degeneration of collagen and elastic fibres were observed.

3. Discussion

In this work we investigated the effect of microbial hyaluronate lyase to the structure and the decomposition of arteriosclerotic plaques in human vessel isolates and the lowering of arteriosclerotic plaques development in WHHL-rabbits. The investigations of the vessels of *Arteria femoralis superficialis* show that treatment with comparatively very high activities of microbial hyaluronate lyase leads to an almost complete disappearance of the mineral calcium deposits both from the plaque and intima surfaces.

A lot of Ca-containing material was released from the treated samples. In parallel the finding of released uronic acid hints strongly to glucuronic acid as the main decay product of hyaluronic acid. The results give evidence that hyaluronic acid is not only together with hyaluronectin a component of the microcrystalline calcium deposits as shown by Levesque et al. (1994) but it also seems to be the main component for anchoring of the calcium deposits on the atherosclerotic surfaces. This is supported by the detection of hyaluronic acid with biotinylated HBP in the border area of atherosclerotic plaques. The position of a hyaluronic acid-containing layer underlying the Ca-deposits as well as its disappearance during treatment with hyaluronate lyase demonstrated clearly that the hyaluronic acid-containing layer anchors the Ca-deposits. Morphological investigations of fibrous-calcified plaques with near-infrared Raman spectroscopy (Rocha et al. 2007) showed the presence of phosphate and carbonate in the accumulated calcium. It is known that hyaluronic acid as polyelectrolyt forms stable complexes with Ca-containing structures as well as with basic proteins (Mapier and Hadler 1978). Hyaluronic acid seems to be a basic linker between the calcified salt conglomerates on the surface and the internal fibrous structure of plaques and could be responsible for physiological resistance against destruction. The role of calcium deposition and its contribution to plaque stability is controversial, but Li et al. (2007) investigated the effect of calcium deposits on the stress distribution within atherosclerotic plaques. The calcification at the thin fibrous cap results in a high stress concentration and a high risk of plaque rupture. The action of hyaluronate lyase could reduce this risk, a disaggregation of the more internal plaque structures by the action of hyaluronate lyase can be expected but was not observed surely.

However it is possible, that the detachment of solid calcium deposit layers on the surface of atherosclerotic plaques can produce the conditions for cellular breakdown pathways of the plaque tissue (Chajara et al. 1996).

The results of the experiments with hyaluronate lyase using WHHL rabbits as *in vivo* model show that under treatment a lower surface area of aorta is covered with atherosclerotic plaques than in the untreated animals even under the consideration that all animals displayed a different degree of aortic atherosclerosis (from aortic arch to iliac bifurcation to pulmonary arteries) as is typical for the respective WHHL rabbit strain. But the aortic preparations of the treated animals showed a substantially lower degree of atherosclerosis than the controls. This result is remarkable because the daily dose of approximately 30,000 IU was clearly higher than any activity of testicular hyaluronidase, which had been used up to now in experiments in animals or humans (Wolff 1972; Grosshenning 1965). There are no major differences in the degree of atherosclerotic plaques in the different aortic localisations. No evidence for morphologically aberrations could be observed. The active hyaluronate lyase was detectable for 2 h in serum of rabbits, and was not immediately inhibited by serum specific hyaluronidase inhibitors as already described by Hertel et al. (2006).

The mechanism responsible for the *in vivo* antiatherosclerotic action of hyaluronate lyase demands further investigation with a larger group of adequate animals of the same age and a close genetic relationship.

The microbial hyaluronate lyase causes destruction of hyaluronic acid from atherosclerotic plaques and release of calcium deposits. The formation of calcium deposits is a relative late event in arteriosclerosis. *In vivo* hyaluronate lyase, like hyaluronidase, exerts an anti-atherosclerotic effect in the initial phase of atherosclerosis, shown by preventing the formation of atherosclerotic plaques. In any case the break of hyaluronic acid by microbial hyaluronate lyase should be of outstanding significance.

4. Experimental

4.1. Hyaluronate lyase

The investigations were performed with purified hyaluronate lyase from *Streptococcus agalactiae* according to Ozegowski et al. (1994). The lyophilized hyaluronate lyase had a specific activity of 400,000 international units/mg (IU/mg). It was stabilized by addition of 5 mg ovalbumin/mg hyaluronate lyase. The activity was determined quantitatively by photometry (Gerlach and Köhler 1972). The purified enzyme was free from proteolytic activities (Ozegowski and Gerlach 1983).

4.2. Treatment of isolated human atherosclerotic plaques with hyaluronate lyase and determination of uronic acid released

The ability of hyaluronate lyase to alter or to decompose atherosclerotic plaques were *in vitro* investigated on isolated human atherosclerotic tissue surgically obtained through necropsy of specimens of *Arteria carotis communis* from a patient with vascular obstruction disease. In the present investigation pieces of neointima (0.3 cm) with arteriosclerotic deposits were used. The samples were suspended in physiological buffer of pH 7.0, containing 10,000 IU/ml hyaluronate lyase and 0.45 µl/ml Proclin 300 (Supelco, Belefonte, PA, USA) in order to prevent bacterial growth. The mixture was incubated for 96 h at room temperature. The destruction of hyaluronic acid was estimated by means of released glucuronic acid determined as uronic acid (Bitter and Muir 1960). For comparison, samples were treated with buffer containing Proclin 300 only. Samples of 100 µl were added to 600 µl of 0.0025 M sodium-tetraborate in concentrated H_2SO_4 and stirred for 10 min at 90 °C. The mixture was cooled to 4 °C and then 20 µl 0.1% carbazol in ethanol were added. The sample was again stirred for 10 min at 90 °C. After staining had developed, the concentration of uronic acid were measured photometrically at 520 nm against distilled water.

4.3. Release of solid material from arteriosclerotic plaques with hyaluronate lyase

Pieces of arteriosclerotic *Arteria femoralis superficialis* (0.8 cm) from a diabetic patient with gangrene were treated with a solution containing hyaluronate lyase (10,000 IU/ml) in physiological phosphate buffer of pH 7.0 and 0.45 µl/ml Proclin 300. The standard solution contained water instead of the enzyme. The samples were incubated for 72 h at 37 °C under slow shaking on a thermo mixer (Thermomixer 5436, Eppenddorf, BRD). Released sediments were collected, two times washed with distilled water and estimated as dry weight. The solid material was analyzed.

4.4. Histomorphological and immunhistological detection of the hyaluronate lyase effect on atherosclerotic plaques

A native part from atherosclerotic *Arteria femoralis superficialis* with 0.8 cm length and macroscopically visible massive atherosclerotic plaques with superficial calcified deposits in lumen was incubated at 37 °C for 72 h by agitating in 2 ml 0.05 M phosphate buffer pH 7.0 with hyaluronate lyase (10,000 IU/ml) and for control with buffer alone and 0.45 µl/ml Proclin 300 (Supelco, Belefonte, PA, USA) in order to prevent bacterial growth. Rinsing in distillated water terminated the incubation. Thereafter the vessels were fixed with 7% buffered formaldehyde, then dehydrated with ethanol ($2 \times 70\%$, $2 \times 96\%$, $2 \times 99\%$) and embedded in paraffin according to standard procedures. Sections of 4 µm on adhesive slides (Superfrost plus, Menzel, Germany) were prepared, deparaffinated and rehydrated ($2 \times$ xylene, $2 \times 99\%$ ethanol, $2 \times$ ethanol 70%, distilled water). The slides were stained with haematoxylin-eosin (HE), Elastica van Gieson (EvG), and von Kossa silver staining and microphotographically documented (Olympus Stereomicroskop, Olympus Digital Camera Camedia C3030, DP-Soft 3.0 Imaging Software).

For the immunhistological localization of hyaluronic acid cryostat sections were incubated with biotinylated hyaluronan-binding protein (HBP, Calbiochem, Merck, Germany) 1 : 50 diluted in 10 mM HEPES, 0.15M NaCl, pH 7.5 for 12 h at 4 °C. After three washes with physiological phosphate buffer (PBS) these sections were incubated with a pre-formed macromolecular complex consisting in avidin and biotinylated peroxidase, which retains biotin-binding sites (ABC-Elite, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Staining was performed by incubation with a solution of 1.5 µM 3.3′-diaminobenzidine, 10 mmol/l H_2O_2, and 0.05 M Tris-HCl buffer (pH 7.6) at 25 °C for 10 min.

After counterstaining in 5% Harris haematoxylin for 5 min slides were dehydrated with ethanol transferred in xylene and mounted with canadabalsam.

Negative controls were performed in HEPES with 1% BSA instead of HBP.

4.5. Administration of hyaluronate lyase in vivo on WHHL rabbits

For the investigations a series of ten WHHL rabbits, which show a high incidence of atherosclerosis, were used. The animals were bred by Charles River Savo (Kisslegg, Germany). The WHHL rabbit has a hereditary hyperlipidaemia due to a congenital low-density lipoprotein (LDL) receptor deficiency and is characterized by elevated serum LDL cholesterol levels (> 400 mg/100 ml plasma) and early atherosclerosis. From birth to the age of one year, WHHL rabbits are characterized by accelerated atherosclerosis with accumulation of lipids in the intima, fatty streaks, foam cell lesions, and atherosclerotic plaques (Watanabe et al. 1985).

Ten rabbits (4 females and 6 males) were 9–11 months old at the beginning of the experiment. The animals were kept in separate metal cages (0.5×1.0 m) in a room maintained at constant temperature (20 ± 2 °C) and humidity (40–60%), and were fed a commercial rabbit chow (sniff, Soest, Germany) and water *ad libitum*. Following a 14-days-period of acclimatization to the conditions of the laboratory, each rabbit was randomly assigned a number from 1–10 and they were divided into two treatment groups of five animals. The animal experiments were performed according to German laws for animal welfare and was approved by the Landesverwaltungsamt Weimar (Free State of Thuringia, Germany).

All animals received 1-hr infusions in 20 ml physiological saline three times per week (Monday, Wednesday and Friday), administered via a marginal ear vein. Each rabbit of the control group received 20 ml saline only during a 1-hr period. Each rabbit of the treated group received 20 ml saline with a daily dose of 30,000 IU hyaluronate lyase in a 1-hr infusion. Within 21 days this infusion dose was given ten times.

4.6. Morphology of the cardivascular system

One day after the last infusion treatment (10^{th} or 20^{th}), the animals were killed after overnight fasting by an intravenous injection of pentobarbital sodium (> 50 mg/kg). Bleeding from a carotid was induced and they were dissected immediately.

The cardiovascular system of each rabbit, including heart, lung, ascending aorta, thoracic and abdominal aorta, and common iliac artery was removed. Afterwards the aorta with major arterial branches from the ascending arch to the iliac bifurcation was prepared. Microphotographs of the inner surface of different aortic segments were taken by Olympus Stereo-Zoom-Microscope equipments Olympus SZ11, Olympus SZPT, and Olympus C-35AD-4, Olympus Optical Co (Europe) GmbH, 20097 Hamburg, Germany).

4.7. Antihyaluronate lyase in serum

After the administration of hyaluronate lyase in WHHL rabbits the antihyaluronate lyase antibodies was estimated according Hill (1976). Hyaluronate lyase solution with 10^3 Units/ml served as test solution.

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