Cell Tissue Res (1991) 263:201–205

Cell and Tissue Research

Localization of hyaluronan in various muscular tissues

A morphological study in the rat

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Accepted August 31, 1990

Summary. The histochemical distribution of hyaluronan (hyaluronic acid, HYA) was analysed in various types of muscles in the rat by use of a hyaluronan-binding protein (HABP) and the avidin-biotin/peroxidase complex staining procedure. Microwave-aided fixation was used to retain the extracellular location of the glycosaminoglycan. In skeletal muscles, HYA was detected in the connective tissue sheath surrounding the muscles (epimysium), in the septa subdividing the muscle fibre bundles (perimysium) and in the connective tissue surrounding each muscle fibre (endomysium). HYA was heterogeneously distributed in all striated muscles. In skeletal muscles with small fibre dimensions (e.g., the lateral rectus muscle of the eye and the middle ear muscles), HYA was predominantly accumulated around the individual muscle fibres. Perivascular and perineural connective tissue formations were distinctly HYA-positive. In cardiac muscles, HYA was randomly distributed around the branching and interconnecting muscle fibres. In comparison, smooth muscle tissue was devoid of HYA.

Key words: Hyaluronan (hyaluronic acid) – Hyaluronic acid-binding protein – Microwave-fixation – Immunohistocytochemistry – Striated muscle – Smooth muscle – Rat (Sprague-Dawley)

Hyaluronan (hyaluronic acid, HYA) is a linear polysaccharide consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine residues. It is present in most body tissues (Reed et al. 1988) but is most abundantly found in loose connective tissue (Comper and Laurent 1978; Laurent 1987; Evered and Whelan 1989). It interacts with other extracellular matrix components to provide stability and elasticity to the extracellular environment. Apart from its various physico-chemical properties, it also exerts important cell biological functions in tissues. It is involved in the control of morphogenesis and differentiation, and it can interact with macrophages, granulocytes and lymphocytes (Laurent 1987). Recently, it has been assigned a role in the mitotic process and has been implicated in malignant growth (see Evered and Whelan 1989).

In rheumatoid arthritis, high molecular weight HYA, accumulated in and around the joints, will immobilize water in the tissues and may act as a mechanical hindrance to movements (Engström-Laurent and Hällgren 1987). The inflammatory process also affects the muscular tissue, giving rise to specific symptoms, e.g. motion pain, stiffness and weakness. The question arises whether changes in the content of HYA in muscles are involved in this and other conditions with restricted power of motion. However, at present little is known about the occurrence and distribution of HYA in muscular tissues.

Recently, techniques for the specific histochemical localization of HYA in sections have been developed (Knudson and Toole 1985; Ripellino et al. 1985; Tammi et al. 1988; Green et al. 1988). These methods utilize the fact that the core of the cartilage proteoglycan is bound to HYA via a hyaluronan-binding protein (HABP). Hardingham (1981) has shown that HABP binds to HYA with high specificity and great affinity, and can thus be used as an affinity probe.

The purpose of the present study made on rat was to localize HYA in normal muscular tissues from various locations using the HABP-histochemical technique.

Materials and methods

Preparation of biotinylated hyaluronan binding protein (HABP-biotin)

The preparation of HABP from cartilage proteoglycans was performed essentially as previously described by Tengblad (1979). The purified HABP was biotinylated after aggregation with HYA in

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order to protect the binding site. The HYA-HABP aggregate (13 mg) was dissolved in 10 ml of 0.1 M $NaHCO_3$ (pH 8.5). This was reacted with 680 µg succinimidylbiotin in 600 µl dimethylformamide, and incubated at room temperature for 30 min. The HYA-HABP-biotin was separated on a Sephadex G15 column. The HABP-biotin was affinity-purified on a HYA-Sepharose column, dialysed in 0.15 M NaCl, and stored at −20° C.

Animals

Six male Sprague-Dawley rats weighing 250–300 g were used. Under sodium pentobarbital anaesthesia, various muscle pieces were collected from each animal and placed immediately in physiological saline.

Tissue preparation and fixation

Within less than 10 min after sacrifice of the animals, the tissue pieces were transferred from the saline to a solution of 2% formalin and 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.35, and fixed in a Miele M-696 microwave oven at a setting of 45° C using 700 watts as described by Hellström et al. (1990), Boon and Kok (1987) and Kok et al. (1988). The microwave oven was stopped when the temperature was reached. After dehydration in ethanol, the tissue pieces were then processed for embedding in paraffin. Serial sections (4 µm thick) were mounted on glass slides for histochemical localization of HYA and for routine haematoxylin and eosin staining.

Histochemical staining for HYA

After deparaffinizing, the slides were incubated with 1.0% bovine serum albumin for 30 min at room temperature to block non-specific binding sites. After washing in PBS, control slides were incubated with 50–100 units/ml *Streptomyces* hyaluronidase (Seikagaku, Kogyo Co., Tokyo, Japan) for 4 h at 37° C. This hyaluronidase specifically degrades HYA and therefore serves as a control showing the specificity of the HABP-biotinylated probe. The slides were next washed in PBS, and then incubated with a fresh solution of 3% H_2O_2 in methanol for 5 min in the dark at room temperature to destroy any endogenous peroxidase activity. After washing, the slides were incubated with 100 µl of the biotinylated-HABP (5–15 µg/ml) at 4° C overnight. Following a PBS wash, the slides were incubated with the Vectastain-Elite avidin-biotin complex (ABC) reagent, 1:200 dilution (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at room temperature. The slides were then incubated with a 0.1% solution of diaminobenzidine tetrahydrochloride (Sigma Co., St. Louis, MO, USA), and 3% H_2O_2 in methanol for 5 min at room temperature, producing a water-insoluble brown precipitate. Finally, the slides were washed in tap water for 5 min and cover-slipped. One set of slides was stained with Mayer's haematoxylin and then cover-slipped. Photomicrographs were made using a Zeiss Axiophot photomicroscope.

An arbitrary grading scheme was devised to semi-quantitate the occurrence of HYA in the various tissue sections as follows: 0=no staining; 1+=faint staining; 2+=intense staining. The histological analysis and grading of the staining intensity of the specimens were performed by four of the authors independently, at two different laboratories.

Results

HYA distribution in skeletal muscles

Fourteen different skeletal muscles were isolated and subsequently stained for HYA. In all muscles examined, HYA was found in the various connective tissue layers, i.e. the epimysium, perimysium and endomysium. This was prominent when the fibres were viewed either in longitudinal or cross section. Generally, the HYA distribution was heterogeneous, but the epimysium and the perimysium contained more HYA, based on the relative staining intensities, compared with the endomysium. However, in muscles with small fibre dimensions (e.g., the lateral rectus muscle of the eye, the tensor tympani and stapedial muscles of the middle ear), HYA staining was very pronounced in the endomysium where it formed dense band-like rings around the individual muscle fibres. Peripheral nerves and perineural connective tissue in muscles with small fibre dimensions also stained intensely. HYA staining obtained in the vastus lateralis and the lateral rectus muscles of the eye are compared in Fig. 1 A, B, respectively. Ten skeletal muscles showed an overall intense HYA staining reaction (2+) according to our grading scheme. The exceptions were the tongue, palatal and intrinsic laryngeal muscles, and the diaphragm; all exhibited a faint (1+) staining reaction.

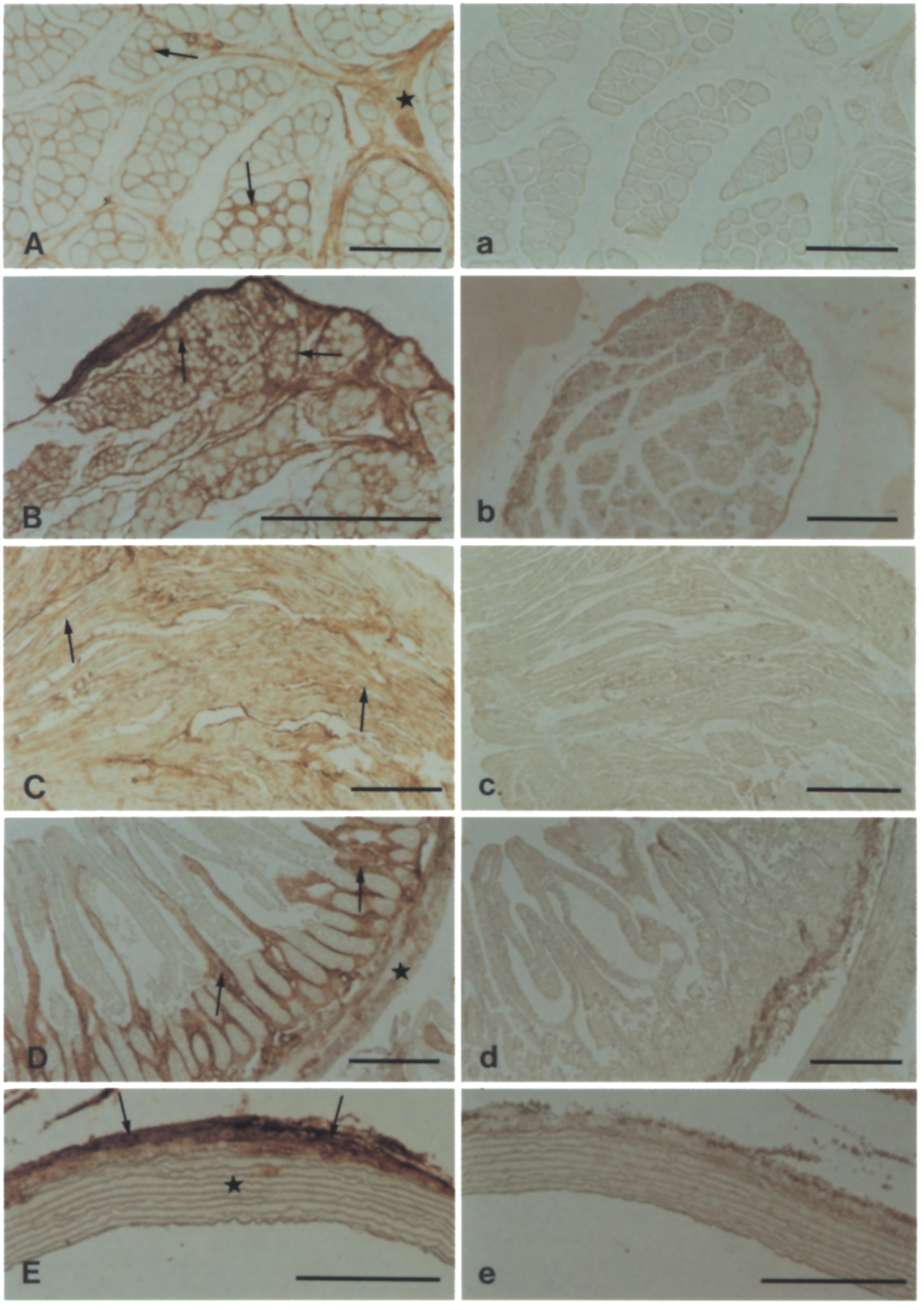
Perivascular connective tissue was HYA-positive in all tissue sections and this region reproducibly stained more intensely than other areas of the connective tissue. All control slides that were first treated with *Streptomyces* hyaluronidase showed no staining, indicating that the HABP specifically reacted with HYA and not with any of the other glycosaminoglycans.

HYA distribution in cardiac muscles

Both the atrial and the ventricular tissue of the heart contained HYA around the branching cardiac muscle fibres. Based on the relative staining intensities, there was more HYA present in the atrium compared with the ventricle. HYA was also abundantly found in the perivascular connective tissue and in the vessel walls of both the atrium and the ventricle. The typical HYA-staining pattern in the atrial tissue is shown in Fig. 1 C.

Color light micrographs of histological sections of various types of muscles showing the staining patterns for hyaluronan (HYA) (**A–E**), and control slides treated with *Streptomyces* hyaluronidase (**a–e**). Length of the *calibration bars:* 200 µm

Fig. 1. A Vastus lateralis muscle. HYA is present in the endomysium surrounding the individual muscle fibres (*arrows*). HYA staining is pronounced in the perivascular and perineural connective tissue (*asterisk*). ×82.5. **a** Control slide of vastus lateralis muscle. ×82.5. **B** Lateral rectus muscle of the eye. Note the dense band-like HYA staining in the endomysium (*arrows*). ×165. **b** Control slide of lateral rectus muscle. ×82.5. **C** Atrial muscle. HYA is present around the branching cardiac fibres (*arrows*). ×82.5. **c** Control slide of the atrium. ×82.5. **D** Jejunal wall. HYA is mainly located in the lamina propria and in the lacteals of the individual villi (*arrows*). The muscularis externa is devoid of HYA (*asterisk*). ×82.5. **d** Control slide of the jejunum. ×82.5. **E** Cross section through the wall of the thoracic aorta. HYA is lacking in the intima and media (*asterisk*). Abundant HYA is seen in the adventitia (*arrows*). ×132. **e** Control slide of the thoracic aorta. ×132


A
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HYA distribution in smooth muscles

Smooth muscle was analysed in tissue sections from jejunum, ileum, ductus deferens, the thoracic aorta and bronchus. In contrast to the large amounts of HYA seen associated with striated muscles, smooth muscles did not contain any detectable HYA after incubation with the specific HABP-biotinylated probe.

Regarding the staining pattern of the small intestines, HYA was found mainly in the connective tissue of the lamina propria associated with the intestinal villus epithelium. HYA was also observed in the lackals (lymph capillaries) of the individual villi. In the irregular connective tissue of the submucosa, HYA was present, but it was absent from the muscularis mucosae and the muscularis externa. No HYA was detected in the serosal lining. The intestinal HYA-staining pattern observed in the jejunum is shown in Fig. 1 D.

A section of the thoracic aorta is shown in Fig. 1 E. The tunica intima and media, which contain an abundance of smooth muscle, did not stain. Only the outermost part of the aortic wall, the adventitia, contained HYA. Perivascular connective tissue and the adventitia of small arteries associated with smooth muscle tissue were also HYA-positive.

Discussion

All physical functions of the body involve muscular activity: skeletal movements, contraction of the heart, contraction of blood vessels, peristalsis of the intestines, etc. In order to perform such diverse movements, each muscle has its own specific morphological characteristics. A description of the distribution of the different connective tissue components in muscles must include an analysis of HYA. Hitherto, however, this glycosaminoglycan has been difficult to trace with classical histochemical methods, which relied on the interaction of basic dyes with the various polysaccharides (Clark 1981). With a specific HABP and avidin biotin/peroxidase-complex staining procedure, we have now been able to describe the normal HYA distribution in various muscles of the rat.

HYA is one of the most water-soluble unlinked polysaccharides in tissue. For histochemistry, HYA has been immobilized at the sites where it is originally present by adding cetyl pyridinium chloride (CPC) to the fixative solution (Ripellino et al. 1985; Tammi et al. 1988; Green et al. 1988). CPC forms an insoluble complex with the HYA molecule and prevents its loss during tissue processing. However, CPC does not penetrate large tissue pieces and might change the original morphology because of precipitation of HYA and similar polyanions in the tissues (see Evered and Whelan 1989). To avoid these problems, we used a microwave-stimulated fixation in aldehydes to secure the localization of HYA without the addition of CPC (Hellström et al. 1990; Boon and Kok 1987; Kok et al. 1988).

Because of the high specificity and affinity of the HABP for HYA, it can be assumed that the relative staining intensities in a tissue section correspond to the amount of HYA that is present. The connective tissue layers associated with striated skeletal and cardiac muscles contain considerable amount of HYA, whereas the muscle fibres themselves remain unstained. It is interesting to note the accumulation of HYA around the individual muscle fibres in muscles with small fibre dimensions (these probably control very discrete movements) compared with the weak HYA staining in the endomysium of skeletal muscles with larger fibre dimensions; these usually exhibit moderately rapid and gross movements. In muscles that have to fulfill the requirement of quick and precise movements, the abundance of HYA could be one factor that facilitates the lubrication between the individual muscle fibres, in the same manner that it has been shown to maintain normal lubrication in connective tissue systems (Swann et al. 1974).

HYA occurs at sites subjected to pronounced mechanical stress, e.g. joint cartilage, synovial fluid and skin (Evered and Whelan 1989). This could also be the situation in the heart, where HYA occurs around the cardiac muscle fibres in both the atria and ventricles. The specific location of HYA in the endomysium makes it tempting to suggest that muscular HYA acts as a cushion separating and lowering the friction between the individual muscle fibres.

The absence of HYA in smooth muscle tissue can be explained by a paucity in connective tissue and its cells. It is feasible that the individual smooth muscle cells need to be tightly bound to each other in order to function and contract as a unit. The occurrence of HYA in the adventitia of the aorta may be associated with the fact that this layer is essentially lacking smooth muscle fibres but is rich in connective tissue, blood vessels and nerves.

We have described the normal HYA distribution in various muscles from the rat. In future investigations, the distribution of HYA in human skeletal muscles should be elucidated. Such studies in humans at rest and exercise are in progress.

Acknowledgements. This study was supported by the Swedish Association Against Rheumatism, Ester Åsberg Foundation, the Gustav the Vth Birthday Fund, the Ragnar and Torsten Söderberg Foundation, the Swedish Medical Research Council (03X-4, 17X-06578), the Arne and Ingalisa Lundberg Foundation and Pharmacia AB. We also thank Cathrine Johansson for excellent technical skills in the histochemical preparations. The biotinylated HABP-probe used in the present study was a kind gift from our recently deceased friend and co-worker Anders Tengblad, Ph.D., Pharmacia AB, Uppsala, Sweden. Alvin F. Wells is a Fogarty International Fellow with support from the Swedish Medical Research Council.

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