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European Journal of Pharmacology 450 (2002) 291–296

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Mystixin peptides reduce hyaluronan deposition and edema formation

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Received 7 March 2002; received in revised form 1 July 2002; accepted 5 July 2002

Abstract

Hyaluronan and its associated water of hydration are the basis of the swelling and edema of acute inflammation. Mystixins are small, synthetic peptides that suppress the acute inflammatory response. Mystixin-7, a prototype of these peptides, has the structure *p*-anisoyl-Arg-Lys-Leu-Leu-D-Thi-Ile-D-Leu-NH_2. As shown previously by this laboratory, the mystixin-7 peptide inhibits edema formation in vivo following intravenous administration at doses of less than 1.0 mg/kg. Mechanisms by which this peptide might suppress edema were examined here in vitro using cultured cells. Normal human dermal fibroblasts normally secrete large quantities of hyaluronan in response to inflammatory stimuli. Mystixin-7 reduced hyaluronan deposition by up to 80% in such cultures. Stimulation of hyaluronidase activity was observed. Mystixins represent a novel class of anti-inflammatory peptides that suppress the edema associated with inflammation. We propose that stimulation of hyaluronidase activity, with a decrease in net hyaluronan deposition and its associated water of hydration, is among the mechanisms of the anti-inflammatory effect of mystixin peptides.

Keywords: Mystixin; Hyaluronic acid; Hyaluronan; Edema; Inflammation

1. Introduction

Hyaluronan is an important component of the edema that accompanies the inflammatory response. Massive accumulations of hyaluronan are documented in the edema fluid found in animal models of inflammation, including a murine model of inflammatory arthritis (Mikecz et al., 1995) and in bleomycin-induced pulmonary interstitial edema (Teder and Heldin, 1997). Hyaluronan is capable of expanding its solvent domain up to 10,000 times its actual polymer volume (Granger et al., 1984). Secretion and release of hyaluronan into the extracellular space by fibroblasts can vastly increase tissue hydration and edema. Hyaluronan levels can be elevated by a number of inflammatory stimuli, including exposure to transforming growth factor β_1 (TGFβ_1). In this study, normal human dermal fibroblasts (Hs27) were cultured in the presence of added TGFβ_1. The effects of a prototype mystixin peptide, mystixin-7, on hyaluronan metabolism were examined in these in vitro studies in an attempt to identify possible molecular mechanisms for the suppression of edema. This follows our in vivo studies that documented edema suppression by mystixin-7 in the thermal injury rat paw model (Baluk et al., 1998; Wei et al., 1988; Wei and Thomas, 1993, 1996).

2. Material and methods

2.1. Handling of tissues

Pathogen-free male F344 rats (200–250 g) were purchased from Simonsen Laboratories (Gilroy, CA). The Committees on Animal Research at the University of California, Berkeley, approved the experimental protocols. The rats were anesthetized and anesthesia-maintained with sodium pentobarbital (50 mg/kg, i.p.). Rats were injected with mystixin-7 (0.8 mg/kg body weight i.v.) or with saline, 1 ml/kg body weight, using a branch of the femoral vein. Ten minutes later, thermal injury was induced by immersing one hind paw, up to the ankle joint, in 58 °C water for 1 min as described previously (Wei et al., 1988). Thirty minutes later, the animals were euthanized with concentrated pentobarbital and both hind paws were removed, with the contralateral, nonheated paw serving as control.

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PII: S0014-2999(02)02073-3

2.2. Immunohistochemistry

The hind paws were fixed in a 10% acid-formalin/70% ethanol. This fixative solution optimizes preservation of tissue hyaluronan (Lin et al., 1997). Hyaluronan is highly water-soluble but becomes largely tissue-bound after exposure to this fixative, even following subsequent immersion in aqueous solutions. The tissue was decalcified, paraffin-embedded and sectioned. Before staining, paraffin was removed by successive washes in xylene, ethanol and saline solutions. All samples were incubated in 5% normal goat serum for 30 min at 37 °C to block nonspecific binding sites. For hyaluronan localization, samples were incubated at 4 °C overnight with 0.3 ml of a biotinylated, hyaluronan-binding protein. Samples were washed in saline solution and reacted with avidin-conjugated horseradish peroxidase. A diaminobenzidine substrate was used for color development. Hematoxylin was used to enhance contrast.

2.3. Human dermal fibroblast cell cultures

Normal human dermal fibroblasts, Hs27, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were routinely used between passages 12 and 20. Cells were cultured at 37 °C in an atmosphere of humidified air with 5% CO_2, and maintained in Dulbecco's Modified Essential medium (DME H-16, 1 g/l glucose, GIBCO/BRL, Long Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (UCSF Cell Culture Facility, San Francisco, CA). The cells were passaged each week with 0.05% trypsin (UCSF Cell Culture Facility). Cells were counted using a Model Z_F Coulter Counter (Coulter Electronics, Hialeah, FL).

Cells were maintained in a logarithmic state of growth (low density, "preconfluent") for hyaluronan determinations by seeding the cells in DME H-16 supplemented with 10% fetal bovine serum at a density of 2×10^5 cells/well in 35-mm diameter polystyrene culture wells on day 1. On day 2, media were aspirated, cultures were washed five times with DME H-16 (serum-free) and media replenished with DME H-16 (serum-free) supplemented with growth factors and peptides. On day 3, samples of cell-conditioned media were collected, after which, cells were trypsinized and cell counts performed. Hyaluronidase determinations were performed on highly dense, "postconfluent" cultures generated by seeding cells, as described above, and replenishing the cultures every third day with fresh DME H-16 supplemented with 10% fetal bovine serum over a 14-day period. On day 14, media was aspirated, cultures were washed five times with DME H-16 (serum-free) and media replenished with DME H-16 (serum-free) supplemented with growth factors and peptides. Samples of cell-conditioned media and corresponding cell layers, retrieved using a rubber cell scraper, were collected on day 15. Enzyme was immunoprecipitated with anti-Hyal-1-coated beads and assayed as described below.

2.4. The mystixin-7 peptide

Mystixins are defined as peptides containing the sequence -Arg-Lys-Leu-(Leu/Met)-X-Ile-(Leu/D-Leu)-NH_2, where X is an anisolylated glutamic acid derivative (A*) or other aromatic residue (Thomas et al., 1993; Wei and Thomas, 1993). The specific peptide utilized was mystixin-7 (*p*-anisoyl-Arg-Lys-Leu-Leu-D-Thi-Ile-D-Leu-NH_2, where D-Thi is β-thienyl-D-alanine). This peptide was synthesized by standard solid-phase methods, purified by high-performance liquid chromatography, and the molecular mass confirmed by mass spectroscopy as described previously (Thomas et al., 1993; Wei and Thomas, 1993, 1996). All references to "mystixin" in this communication are to mystixin-7 (MW 1.041 kDa). Commercial human recombinant $TGF\beta_1$ (GIBCO/BRL) was added to cultures in order to up-regulate hyaluronan production in the cultured fibroblasts.

2.5. Hyaluronan determinations

Levels of hyaluronan were quantified using a microtiter-based assay (Fosang et al., 1990), which relies upon adsorption of hyaluronan to wells in 96-well microtiter plates (Costar, Cambridge, MA). Sample aliquots were incubated with the biotinylated hyaluronan-binding protein and then added to the microtiter plates in triplicate. After blockade of nonspecific adsorption, adsorbed protein was detected using peroxidase-conjugated biotin and avidin (Vector Labs, Burlingame, CA). Bound material was detected using an *o*-phenylenediamine substrate color reaction (CalBiochem, La Jolla, CA). Color intensity following development was read on a Titertek Multiskan Plus ELISA plate reader (ICN, Aurora, OH) at 492 nm. A standard curve for hyaluronan using known concentrations of commercially available hyluronan (ICN) was performed routinely in each experimental plate. Interassay variation was less than 5%.

2.6. Measurement of hyaluronidase activity

Hyaluronidase activity was determined using a microtiter-based assay (Stern and Stern, 1992; Frost and Stern, 1997). Enzymatic activity was expressed in rTRU (relative turbidity-reducing units), a unit described previously (Tolksdorf et al., 1949). Recombinant Hyal-l, also known as human plasma hyaluronidase (Frost et al., 1997), was used as a control.

2.7. Immunoaffinity beads

For the immunoaffinity procedure, purified immunoglobulin (IgG) from the hybridoma clone 4D5 was used (Frost

et al., 1997). This hybridoma is now deposited with the American Type Culture Collection, as required by N.I.H. regulations. Three milligrams of purified IgG was coupled to 1 mg of Protein A Sepharose 4B (Zymed, South San Francisco, CA) to produce the anti-Hyal-1 beads.

2.8. Hyaluronan-substrate gel zymography

A hyaluronan-substrate gel zymography procedure was also utilized to evaluate hyaluronidase activity. Such gels were doubly stained using Alcian blue, followed by Coomassie blue (Guntenhoener et al., 1992). Experimental samples containing hyaluronidase activity left a clear band that corresponded to degradation of the hyaluronan embedded within the gel. Recombinant Hyal-l (Frost et al., 1997) was used here also as a control.

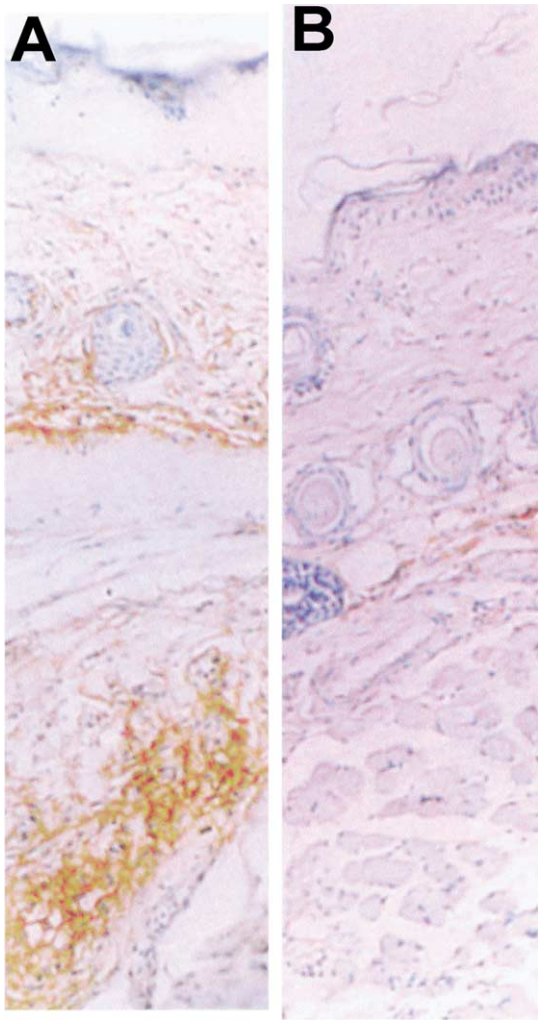

Fig. 1. Histolocalization of hyaluronan in rat paws exposed to thermal stimulation. (A) Saline solution was administered intravenously prior to thermal stimulus. Increased levels of extracellular hyaluronan are observed surrounding stromal fibroblasts. (B) Mystixin (0.8 mg/kg body weight) was administered intravenously prior to thermal stimulus. The hyaluronan deposition now resembled the normal paw more closely (not shown), demonstrating that mystixin administration inhibited hyaluronan deposition during the acute inflammatory response to thermal injury.

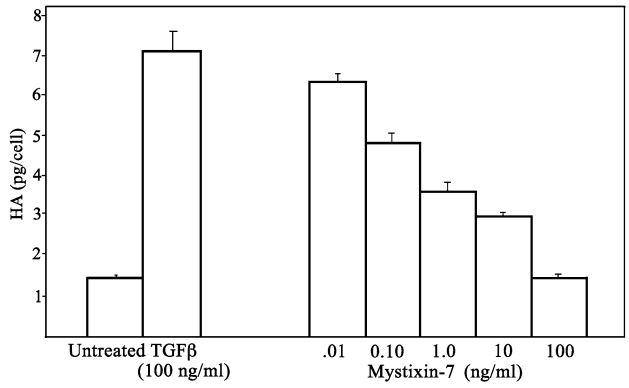

Fig. 2. Quantification of hyaluronan deposition using a microtiter assay. TGFβ_1-stimulated normal human dermal fibroblast cells were incubated with increasing doses of mystixin-7 at 0.01, 0.1, 1, 10 and 100 ng/ml. A dose response was observed: increasing levels of mystixin-7 caused increasing inhibition of fibroblast hyaluronan deposition ($P<0.05$ using one-way analysis of variance; values are $\pm$ S.E.M.; $n=5$ experiments per dose).

3. Results

3.1. Histochemical detection of hyaluronan

Administration of mystixin-7 suppressed the edema induced by thermal injury, as measured by paw weight. In the unheated paw, minimal amounts of dermal hyaluronan were observed (not shown). Following heat injury, there was a marked increase in extracellular hyaluronan deposition that separated dermal fibroblasts (Fig. 1A). Such hyaluronan accumulation was much reduced in heated paws of animals pretreated with mystixin-7 (Fig.

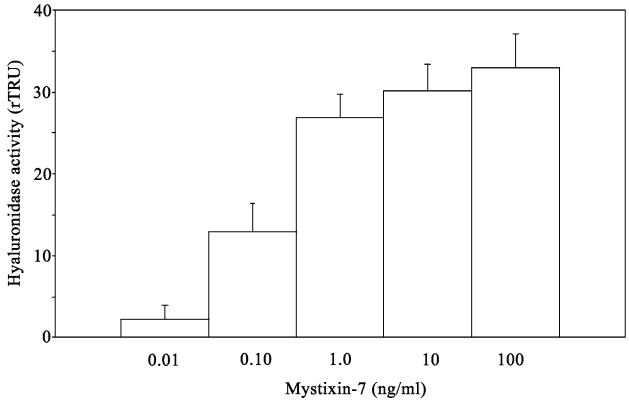

Fig. 3. Detection of hyaluronidase activity in response to the mystixin-7 peptide, quantified using a microtiter-based assay. A dose-dependent relationship was observed between mystixin-7 at 0.01, 0.10, 1, 10 and 100 ng/ml, and levels of hyaluronidase activity ($P<0.05$ using one-way analysis of variance; values are $\pm$ S.E.M.; $n=4$ experiments per dose).

1B). The hyaluronan deposition now resembled that of the unheated control paw.

3.2. Hyaluronan formation in fibroblasts

Cultures of human dermal fibroblasts, Hs27 secreted hyaluronan into the culture medium at a basal rate of 1.5 pg/cell in a linear fashion over a 24-h period (data not shown). After stimulation with TGFβ_1, the amount of hyaluronan secreted increased to 7 pg/cell. The hyaluronan levels in these cultures were examined following a 24-h exposure period.

The effect of mystixin-7 was examined in fibroblasts incubated in the presence of TGFβ_1. The peptide inhibited hyaluronan production in a dose-dependent manner (Fig. 2). Values shown are means ± S.E.M.; $P < 0.05$ represents one-way analysis of variance. The experiments were repeated five times at each dose.

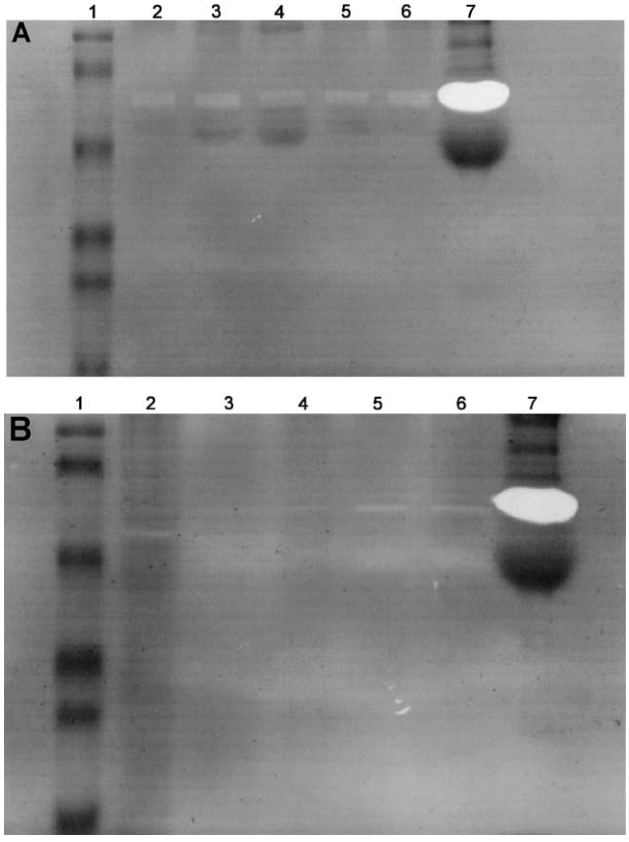

Fig. 4. Detection of fibroblast hyaluronidase activity using hyaluronan-substrate gel zymography. Samples of (A) fibroblast-conditioned media and (B) fibroblast cell layers in the presence and absence of mystixin-7 were subjected to HA-impregnated SDS gel electrophoresis. Hyaluronidase activity was detected by the presence of clear bands, reflecting degradation of hyaluronan within the gel. The gels were incubated at pH 3.7 and hyaluronidase activity was detected at 57 kDa, identified as Hyal-1. (A) media and (B) cell layers. Lane 1 = molecular weight markers; lane 2, vehicle (negative) control; lane 3, TGFβ_1, 100 ng/ml only; lane 4, TGFβ_1 and mystixin, 100 ng/ml; lanes 5 and 6, mystixin, 100 ng/ml; lane 7, purified plasma hyaluronidase. The molecular weight markers are in descending order: 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa.

3.3. Hyaluronidase enzyme activity

The TGFβ_1-treated fibroblasts were incubated with mystixin-7 at five concentrations, at 0.01, 0.10, 1, 10 and 100 ng/ml for 24 h. The peptide induced a dose-dependent increase in hyaluronidase activity. Hyaluronidase activity, quantified using the microtiter plate-based assay, is shown in Fig. 3. Activity is reported as the percentage of activity of human plasma hyaluronidase, used as standard controls. Values are means ± S.E.M.; $P < 0.05$ represents one-way analysis of variance; n = four experiments per dose. Hyaluronidase activity was confined to the culture medium and could not be detected in any of the cell layer samples hyaluronidase inhibitors (Mio et al., 2000).

3.4. Hyaluronidase activity as reflected by hyaluronan-substrate gel zymography

An alternative technique for detection of hyaluronidase activity is hyaluronan-substrate gel zymography. Fibroblast cultures were treated with 100 ng/ml of TGFβ_1 and 100 ng/ml mystixin-7 for 24 h. The hyaluronidase activity was detected in both culture media (Fig. 4A) and cell layer (Fig. 4B), with the highest level of activity occurring in the mystixin-treated samples. The mystixin-treated cell layer samples had an additional band of activity at approximately 46 kDa, representing a processed form of hyaluronidase as described previously (Csoka et al., 1997). Mystixin-treated cell layer samples (lanes 5 and 6, Fig. 4B) contained higher levels of hyaluronidase activity (lane 1, molecular weight markers; lane 2, untreated; lane 3, TGFβ_1, 100 ng/ml; lane 4, TGFβ_1 and mystixin, 100 ng/ml each; lanes 5 and 6, mystixin, 100 ng/ml; and lane 7, plasma hyaluronidase).

4. Discussion

Soft tissue injury is accompanied by lowering of interstitial fluid pressure, plasma protein extravasation and edema. Mystixins are synthetic peptides that inhibit plasma leakage after tissue injury. In an earlier study (Baluk et al., 1998), we sought to determine the mechanism of the antileakage effect of mystixins, with particular reference to the formation of endothelial gaps in postcapillary venules, but no direct effects on gap formation were found.

The effect of the mystixin peptide, mystixin-7, on hyaluronan deposition was examined both in vivo, in a thermally injured rat paw model, and in vitro, using cultured human dermal fibroblasts. Mystixin-7 pretreatment suppressed the increase in hyaluronan that occurred in vivo in response to thermal injury. Suppression of hyaluronan was also observed in the cultured fibroblasts pretreated with TGFβ_1.

Hyaluronan is a large sugar polymer of the extracellular matrix. It participates in many important functions, including maintaining interstitial fluid volume. Hyaluronan can take on a large water of hydration due to its physiochemical

composition. This facilitates processes such as angiogenesis that requires tissue expansion. Large and variable quantities of hyaluronan are distributed throughout the body's tissues and fluids. Approximately one-half of this hyaluronan is localized within the dermal layer (Reed et al., 1988). In this study, hyaluronan served as a biomarker of cutaneous edema during thermally induced acute inflammation. Exposure to heat caused increases in hyaluronan concentration within the skin. Pretreatment with mystixin-7 was effective in suppressing the accumulation of hyaluronan.

The preventive use of mystixin-7 was examined here, in preventing subsequent hyaluronan deposition and edema following heat shock. More relevant for anti-inflammatory drug development would be an examination of the effect of mystixin-7 administration at various time periods following injury in vivo. In the current experiments, the TGFβ_1 and mystixin-7 peptide were added simultaneously. Similarly, the effect on the cultured fibroblasts of mystixin-7 addition at various time periods following TGFβ_1 on hyaluronan deposition and hyaluronidase expression addition would be of intrinsic interest. Such experiments are being planned.

The mystixin peptides possess a certain degree of structural similarity to corticotropin-releasing factor, a neuropeptide with endogenous anti-inflammatory properties. Preliminary observations indicate that corticotropin-releasing factor and other peptides of the corticoliberin family, such as sauvagine and urocortin, are able to inhibit the deposition of fibroblast HA, although to a lesser extent than mystixin-7. To date, mystixin-7 is the most potent of the mystixin analogs tested in the human fibroblast system; others are proven to be less effective in suppressing hyaluronan deposition.

CD44 is a type I transmembrane protein receptor for which hyaluronan is the primary ligand. CD44 localizes to microdomains within the plasma membrane of certain cells, including fibroblasts (Oliferenko et al., 1999). These microdomains, called lipid rafts, contain a lipocortin called annexin II at their cytoplasmic face. Caveolae, noncoated invaginations of the plasma membrane, constitute one type of these cholesterol-rich microdomains. Some vertebrate hyaluronidases are glycosyl-phosphatidylinositol-anchored, and most glycosyl-phosphatidylinositol-anchored proteins cluster into caveolae. Certain anti-inflammatory substances utilize the lipocortin family of proteins to inhibit the generation of phospholipase A_2 and, thus, retard the inflammatory response. There is a sequence homology between lipocortin and sauvagine. Mystixins may possibly be interacting with CD44, regulating the metabolic fate of hyaluronan and inhibiting acute inflammation by acting either directly or indirectly as mediators of arachidonic acid production by way of a lipocortin-like ligand.

Degradation of hyaluronan is achieved through the coordinated action of hyaluronidases, a family of β-endoglycosidases. The ability of mystixin to reduce hyaluronan content in inflamed tissues may in part be due to increased activity of hyaluronidase, the endogenous enzyme that degrades HA.

Hyaluronidase activity is regulated at the genetic level by the HYAL family of hyaluronidase genes. The breakdown and renewal of hyaluronidase in the body and the skin can occur very rapidly. Half of the hyaluronan in the skin is turned over within hours. (Reed et al., 1988, 1990). Recently, a dermal source of hyaluronidase has been identified in human fibroblast cells (Stair-Nawy et al., 1999). This local source of degradative activity may provide a partial explanation for the rapidity with which mystixin is able to elicit its anti-edema effects.

In conclusion, mystixin-7 is an effective inhibitor of hyaluronan deposition both in vivo and in vitro. The mechanism whereby mystixin modulates hyaluronan metabolism involves in part, the stimulation of hyaluronidase activity. Identification of additional molecular targets of the mystixin peptides may provide further insights into the mechanisms of this class of new anti-inflammatory peptides.

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