International Journal of Pharmaceutics, 85 (1992) 51–56

IJP 02767

Evaluation of hyaluronan as a vehicle for peptide growth factors

Per T. Prisell [a,b], Ola Camber [c], Johan Hiselius [c] and Gunnar Norstedt [a]

[a] *Center for Biotechnology and* [b] *Department of Orthopaedic Surgery, Huddinge University Hospital, Karolinska Institute, NOVUM, S-141 57 Huddinge (Sweden) and* [c] *KabiPharmacia AB, S-751 82 Uppsala (Sweden)*

(Received 11 November 1991)
(Accepted 20 January 1992)

Key words: Hyaluronan; Insulin-like growth factor; Slow release mechanism; Tissue regeneration; Wound healing

Summary

Sodium hyaluronate, or hyaluronan (HA), was investigated as a vehicle for high molecular weight drugs given subcutaneously. Human recombinant insulin-like growth factor-I (hIGF-I), a peptide showing diverse anabolic cellular effects, was dissolved in different concentrations (0.0–2.0%) of hyaluronan. Three kinds of experiments were performed: a diffusion study, an in vitro release study and an in vivo release study in the rat. The diffusion study showed the diffusion rate of the peptide to be extremely low in hyaluronan, indicating an interaction between vehicle and drug. The in vitro release study showed that the release rate of hIGF-I from the vehicle is dependent on the vehicle concentration; the higher the concentration of hyaluronan the lower the release rate. This result was confirmed in the in vivo release study, where the dorsal rat hind foot was injected with ^{125}I-labelled hIGF-I dissolved in vehicles containing 0.0, 0.5, 1.0 or 2.0% hyaluronan, followed by determination of remaining radioactivity at the injection site. We conclude that HA, a substance which occurs naturally in the body, can retard the release of peptide growth factors.

Introduction

Hyaluronan (Balazs et al., 1986) is a high molecular weight glucosaminoglycan with repetitive units of *N*-acetylglucosamine and gluconuronic acid. It occurs naturally in the body in synovial fluid, corpus vitreum, skin and in the umbilical cord (for reviews see Balazs et al., 1986; Laurent and Fraser, 1986; Laurent, 1987). The in vivo function of hyaluronan is still obscure, but it is believed to serve as a stabilizer and as a transport system for nutrients and metabolites, for example, in the vitreous body of the eye and in the synovial fluid in joints. Hyaluronan is currently and preferentially used clinically in eye surgery, where a 1% solution of hyaluronan is commonly used (e.g., Healon®). Further beneficial effects, resulting from locally administered hyaluronan, have been shown during wound healing (King et al., 1991). Since hyaluronan is a natural and non-irritating substance with a very high water binding capacity, interest has been focused on its use as a vehicle for local delivery of drugs (Camber et al., 1987; Camber and Edman, 1989; Ludwig and Van Ooteghem, 1989).

Correspondence to: P.T. Prisell, Center for Biotechnology, Karolinska Institute, NOVUM, S-141 57 Huddinge, Sweden.

Growth factors (GFs), peptides with growth promoting/anabolic effects, are produced by a variety of tissues. The mode of action of growth factors can be paracrine, endocrine, autocrine or matricrine. The insulin-like growth factors (IGFs) represent one example of this type of peptides. Two subgroups of IGFs have been described, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) (for reviews see Daughaday and Rotwein, 1989; Humbel, 1990). Both are peptides with molecular masses of approx. 7500 Da. Based on its mitogenic properties and its role in tissue repair (Edwall et al., 1989, 1992), the administration of GF, including the IGFs, has been proposed to have a therapeutic potential in the facilitation of tissue regeneration (Howes et al., 1988; Aspenberg and Lohmander, 1989; Lynch et al., 1989; Jingushi et al., 1990).

The short biological half-life of the peptides must be taken into consideration, as well as the concept that the action of growth factors is mediated mainly through autocrine and/or paracrine pathways from locally activated/regenerating cells. An increased local bioavailability of growth factors in a slow release form therefore could be of potential interest in future pharmaceuticals. The combination of HA and active pharmaceuticals is already experimentally in use in topical ophthalmic drug delivery systems (Camber et al., 1987; Camber and Edman, 1989; Ludwig and Van Ooteghem, 1989). The aim of these combined in vitro and in vivo studies was to investigate the influence of HA on the release of IGF-I.

Materials and Methods

Chemicals

Hyaluronan (HA), derived from rooster combs, was obtained from KabiPharmacia AB, Sweden. [^{14}C]Hyaluronan (485 kBq/mg) was purchased from Biotechnology General Ltd, Israel. Just prior to the experimental run, the molecular mass of [^{14}C]hyaluronan was determined by size exclusion chromatography (Sephacryl HR, Pharmacia AB, Sweden) and was found to be 5.82×10^5 Da. Recombinant human insulin-like growth factor-I (hIGF-I), a generous gift from KabiGen AB, Sweden, was iodinated ([^{125}I]iodine, Amersham, Bucks, U.K.) by the Iodogen method (Fraker and Speck, 1978) and a specific activity of 120 Ci/g was obtained.

In vitro assays for IGF-I diffusion

[^{125}I]Iodine or ^{125}I-labelled hIGF-I was added to solutions of HA. Each mixture was then agitated slowly at room temperature for 24 h. The homogeneity of each solution was checked and confirmed by radioactivity determinations in multiple samples after the mixing procedure. Upright plastic tubes were used in the study of IGF-I diffusion in HA. The tubes (length, 70 mm; diameter, 5 mm) were filled with 1% hyaluronan. The experimental zero run-time started with the loading of 120 μl [^{125}I]iodine in HA or ^{125}I-labelled hIGF-I in HA solutions, into the top of each HA-filled tube. After 140 h, in an isolated, temperature-controlled room, the tubes were frozen in liquid nitrogen and cut into 8 mm slices. The radioactivity was thereafter determined in the segments of each tube. The diffusion of [^{14}C]hyaluronan in 1% HA was studied in a similar way.

The release of ^{125}I-labelled hIGF-I into another medium, phosphate-buffered saline (PBS), was also studied. A diffusion chamber was used containing two compartments (donor/receiving) separated by a 10 μm nylon filter (Nytal HD 10). The filter was in constant contact with the lower (receiving) compartment, which was filled with PBS. Each donor compartment containing either ^{125}I-labelled hIGF-I in HA or ^{125}I-labelled hIGF-I in PBS. Aliquots of 150 μl PBS could be removed from the lower compartment (constantly stirred at a slow speed with a magnetic stir bar) through a separate tube and analyzed for ^{125}I radioactivity. Each sample volume (150 μl) was replaced with PBS. Before each experimental run all solutions were mixed by slow rotation for 24 h.

Equilibrium was determined when sampled temporal changes of radioactivity were zero (48 h in all cases).

In vivo experiments

In vivo release of ^{125}I-labelled hIGF-I, dissolved as described above in HA or PBS, was evaluated by monitoring local radioactivity de-

cline subsequent to subcutaneous injection. Male Sprague Dawley rats of approx. 250 g body weight were used. During ether narcosis, the ^{125}I-labelled + unlabelled hIGF-I in HA or PBS was injected subcutaneously (50 μl) into the distal, dorsal right hind foot. After different time intervals the radioactivity was determined at the injection site. Two different experimental evaluations were used, either an invasive or a non-invasive method. When using the invasive method, rats were killed at each time point using CO_2. The feet (from four rats in each group and time point) were amputated at the ankle joint level and radioactivity was determined using a scintillation counter. In the non-invasive method each group contained three to six animals. The injected region was localized, firmly kept in position and the radioactivity was determined, at different time points, using a GM tube (Na I-detector, J&P Engineering Ltd).

Calculation of results

Means and standard deviations were calculated and/or Kruskal-Wallis one-way analysis of variance by ranks was/were performed for the various groups.

Results

In vitro experiments

Diffusion of ^{125}I-labelled hIGF-I in HA was examined initially. Fig. 1 shows the diffusion of hIGF-I in HA 72 h after the application of a small volume of ^{125}I-labelled hIGF-I into the top of a tube containing 1% HA. Radioactivity determinations in sections of the tube showed the majority of radioactivity to be near the site of application of the peptide, indicating the slow diffusion of hIGF-I in HA. The diffusion of ^{125}I-labelled hIGF-I was different from that of [^{125}I]iodine, as the latter was more evenly distributed in all sections of the tube. The slow diffusion of ^{125}I-labelled hIGF-I appeared to have properties similar to those for diffusion of [^{14}C]HA (Fig. 1).

Release chambers were used where ^{125}I-labelled hIGF-I dissolved in HA was loaded on a

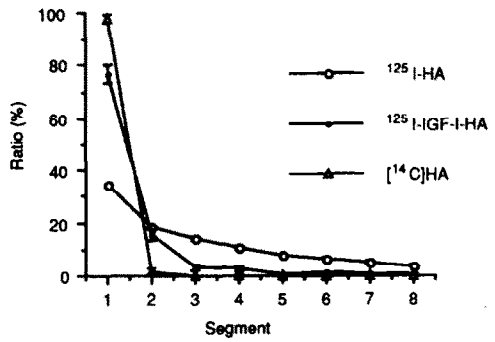

Fig. 1. In vitro diffusion of I, hIGF-I and hyaluronan in hyaluronan. [^{125}I]Iodine (500000 cpm), ^{125}I-labelled hIGF-I (100000 cpm) or [^{14}C]hyaluronan (800000 cpm) were mixed for 24 h in 1% HA. Aliquots of 120 μl of each solution were subsequently loaded into the top of tubes containing 1200 μl 1% HA. After 140 h the tubes were frozen in liquid N_2 and cut into 8 mm sections. The abscissa indicates the section number, starting from the loading site. The ordinate shows the radioactivity expressed as the ratio of total radioactivity in each tube. The data shown are means ± SD, $n = 3$.

filter separating the two compartments. The filter was in contact with the lower (receiving) compartment containing PBS. Analysis of release rates of ^{125}I-labelled hIGF-I into PBS is shown in Fig. 2.

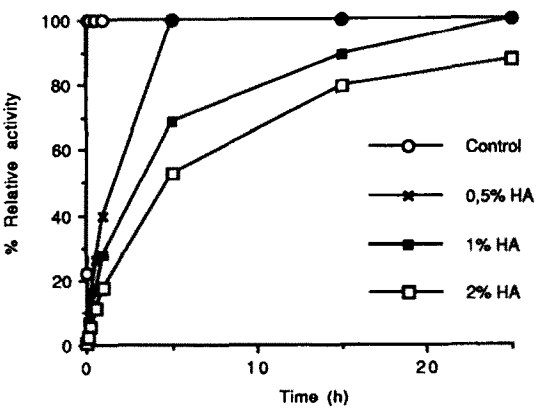

Fig. 2. In vitro release of hIGF-I. ^{125}I-labelled hIGF-I was mixed, by slow rotation for 24 h, with control (PBS) or 0.5, 1 or 2% HA, respectively. A 1 ml aliquot of each mixture (with approx. 270000 cpm) was deposited in the donor compartment of a release chamber. The two compartments were separated by a nylon filter with a pore size of 10 μm. The abscissa indicates each sampling time point and the ordinate shows the ratio of radioactivity in the lower (receiving) PBS-containing compartment compared to the amount of radioactivity found at steady state after 48 h. Each sampling volume consisted of 150 μl and was replaced by PBS. The data are from one representative experiment repeated twice.

Dissolution of hIGF-I in HA retarded the release of hIGF-I compared to dissolving the compound in PBS. The time point at which 50% of the radioactivity was recovered in the lower, PBS-containing (receiving) compartment was less than 30 min for hIGF-I dissolved in PBS and approx. 3 h for hIGF-I dissolved in 1% HA. The capacity of HA to achieve a slow release of hIGF-I was furthermore dependent on the concentration of HA; hIGF-I in a 2% HA solution released 50% of the radioactivity in approx. 5 h.

From the above experiments, it clearly appears that mixing hIGF-I in HA results in an association to give hIGF-I-HA. This process of association shows slow release characteristics for hIGF-I.

In vivo experiments

The release properties of ^{125}I-labelled hIGF-I + 6 μg hIGF-I in 1% HA or in PBS, mixed for 24 h, were investigated in rats after injection into the dorsal right hind foot. Using the invasive method, we observed a pronounced slow release among rats with HA as vehicle as compared to those with PBS (Fig. 3). After 8 h the ^{125}I activity in the control group had declined to approx. 6%,

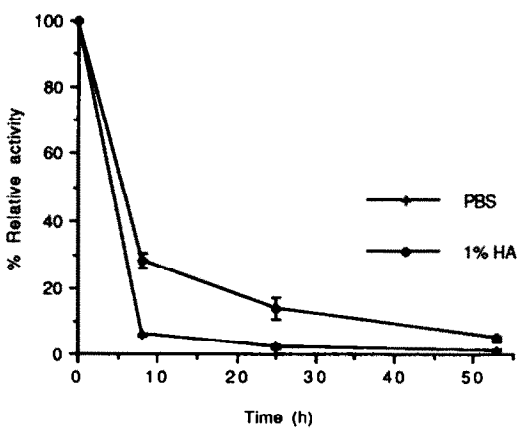

Fig. 3. In vivo release following subcutaneous injection of radiolabelled hIGF-I in 1% HA. Each rat was injected (50 μl) in the right dorsal hind foot with ^{125}I-labelled hIGF-I + 6 μg hIGF-I, which was previously mixed for 24 h in 1% HA or PBS. Radioactivity in amputated rat feet was analyzed at the time points indicated on the abscissa. The ordinate shows the radioactivity expressed as a ratio of total deposited radioactivity (3200 ± 290 cpm). Each point represents the mean ± SD of four animals.

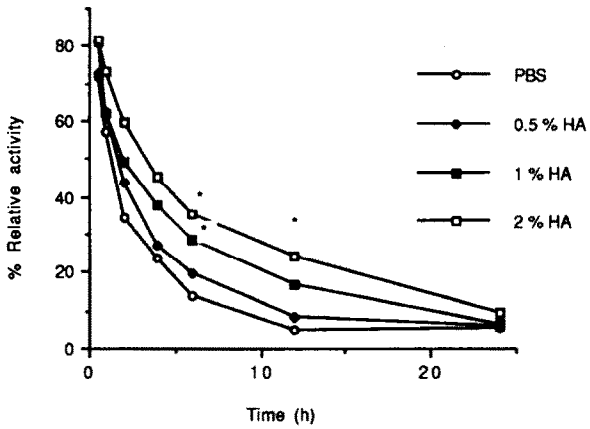

Fig. 4. In vivo release following subcutaneous injection of radiolabelled hIGF-I in 1% HA. Rats were injected (50 μl) in the dorsal right hind foot with ^{125}I-labelled hIGF-I + 2 μg hIGF-I, which was mixed for 24 h in PBS or 0.5, 1 or 2% HA, respectively. The amount of radioactivity at the site of injection was analyzed, using a GM tube, at the time points indicated on the abscissa. The ordinate shows radioactivity found as a ratio of total deposited radioactivity (approx. 5000 cpm). Each time point represents three to seven animals (mean ± SD). Kruskal-Wallis one-way analysis of variance by ranks was performed and a significance of $p < 0.05$ as compared to control was found at 6 h for both 1 and 2% HA and at 12 h for 2% HA.

whereas 28% remained in the HA group. After 24 h the ^{125}I activity was approx. 3 and 15%, respectively.

In the non-invasive method, ^{125}I-labelled hIGF-I + 2 μg hIGF-I, mixed for 24 h in PBS or 0.5, 1.0, or 2.0% HA, respectively, was administered. The duration of radioactivity decline at the site of injection was significantly ($p < 0.05$) longer in animals that received hIGF-I dissolved in HA compared to those which received hIGF-I dissolved in PBS (Fig. 4). After 6 h, approx. 26 and 35% of radioactivity remained at the site of injection in animals receiving 1 and 2% HA, respectively, corresponding to 12% for the group injected with ^{125}I-labelled hIGF-I + 2 μg hIGF-I in PBS. After 24 h there was no statistical difference among tested groups.

Discussion

In this study, we have described experiments which show that dissolving hIGF-I in hyaluronan

results in the slow release of this peptide growth factor in vivo and in vitro. The extent of slow release was dependent on the concentration of hyaluronan; a higher concentration of HA reduced the release rate. The diffusion of hIGF-I in HA was similar to that of [^{14}C]HA in HA itself, indicating the possibility of an interaction between HA and the peptide hIGF-I. The nature of this interaction is unknown but one may speculate that the capacity of HA to slowly release a peptide may be due to the complexity of the long chains of HA and/or ionic interactions between HA and the peptide. The contention that hIGF-I dissolved in HA results in the slow release of the peptide was also supported by in vivo experiments. The kinetics of radioactivity decline was somewhat different depending on the technique used for radioactivity determinations. The reason for this remains unclear.

In other studies, methyl cellulose has been used as a vehicle/slow release carrier for peptides (Aspenberg and Lohmander, 1989; Lynch et al., 1989) and this has resulted in the promotion of peptide effects, as compared with earlier studies not using slow release systems. Previous investigations have indicated the potential use of HA as a slow release vehicle for compounds with lower molecular mass, for example pilocarpine (Camber and Edman, 1989; Camber et al., 1987; Ludwig and Van Ooteghem, 1989). In the present study, we have not investigated the biological activity of hIGF-I when dissolved in hyaluronan. Assuming that IGF-I is biologically active in HA, a slow release of IGF-I could have several applications. One potential situation in which slow release administration of IGF-I could be of value is during muscle and bone regeneration. In experimental injuries of bone and muscle the endogenous production of IGF-I is locally and transiently activated during the early phase of tissue repair (Edwall et al., 1989, 1992). In addition, administration of IGF-I and platelet-derived growth factor (PDGF) dissolved in methyl cellulose gel increases the wound healing rate (Lynch et al., 1989).

In conclusion, one may speculate that a non-irritating, biodegradable, naturally occurring substance which allows the localized slow release of active growth factors, such as that discussed here, may in the future find application in several conditions, including diseases of bone and cartilage and other situations involving tissue regeneration and wound healing. Growth factor-HA combinations therefore could provide one answer to the recent demands for 'growth factors in appropriate delivery systems' (Bessho et al., 1991).

Acknowledgements

This work was supported by the Swedish Medical Research Council (Project No 7762) and by a grant from KabiPharmacia AB.

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