Available online at www.sciencedirect.com
ScienceDirect
Journal of Controlled Release 114 (2006) 230 – 241
journal of controlled release
www.elsevier.com/locate/jconrel

A recombinant human enzyme for enhanced interstitial transport of therapeutics

L.H. Bookbinder [a], A. Hofer [a], M.F. Haller [a], M.L. Zepeda [b], G.-A. Keller [a], J.E. Lim [a], T.S. Edgington [c], H.M. Shepard [d], J.S. Patton [e], G.I. Frost [a,*]

[a] *Halozyme Therapeutics, San Diego, CA 92121, United States*
[b] *Schering-Plough Research Institute (SPRI), San Diego, CA 92121, United States*
[c] *Department of Immunology and Vascular Biology, Scripps Research Institute, La Jolla, CA 92037, United States*
[d] *Receptor Biologix, South San Francisco, CA 94080, United States*
[e] *Nektar Therapeutics, San Carlos, CA 94070, United States*

Received 16 March 2006; accepted 24 May 2006
Available online 7 June 2006

Abstract

Subcutaneously injected therapeutics must pass through the interstitial matrix of the skin in order to reach their intended targets. This complex, three-dimensional structure limits the type and quantity of drugs that can be administered by local injection. Here we found that depolymerization of the viscoelastic component of the interstitial matrix in animal models with a highly purified recombinant human hyaluronidase enzyme (rHuPH20) increased the dispersion of locally injected drugs, across a broad range of molecular weights without tissue distortion. rHuPH20 increased infusion rates and the pattern and extent of appearance of locally injected drugs in systemic blood. In particular, rHuPH20 changed the pharmacokinetic profiles and significantly augmented the absolute bioavailability of locally injected large protein therapeutics. Importantly, within 24 h of injection, the interstitial viscoelastic barriers were restored without histologic alterations or signs of inflammation. rHuPH20 may function as an interstitial delivery enhancing agent capable of increasing the dispersion and bioavailability of coinjected drugs that may enable subcutaneous administration of therapeutics and replace intravenous delivery.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Hyaluronan; Hyaluronidase; Subcutaneous drug delivery; Subcutaneous bioavailability; PH20; Monoclonal antibodies; Interferon; Adenovirus

1. Introduction

Following subcutaneous injection, most drugs must pass through the interstitial matrix, traverse capillaries or lymphatics, and reach the vascular compartment in order to reach the final site of pathology [1]. The rate by which drugs permeate the interstitium is regulated by both diffusion and convective fluid flow. The interstitial matrix is a complex three-dimensional dynamic structure that acts as a filter controlling the rate of drug flow. It is comprised of numerous structural macromolecules including collagens, elastin, and fibronectin, in which glycosaminoglycans and proteoglycans form a hydrated gel-like substance. Collagens are the predominant fibers that hold tissues in place and maintain tissue integrity. Glycosaminoglycans such as hyaluronan contribute to the "ground substance" by creating a barrier to bulk fluid flow through the interstitial collagenous matrix by way of their viscosity and water of hydration [2]. Hyaluronan is a mega-dalton molecule consisting of repeating disaccharide units that allows the extracellular matrix to resist compressive forces. While hyaluronan is found at only 1% the concentration of collagen in the skin, it occupies a fluid exclusion volume 10-fold higher than that of collagen on a mL H_2O/mg basis [3]. In contrast to collagen, which has a half-life approaching 15 years [4], hyaluronan is rapidly turned over in the body with a half-life of 15–20 h in the skin [5,6].

"Spreading agents" derived from animal testes extracts containing interstitial matrix-degrading enzymes have been used clinically for over 50 years to facilitate the dispersion and absorption of other drugs [7]. The active agent in these testes

* Corresponding author. Tel.: +1 858 794 8889.
E-mail address: gfrost@halozyme.com (G.I. Frost).

0168-3659/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2006.05.027

extracts was later associated with hyaluronidase activity [8]. Hyaluronidases are a family of glycosaminoglycan-degrading enzymes that nature has utilized repeatedly as a delivery agent to facilitate the dispersion of venoms, toxins, bacteria and spermatozoa through interstitial spaces [9]. PH20 is the predominant hyaluronidase in mammalian testes. Among the six hyaluronidase-like genes in the human genome, only the PH20 gene product is a neutral pH-active hyaluronidase and degrades glycosaminoglycans under physiologic conditions. The human enzyme is a 509 amino acid glycoprotein anchored to the plasma membrane through a glycosyl-phosphatidylinositol (GPI) moiety that facilitates penetration of spermatozoa through the cumulus cells to enable fertilization [10].

While animal testes-derived hyaluronidase extracts have been used extensively in the clinic to disperse other injected drugs, they have generally been limited by both immunogenicity and impurity profiles, typically comprising less than 1% enzyme per mg total protein. Such preparations are frequently contaminated with proteases, immunoglobulin and factors that increase capillary permeability and can also give rise to IgE mediated allergic reactions upon repeat administration [11–13], generally precluding their repeated use.

To investigate whether a purified soluble recombinant human PH20 enzyme could mediate the same spreading properties described in the animal sourced hyaluronidase extracts, we generated a soluble form of human hyaluronidase (rHuPH20) lacking the GPI membrane attachment motif. We provide evidence that the enzyme enhanced the infusion rates and penetration of molecules up to 200 nm in diameter up to 20-fold without eliciting inflammation, vascular permeability, immunogenic or allergic reactions. In addition to increasing infusion rates and dispersion across a broad range of molecular weights, rHuPH20 was found to enhance the systemic bioavailability of locally-injected biotherapeutics such as alpha interferons and monoclonal antibodies with an absolute bioavailability comparable to the levels obtained by intravenous delivery. By increasing infusion rates, rHuPH20 also permitted injection of large volumes with minimal distortion and tearing of the subcutaneous fascia. Finally, we show that the effects of rHuPH20 were temporary and fully reversible within 24 h of injection.

2. Materials and methods

2.1. Engineering, purification, and characterization of recombinant neutral-active soluble human PH20 hyaluronidase

A construct encoding a soluble, neutral-active form of the human PH20 gene was identified from a full length human PH20 cDNA EST clone by screening progressive carboxy terminal truncations for soluble neutral active enzyme activity when transfected into CHO cells. Enzyme activity in culture supernatants was measured as described previously [14]. An expression system was constructed with a CMV-based bi-cistronic cassette driving both the soluble rHuPH20 domain (amino acids 1–447) and the murine DHFR gene separated by an internal ribosomal entry site. The selected clone was electroporated into DHFR$^{-/-}$ CHO cells (DG44), previously adapted to grow in a chemically defined, animal product-free medium (Invitrogen). Cells were cloned by limiting dilution and amplified in methotrexate.

Recombinant human PH20 was purified from conditioned media through a series of ion exchange, hydrophobic interaction, aminophenylboronate, and hydroxyapatite chromatography to greater than 90% purity based upon specific activity (>100,000 USP Units/mg). CHO host protein contaminants were found to be less than 1% by ELISA, and preparations had endotoxin content of less than 0.5 EU/mg protein. Enzyme potency in bulk preparations was determined by turbidimetric assay [15] with USP bovine hyaluronidase reference standard. Analysis of hyaluronan fragment sizes following digestion with rHuPH20 was performed as described previously [16].

2.2. Interstitial dispersion experiments

All animal studies were approved by IACUC. Unless specified, outbred $^{Nu/Nu}$ mice (Harlan) were anesthetized by intraperitoneal injection of Ketamine (150 mg/kg) and Xylazine (10 mg/kg) followed by injection of test article or carrier control (10 mM Hepes, 1 mM Calcium, 130 mM NaCl, 1 mg/mL Human Serum Albumin) in a 0.5 mL syringe with a 27 gauge needle in a final volume of 50 μl. Test articles and controls were isotonic and ranged from pH 6.5 to 7.5. Following injection, dye areas were measured at the respective time points, under normal light (trypan blue) or black light (fluorescence) using microcalipers and measured in 2 dimensions, with areas calculated using the formula Area$=((D_1 \times D_2) \times \pi/4)$ (where D_1 and D_2 are the diameters of the dye front). A minimum of four injection sites per dose were tested and compared by single factor ANOVA with controls for each time point. For dermal barrier reconstitution experiments, rHuPH20, USP hyaluronidase reference standard or carrier control were injected and marked with a pen followed by injections of 50 μL of trypan blue dye at 0.5, 1, 24, and 48 h.

2.3. Effects of rHuPH20 on IL-8 expression by microvascular endothelial cells

Human microvascular endothelial cells were grown as recommended by the supplier (Cascade Biologics), Endotoxin (purified fraction from *Escherichia coli* LPS) was purchased from Charles River Endosafe (lot EX32952), and Peptidoglycan from Staph A from Sigma. Cells were seeded into 96 well plates at 2000 viable cells/well for 16–24 h before adding test articles. Test articles were incubated with cells for 24 h followed by detection of IL-8 using a capture ELISA kit (Pharmingen).

2.4. Toxicity study of rHuPH20 administered by subcutaneous injection to non-human primates

Toxicology studies were performed at Charles River Laboratories, Inc. (Sierra Division, Sparks, NV). Forty-eight experimentally naive rhesus monkeys, 24 males, 2.6 to 8.1 years of age, weighing 3.6 to 8.6 kg and 24 females 3.2 to 7.1 years of age weighing 3.3 to 6.3 kg at the outset (Day 1) of the study were dosed with 0, 130, 3,800, and 38,000 Units of rHuPH20 in 1 mL of 10 mM Phosphate, pH 7.4 with 1 mM EDTA, 1 mM CaCl2,

and 144 mM NaCl with 1 mg/mL human serum albumin via subcutaneous injection on Day 1 (scapular region). Some animals received an additional dose on Day 8. Blood samples were collected for evaluation of clinical pathology from all animals 48 h after the initial dose (Day 3) and on Days 10 and 29, and for antibody analysis on Day 21 and 28. At termination, a full necropsy was conducted on all animals. A standard list of tissues including the injection sites was collected at Days 3, 10, and 29, processed for histology and evaluated by a pathologist certified by the American College of Veterinary Pathologists.

2.5. Measurement of neutralizing antibodies in non-human primates

Neutralizing antisera and dose solution testing were performed at Microconstants (San Diego, CA). Neutralizing antibodies to rHuPH20 were measured by spike recovery in a modified hyaluronidase turbidometric assay using hyaluronan as a substrate. Recovery of rHuPH20 enzyme activity was tested in the sera of animals taken on Days 21 and 28. Hyperimmune sera from rabbits immunized against rHuPH20 were added as a positive control to neutralize spike recovery. The method is capable of detecting the neutralization of rHuPH20 from 5 ng/mL in primate sera. This represents less than 1% of the neutralizing activity found in the sera of rabbits hyperimmunized to rHuPH20.

2.6. Determination of channels size in the skin of mice treated with rHuPH20

Anesthetized mice received either 25 U rHuPH20 in 10 mM Hepes, 1 mg/mL Human serum albumin, 130 mM NaCl, or the carrier control (buffer without rHuPH20) in a 25 μL dose by intradermal injection in opposing midline lateral sides. Ten minutes later, mice were injected with 25 μL of either 2 million Da dextran (Sigma) or fluorescent latex beads of increasing size: 20 nm, 100 nm, 200 nm, 500 nm, and 1 μm (Molecular Probes, Carlsbad, CA). Mean areas ($n=4$ animals per group/time point) were compared between the rHuPH20 injected and placebo injected groups. Statistical analysis of the dye area at each time point and particle size was performed with a 2-tailed Student's *t*-test using an exploratory design without compensation for potential Type I error. Particle sizes and time points significantly different ($P<0.05$) between rHuPH20 and control are represented with an asterisk.

2.7. Measurement of infusion rates

Human serum albumin (MW = 68 kDa) (Buminate, Baxter Healthcare, Glendale, CA) diluted to 50 mg/mL in PBS was filled into a 10 mL reservoir connected to an 18 gauge needle through 0.5 mm diameter tubing. The needle was placed into the dermis of the flank of an anesthetized nude mouse and flow rates were established by tracking microbubble movement introduced into the tubing. The flow rates for each animal were measured for 100 μL at 20, 30 and 40 cm H_2O of pressure with and without 100 U rHuPH20. Three animals per group were used to establish flow rates at each pressure.

2.8. Effects of rHuPH20 on adenovirus delivery

rAd-GFP viral construct is an E1, E3, and pIX deleted adenoviral vector containing the EGFP expression cassette (Clontech, Palo Alto, CA) driven by a human cytomegalovirus (CMV) promoter. Homologous recombination in 293 cells (Microbix Biosystems, ON, Canada) was used to generate infectious viral DNA, which was subsequently transfected into 293 cells to generate virus, purified by column chromatography [17] and quantitated by anion-exchange HPLC. Under sterile conditions, mice were pre-injected with either 50 μL of vPBS (DPBS solution containing 3% sucrose, pH 7.4) or 50 μL of rHuPH20 diluted in vPBS to a final concentration of 10 U/injection (200 U/mL). Pre-treatment injections occurred 0.75, 1.5, 6, or 24 h prior to adenoviral administration. For co-administration, rHuPH20 was mixed with rAd-GFP and vPBS to a final concentration of 10 U/injection (200 U/mL), with a final virus concentration of 1.5×10^{10} particles (PN)/injection and a total injection volume of 50 μL. Injections were made 2 and 4 cm above the tail base along the midline of the animal. The perimeter of the injection sites were then marked with a permanent marker to delineate the initial injection areas. Following the pre-treatments, 1.5×10^{10} PN of rAd-GFP was injected in the same location as the previously marked pre-treatment injection sites, in a final volume of 50 μL/injection. Following adenovirus injection, animals were anesthetized under isofluorane gas and GFP expression was recorded at 0.25, 0.75, 1, 2, 3, 5, and 6 days under identical camera settings. The digital SLR camera (Nikon, Melville, NY) was calibrated to a fixed reference focal distance to the injection site. Digital images were then quantitatively analyzed for total area of fluorescence signal using the Image Pro Plus software™ (Media Cybernetics, MD).

2.9. Pharmacokinetics and bioavailability of a pegylated-cytokine and therapeutic monoclonal antibody

Anesthetized female Sprague–Dawley rats (6 per group) were injected intravenously (group 1), locally (group 2) and locally with 100 Units rHuPH20 (group 3) in a 150 μL volume with either pegylatedinterferon alfa-2b (1.5 μg/kg, Schering Plough, NJ) or a Infliximab (10 mg/kg, Centocor, PA) labeled with 125-Iodine to specific activities of 55 μCi/μg and 10.3 μCi/mg, respectively. Radiolabeling was performed by Amersham Biosciences, NJ. 300 μL of blood was collected via tail bleed at 0.5, 1, 2, 3, 6, 8, 12, 24, 36 and 48 h post-dose from rats receiving pegylatedinterferon alfa-2b at 0.5, 1, 2, 4, 8, 24, 48, 72, 96 and 120 h post-dose from animals receiving infliximab. Activity was measured by gamma counting (IsoData model #20-10 gamma counter, Isodata, IL) and expressed as CPM/g blood. Pharmacokinetic parameters were established using the linear trapezoidal method with integration from T_0 to infinity based upon the elimination rate constants established from the intravenous doses.

2.10. Measurement of vascular permeability

Vascular permeability was tested in nude mice using the Miles Assay [18]. Recombinant murine vascular endothelial growth

factor (VEGF) positive control (100 ng) (Sigma) or 100 ng rHuPH20 (10 U) was injected 15 min after intravenous injection of 100 μL of 0.5% Evans blue dye (Mallinckrod Baker, Inc. NJ) into female nude mice. Dye extravasation was photographed 15 min after the injection of test article.

3. Results

3.1. Characterization of recombinant human PH20 hyaluronidase

Expression of DNA encoding a 447 amino acid human PH20 sequence devoid of the GPI anchor attachment motif resulted in a catalytically active soluble variant of the human rHuPH20 enzyme capable of degrading hyaluronan under physiological conditions. The 61 kDa glycoprotein was secreted with 6 N-linked glycans and a properly processed signal peptide. Production of rHuPH20 in Chinese Hamster Ovary (CHO) cells without animal products such as fetal bovine serum allowed purification of the protein to high specific activity (>100,000 USP Units/mg protein). The rHuPH20 preparation was approximately 100-times greater purity than the bovine testes-derived hyaluronidases used clinically for many years by specific activity. As shown in Fig. 1A, whereas the purified rHuPH20 migrated on SDS PAGE as a homogeneous preparation, a commercial bovine testes-derived hyaluronidase extract showed multiple impurities. Only one minor 75 kDa band in the smear of proteins of the bovine preparation cross-reacted strongly with a monoclonal antibody to anti-ram PH20 by Western blot, confirming that the hyaluronidase enzyme is a minor component of the animal derived hyaluronidase preparations. Previous studies have demonstrated that when purified to homogeneity, bovine and ovine PH20 preparations also possess high specific activities comparable to recombinant human PH20 [19–21]. Hyaluronan is a highly polymerized molecule consisting of alternating units of N-acetylglucosamine and glucuronic acid. Its molecular weight ranges from 400 Da to over 6 million Da. As shown in Fig. 1B, high molecular weight hyaluronan (approximately 1×10^6 Da) could be completely depolymerized *in vitro* with nanogram quantities of rHuPH20 (1 USP Unit, ~10 ng) as determined by electrophoretic analysis of digestion products over time followed by staining with Alcian Blue.

3.2. Effects of rHuPH20 are dose dependent in vivo

Spreading factors can be measured by their ability to increase the area of locally injected tracer dyes following intradermal co-administration. As shown in Fig. 2, dose response studies with rHuPH20 in a fixed volume (50 μL) measuring dye front area in two dimensions from 2 to 20 min post injection demonstrated significantly increased tracer dye area relative to carrier control in a dose dependent fashion. At higher concentrations of enzyme, the initial injection bleb collapsed rapidly before the initial time point could be measured. Thermal inactivation of the enzyme at 90 °C for 10 min completely abolished both enzyme activity *in vitro* and spreading activity *in vivo*, confirming specificity of the enzyme. Comparison of dye area generated by rHuPH20 to a bovine testes extracted hyaluronidase preparation revealed that the two enzymes were equipotent on a unit per unit basis when calibrated by catalytic activity *in vitro* against a hyaluronan substrate.

3.3. Tissue changes following administration of rHuPH20 hyaluronidase are transient, reversible and non-inflammatory

The principal substrate for rHuPH20, hyaluronan, displays a remarkably short half-life *in vivo* compared to other extracellular matrix components found in the skin. Thus, provided that the enzyme itself was inactivated within a short period of time, it would be expected that the effects of rHuPH20 hyaluronidase on

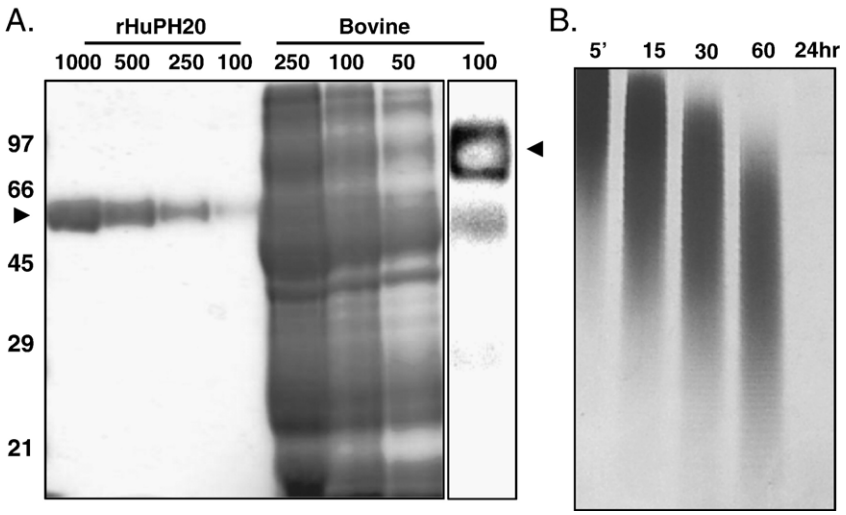

Fig. 1. Characterization of rHuPH20—(A) CHO-derived rHuPH20 1000, 500, 250, 100 Units per lane loaded compared to 250, 100, 50 Units bovine testes hyaluronidase. Western blot analysis of bovine hyaluronidase preparations with the 1D6 mouse anti-ram hyaluronidase monoclonal antibody (Babraham Bioscience Technologies, UK) reveals a single immunoreactive hyaluronidase band in bovine hyaluronidase preparations. (B) rHuPH20 mediates depolymerization of hyaluronan. Umbilical cord hyaluronan incubated with 1 Unit of rHuPH20 for indicated times from 5 min to 24 h. Electrophoresis shows that rHuPH20 completely digests the substrate to undetectable fragments by 24 h.

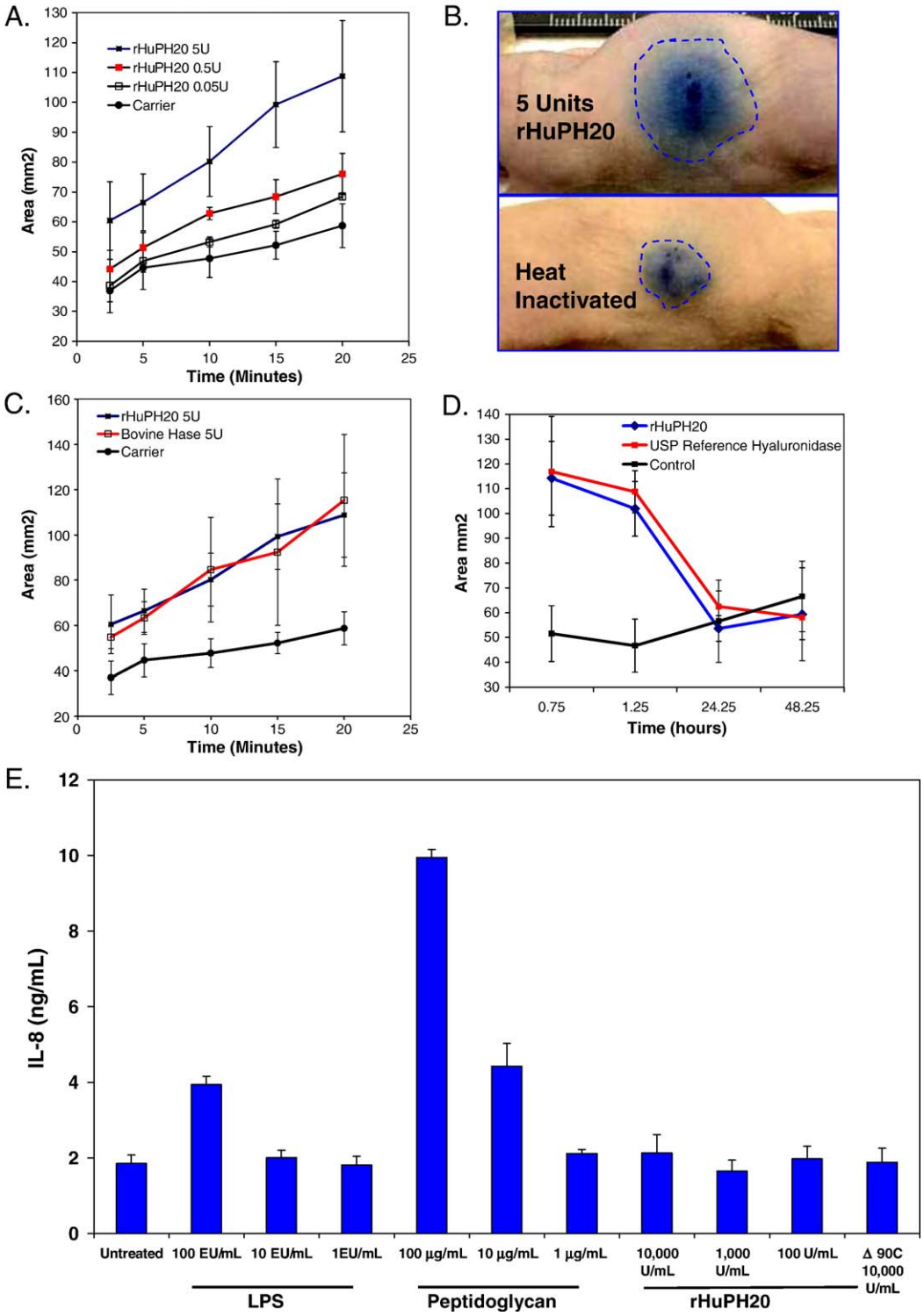

Fig. 2. rHuPH20 is a potent spreading factor *in vivo* but does not activate inflammatory mediators. (A) rHuPH20 (5.0, 0.5, 0.05 Units) or carrier control was co-injected locally with trypan blue dye in mice (50 µL). (B) Heat inactivation of rHuPH20 (90 °C, 10 min) destroys both enzymatic and dye spreading. (C) Comparison of spreading activity of 5 Units rHuPH20 and 5 Units bovine hyaluronidase extract shows equivalent dye dispersion over time. (D) Reversibility of the action of rHuPH20 on the interstitium. 5 Units of rHuPH20 or 5 Units USP Hyaluronidase was injected in the skin followed by injection trypan blue dye at the same site from 0.5 to 48 h later. The effects of rHuPh20 are reversed by 24 h post-injection ($n=4$ mice per group for each study). (E) Lack of IL-8 expression by human microvascular endothelial cultures (HMVECs) exposed to rHuPH20 compared to LPS or peptidoglycan. Cultures of HMVEC's were cultured for 24 h in the presence of the respective test agents and assayed for IL-8 levels in the conditioned media by ELISA. rHuPH20 from 100–10,000 Units failed to increase IL-8 expression.

the dispersion of drugs would be transient and reversible. To examine the pharmacodynamics of rHuPH20, 5 Units (50 ng) of purified rHuPH20 hyaluronidase and 5 Units of a bovine testes hyaluronidase preparation (approximately 7 μg) or carrier control were injected by intradermal injection in nude mice. 50 μL of trypan blue dye, a 960 Da molecular weight tracer used to visualize the extent of diffusion, were administered at the same site from 30 min to 48 h following injection of enzyme or carrier. The dispersion area of trypan blue dye was significantly increased relative to carrier controls at 30 and 60 min post-injection using either enzyme. However, by 24 h post-hyaluronidase injection, the tracer dye area was indistinguishable from that generated at the carrier control injection sites, demonstrating that the changes induced by rHuPH20 in the interstitial matrix were reversible and occurred within a time frame similar to the reported half-life of hyaluronan in the skin (Fig. 2D).

Enzymatic depolymerization of hyaluronan with rHuPH20 could, in theory, produce digestion products that reportedly induce inflammatory mediators through the hyaluronan receptor, CD44 and/or the Toll-Like Receptor (TLR) family [22,23]. Other studies suggest that co-purified materials in the hyaluronan preparations mediate their pro-inflammatory activity [24]. As hyaluronan fragments reportedly induce IL-8 secretion by human microvascular endothelial cultures (HMVEC) through TLR-4 (23), primary HMVEC cultures were exposed to rHuPH20 or heat inactivated control enzyme and IL-8 secretion measured by ELISA. LPS and peptidoglycan, two bacterially derived molecules known to activate the TLR2 and TLR4 pathways were also tested as positive controls. As shown in (Fig. 2E), whereas peptidoglycan and LPS gave rise to dose dependent increases in IL-8 secretion, no such response was found with rHuPH20 over a broad dose range compared to heat inactivated enzyme control. Thus, rHuPH20 does not appear to elicit the inflammatory responses reported previously with human microvascular endothelial cultures.

To examine whether subcutaneous injection of rHuPH20 might lead to the extravasation and accumulation of leukocytes in the vicinity of the injection site, male and female rhesus primates were injected subcutaneously with rHuPH20 from 130 to 38,800 Units with repeat dosing over 28 days. No remarkable inflammatory reactions or infiltrates to rHuPH20 were identified by clinical or histological evaluation of the injection site across a 3 log dose range relative to carrier control (Fig. 3A) in animals sampled from 24 h to 28 days post injection. Furthermore, no adverse local, electrocardiographic, hemodynamic, clinical, or anatomic pathological changes were noted throughout the 28-day observation period of the study.

Animal-derived hyaluronidase preparations are also reportedly allergenic and immunogenic following repeat administration [25]. This could result from either the protein contaminants in the

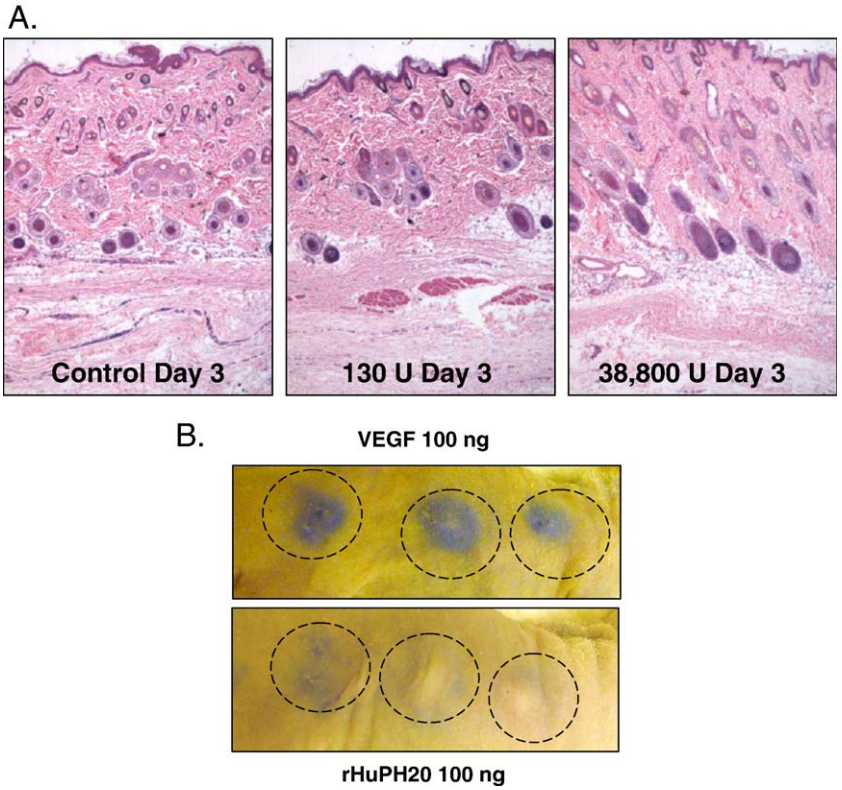

Fig. 3. rHuPH20 does not induce inflammatory responses or vascular permeability *in vivo*. (A) Representative H&E stained slide photomicrographs of rhesus primate injection sites from the scapular region after subcutaneous injection of control, 130 or 38,000 Units of rHuPH20 in 1mL (n=4/sex/group). Similar histology was noted on animals evaluated on day 11 and 28. (B) Intradermal injection of 10 U (100 ng) rHuPH20 does not induce vascular permeability and plasma leakage following intravenous injection of Evan's Blue dye compared to intradermal injection of 100 ng VEGF positive control. Dye extravasation was photographed at 15 min post injection.

bovine preparations or the bovine hyaluronidase enzyme itself, which shares only 65% identity with the human protein. Therefore, sera from male and female rhesus monkeys injected subcutaneously with 3,800 and 38,800 Units of rHuPH20 were tested for the presence of neutralizing antibodies to rHuPH20 on days 21 and 28 by a modified turbidometric enzyme assay and compared to a rabbit antihuman rHuPH20 neutralizing antisera used as a positive control. No neutralizing antibodies were detected at day 21 or day 28 of the study.

3.4. rHuPH20 hyaluronidase does not increase vascular permeability

Testicular hyaluronidase preparations have been reported to modify capillary permeability in rat skin [26]. We therefore measured whether rHuPH20 modifies vascular permeability using the Miles assay with Evans blue dye, a molecular dye that binds to endogenous serum albumin, as a tracer to assay permeability in peripheral vessels, and recombinant vascular endothelial growth factor (rHuVEGF165) as a positive control for mediated vascular leakage. Mice received an intravascular injection of 100 μL of 0.5% Evans blue dye via the tail vein. Fifteen minutes later they were injected intradermally with 100 ng (10 Units) rHuPH20 or 100 ng of VEGF at three different sites on the flank and were photographed 15 min post injection to document any leakage of the dye into the dermal tissue. As shown in Fig. 3B, VEGF but not rHuPH20, prompted a marked increase in vascular permeability that promoted extravasation of plasma proteins, indicating that, while rHuPH20 can facilitate the dispersion of locally injected drugs within the interstitium, it does not appear to perturb the integrity of the vasculature.

3.5. Co-injection of rHuPH20 hyaluronidase enhances the dispersion of molecules up to 200 nm in diameter

Due to the specificity of its substrate, hyaluronidase does not degrade the predominant protein-based structural matrix components in skin, such as the collagen network. The increased infusion rates should therefore be limited to particles small enough to flow through the collagen fibrils remaining in the extracellular matrix following hyaluronan degradation by rHuPH20 [27]. To determine the size of the channels generated by rHuPH20, fluorescently-labeled dextrans of different molecular weights and latex beads of increasing diameter were injected with rHuPH20 in mice, and areas of spreading measured over time. Fig. 4 shows that dextrans of 2,000,000 MW were effectively dispersed with rHuPH20 compared to dextran alone. Injections of fluorescinated latex beads ranging in size from 20 nm to 1 μm in diameter displayed a significantly increased dispersion area for beads up to 200 nm in diameter relative to beads injected with the carrier control alone. However, the dispersion of 500 nm and 1 μm diameter beads in the presence of rHuPH20 was not significantly different from carrier controls up to 4 h post-injection, indicating that rHuPH20 treatment did not improve the diffusion of agents larger than 200 nm.

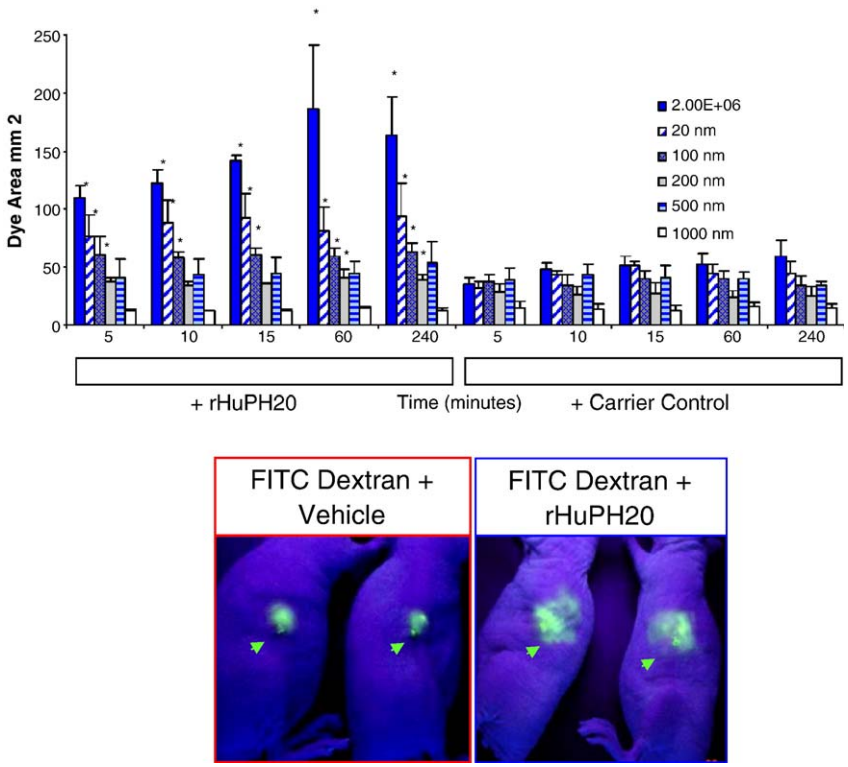

Fig. 4. Determination of the gap sizes generated by rHuPH20. Mice injected locally with 25 Units rHuPH20 or carrier control with molecules of increasing size in 50 μL (FITC Dextran 2 million Da or fluorospheres from 20 to 1000 nm diameter) demonstrated significantly increased infusion rates for particles up to 200 nm in diameter over time.

Modified adenoviruses have been tested as vectors for gene transfer in clinical trials for the treatment of various diseases. Adenoviruses are particles with a diameter of approximately 35 nm. The effects of rHuPH20 hyaluronidase on the dispersion of recombinant adenovirus encoding green fluorescent protein (GFP) were evaluated in mice as a model large particle. GFP recombinant adenovirus was injected intradermally into the backs of mice in the absence or presence of 10 Units of rHuPH20 hyaluronidase. Animals were imaged from 24 h to 7 days post-injection and images were quantitatively analyzed for total area of fluorescence signal. Visualization of GFP gene expression confirmed that the adenoviruses spread to a significantly larger surface area of the skin when co-injected with rHuPH20, allowing a significantly greater area of tissue to be infected (Fig. 5). The increased dispersion of adenovirus co-injected with rHuPH20 was similar to that of trypan blue dye, being effective only when injected within 1 h post administration. Fluorescence remained visible in the skin up to 7 days post-infection in both the rHuPH20 and control groups (Fig. 5).

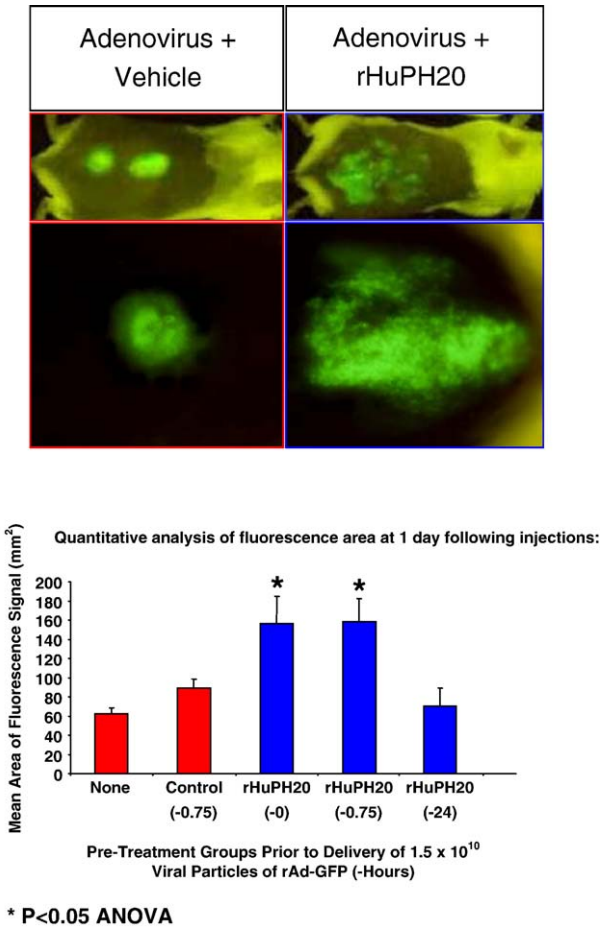

Fig. 5. Effects of rHuPH20 on the dispersion of adenoviral particles. rAd-GFP is more effectively dispersed in balb/c mice co-injected with rHuPH20 relative to carrier controls when injected within 45 min of rHuPH20 injection. Tissue expression of GFP was quantified by integrating total pixel area 24 h after injection of adenovirus. The effects of rHuPH20 on viral dispersion were significant ($P \leq 0.05$) when injected within 45 min of virus injection. Fluorescence remained visible in the skin up to 7 days post-infection in both the rHuPH20 and control groups ($n = 4$ mice per group for each study).

3.6. Co-injection of rHuPH20 increases the infusion rates and permits injection of large volumes without tissue distortion

Subcutaneous and intramuscular injections are typically limited to small volumes of less than 2 mL, largely due to tissue distortion, tearing, and pain that occur when larger volumes are rapidly injected. rHuPH20 was therefore tested to establish whether large volumes of solutions could be infused without causing tissue distortion, and to measure the increase in infusion rates. As shown in Fig. 6A, the infusion of saline at 300 μL per min in mice in the rear flank resulted in significant tissue distortion in control tissues that persisted for at least 10 min post infusion. In contrast, rHuPH20-infused sites were uniformly dispersed without tissue distortion and swelling.

Some biopharmaceuticals such as monoclonal antibodies are formulated as highly concentrated solutions. For example, adalimumab (Abbott Laboratories, IL) is administered subcutaneously as a 0.8 mL of a 50 mg/mL solution. Therefore, to examine whether a viscous fluid could also be injected at rapid rates, an infusion of 50 mg/mL of human serum albumin was tested in nude mice. The infusion rates of concentrated serum albumin were measured in the dermis over 100 μL increments up to a total volume of 600 μL per mouse with increasing pressure. As shown in Fig. 6B, injection of rHuPH20 with 50 mg/mL protein solution increased infusion rates up to 20-fold relative to control infusions without rHuPH20, indicating that rHuPH20 significantly increased infusion rates even with high concentration protein solutions.

3.7. Recombinant hyaluronidase alters the serum pharmacokinetic profiles and bioavailability of large molecule therapeutics injected into the interstitium

Increasing the exposure of therapeutics to the capillary beds and lymphatics could alter their pharmacokinetic profile. As protein therapeutics can be degraded by cells of the interstitial matrix, reducing the residence time in this compartment may increase systemic bioavailability of biotherapeutics. To test this concept, we assessed the effects of rHuPH20 on the pharmacokinetics and bioavailability of a 31 kDa pegylated cytokine, peginterferon alfa-2b, and a 149 kDa monoclonal antibody, infliximab, as two model biotherapeutics in a rodent model. Three groups of 6 rats each received 1.5 μg/kg of 125-I labeled peginterferon alfa-2b administered either IV or locally with and without 100 Units of rHuPH20. Serum levels of peginterferon alfa-2b were measured over 48 h post injection. As shown in Fig. 6C, local co-injection with rHuPH20 increased the absolute bioavailability of the cytokine from 61 to 108% ($P<0.001$) and C_{max} from 61,782 to 121,750 CPM/g ($P<0.0003$). To evaluate a biopharmaceutical that requires larger volume dosing such as a therapeutic antibody, 125-I labeled infliximab (149 kDa) was administered to rats in a comparable experiment at a concentration of 10 mg/kg. As shown in Fig. 6D, the absolute bioavailability of the monoclonal antibody increased from 59% to 94% ($P<0.00001$), and C_{max} increased from 531,866 CPM/g to 875,369 CPM/g ($P<0.00002$). Furthermore T_{max} decreased from 48 to 18.7 h ($P<0.002$), indicating that rHuPH20 markedly improves the pharmacokinetic profiles of large molecule biopharmaceuticals administered via

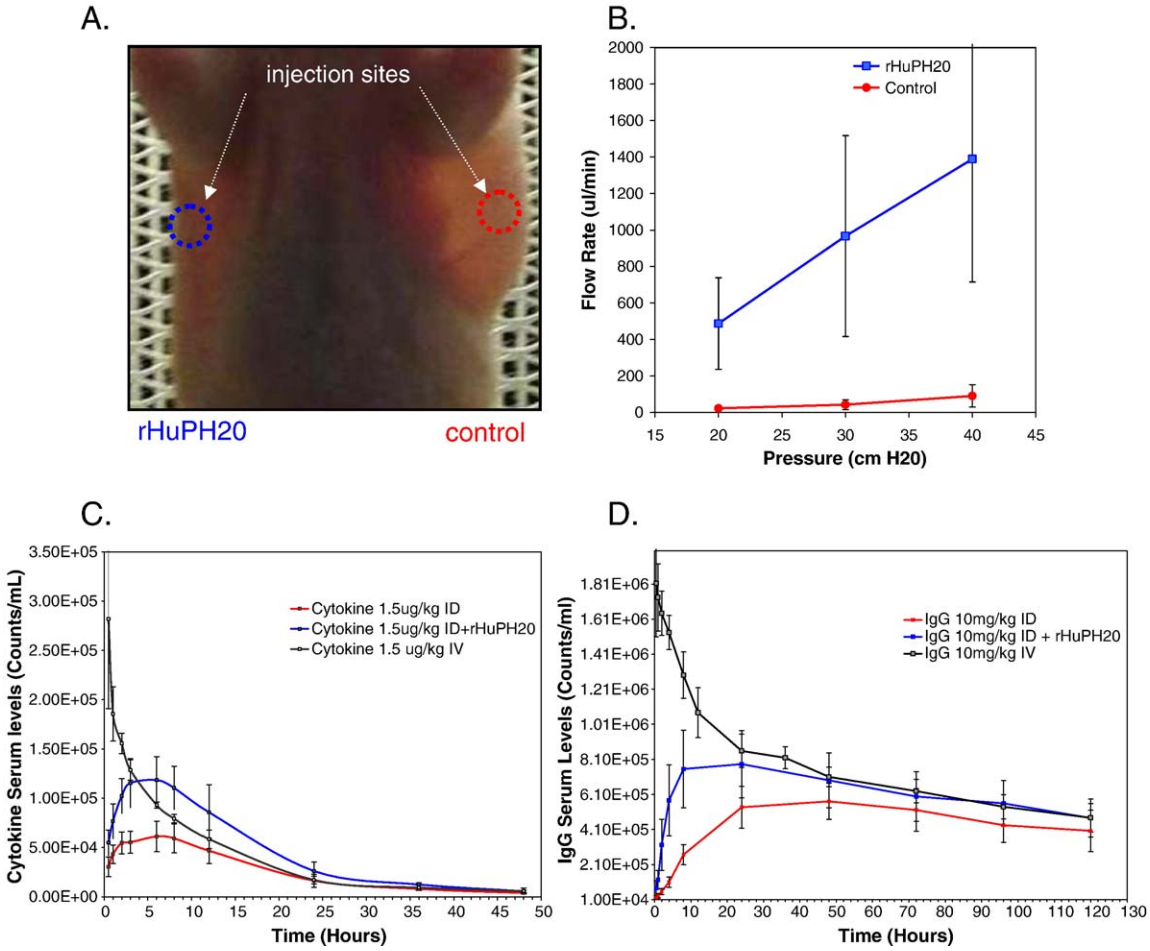

Fig. 6. rHuPH20 increases local infusion rates and speeds the appearance of locally injected drugs into the bloodstream. (A) Reduction of tissue distortion following injection of rHuPH20. Injection of 50 μL rHuPH20 (5U) or carrier control followed by saline infusion at 300 μL/min for 2 min in both sites by infusion pump demonstrates reduced tissue distortion with rHuPH20 compared to carrier control. (B) rHuPH20 increases infusion rates up to 20-fold in concentrated protein solutions ($P \leq 0.05$). Human serum albumin (50 mg/mL) was infused at pressures from 20–40 cm H_2O with and without hyaluronidase. Flow rates were measured at 0.1 mL intervals at each pressure ($n=3$ animals per group). (C) rHuPH20 increases C_{max} and bioavailability of a 125-I labeled cytokine injected locally. Pegylated cytokine (1.5 μg/kg) was injected intravenously (IV) intradermally (ID) or intradermally with 100 Units rHuPH20 (ID+rHuPH20). Absolute bioavailability increased from 61% to 108% ($P<0.001$) and C_{max} increased from 61,782 to 121,750 CPM/g ($P<0.0003$). (D) rHuPH20 decreases T_{max}, increases C_{max} and bioavailability of a therapeutic monoclonal antibody. IgG1 (10 mg/kg) was injected intravenously (IV) intradermally (ID) or intradermally with 100 Units rHuPH20 (ID+rHuPH20). Absolute bioavailability increased from 59% to 94% ($P<0.00001$), T_{max} decreased from 48 to 18.7 h ($P<0.002$) and C_{max} increased from 531,866 CPM/g to 875,369 CPM/g ($P<0.00002$).

the interstitial route and drives the pharmacokinetic profile towards an intravenous administration.

4. Discussion

While a spreading factor was first described in testes extracts over 70 years ago, its association with hyaluronidase activity in these preparations was based upon preparations containing less than 1% enzyme protein. Animal hyaluronidase containing extracts from cattle testes have been used for over 50 years to increase tissue permeability and promote the spread or dispersion of other co-injected drugs, most often in combination with local anesthetics in the setting of ophthalmic surgery. Despite their relative safety and potential for improving drug delivery, such preparations have had limited application so far, mostly due to the fact that they contain impurities in greater abundance than the animal hyaluronidase enzyme, all of which are foreign antigens. The confounding activities of contaminants present in crude hyaluronidase preparations such as proteinases [28], anti-coagulants [29], growth factors [30], vasopermeability factors [9] immunoglobulins [31] and other unidentified components may in fact contribute to the various biological properties attributed to hyaluronidases, such as being pro-inflammatory or pro-angiogenic. To overcome the shortcomings of animal derived extracts, we generated a recombinant human form of PH20 in Chinese hamster Ovary cells. The recombinant enzyme is a soluble single chain polypeptide with N-linked glycosylation structures lacking the GPI-anchor that, like the bovine PH20, degrades hyaluronan by hydrolysis of the β 1,4 linkage between the C1 position of N-acetyl glucosamine and the C4 position of glucuronic acid. We found that rHuPH20 hyaluronidase is equally potent in its spreading activity on a Unit per Unit basis relative to the USP bovine hyaluronidase

reference standard that contains less than 1% hyaluronidase based upon specific activity.

The effects of rHuPH20 on the dispersion of other molecules in the interstitium are not anticipated from the relative abundance of glycosaminoglycans, compared to other molecules in the skin such as collagen. However, hyaluronan, through its volume of hydration and viscoelastic properties, can prevent bulk fluid flow through the collagenous interstitium [36]. By depolymerizing hyaluronan, rHuPH20 rapidly reduces its viscosity, changing the interstitial glycosaminoglycans from a gel-like phase to a fluid liquid phase, thereby making increased bulk fluid flow possible through the collagen fibers. The human body turns over nearly 1/3 of its hyaluronan (approximately 5 g) in the skin each day. As rHuPH20 has a very short half life, at least in the vascular compartment ($T_{1/2}<1$ min), the reconstitution of hyaluronan in the interstitium within 15–20 h after injection of enzyme fits well with this model.

The clinical preparations used to date are non-human, unpurified, and can elicit immune reactions with repeat administration [12,13]. Some of the bovine preparations were reported to elicit allergic responses in as much as 10% of a naïve population [32]. In contrast to the side effects observed with testes-derived preparations, we established in cynomolgus monkeys, whose PH20 is 87% identical to the human enzyme, that rHuPH20 was well tolerated and did not elicit neutralizing antibodies. Repeated subcutaneous injections of rHuPH20 were neither inflammatory nor immunogenic based upon gross anatomy, histological and immunologic criteria, even when delivered at a dose over 500 fold above the therapeutic dose. As a result, the favorable safety profile of rHuPH20 opens the possibility to investigate co-formulation of the enzyme with therapeutic agents for the treatment and management of a range of diseases.

There is little published information regarding the spreading activity of even partially purified PH20 hyaluronidase from any species. Early studies on the mechanism of action of PH20 carried out with crude hyaluronidase enzyme preparations concluded that hyaluronidase acts principally by way of increasing hydraulic conductivity, or bulk fluid flow [33–35]. Hydrolysis of high molecular weight hyaluronan polymers by PH20 reduces their viscosity in the interstitium [36], thereby increasing the infusion rates in the interstitial matrix and facilitating bulk fluid flow. While increasing the bulk fluid flow appears to be the prevailing effect of rHuPH20 treatment, there are clearly sieving effect limitations, as shown by the results of injection of fluorescent particles of increasing size. We found that the size of the channels created in the interstitial matrix by the depletion of hyaluronan allowed the diffusion of particles up to 200 nm in diameter. Administration of PH20 does not affect the organization of interstitial matrix fibers as they are largely degraded by specific matrix metalloproteinases, including collagenases. One plausible explanation for the observed particle size limitations is that the degradation of interstitium hyaluronan by rHuPH20 results in the widening of the channels that typically exist between individual collagen fibers. The dimensions of these channels well exceed the diameter of high molecular weight therapeutic proteins (3–5 nm), monoclonal antibodies (approximately 10 nm) and gene therapy vectors (35 nm), supporting a wide range of molecules from conventional small molecule drugs to large polypeptide therapeutics to be more consistently and predictably absorbed.

4.1. Implications for drug delivery

Many drugs must be injected intravenously due to limitations on drug solubility, bioavailability or tissue irritation. Tissue distortion, injection site pain and irritation negatively influence patient compliance for repeat subcutaneous injections. Historically, the use of crude hyaluronidase preparations was limited to acute use or emergency situations such as periocular anesthesia, paravenous injections of toxic substances and emergency fluid hydration [37]. By increasing the infusion rates, co-injection with rHuPH20 reduced tissue distortion during injection and dispersed injected drugs into the interstitial matrix for systemic adsorption, potentially enabling subcutaneously injections of intravenous dosing volume.

Our studies also established that the pharmacokinetic profiles of macromolecule drugs injected subcutaneously could be significantly improved by co-administration with rHUPH20. We selected two biologics for their therapeutic relevance among the many therapeutic proteins that are presently marketed. In one example, we tested a long lasting peginterferon alfa-2b approved for the treatment of chronic HCV infection. Peginterferon alpha-2b is administered subcutaneously as a monotherapy or in combination with small molecular weight drugs once a week. In the second example, we tested a therapeutic antibody that blocks TNF-alpha. Infliximab is approved for the treatment of the inflammation of Crohn's disease, rheumatoid arthritis, and psoriatic arthritis. The antibody is administered by intravenous infusion. Comparison between the areas under the curve of either peginterferon or infliximab delivered intravascullarly or locally shows that the pharmacokinetic profile and absolute bioavailability of both therapeutics were dramatically improved when co-administered with rHuPH20. As a result, peginterferon bioavailability increased from 60% to levels similar to those achieved by intravascular delivery. Similarly, Infliximab co-injected with rHuPH20 exhibited pharmacokinetic profiles that were more similar to intravenous dosing.

Importantly, the co-injection with rHuPH20 hyaluronidase permitted the administration of up to five times more volume than normally feasible for subcutaneously injected drugs, e.g., 5–10 mL vs. 1–2 mL, respectively. This may allow the conversion of intravenously-administered pharmaceuticals to more desirable local routes, and may make it possible for patients with chronic diseases to self-administer injectable medicines, thus reducing the need for hospital visits. It is also of interest to note that animalderived hyaluronidase preparations have been historically used as an antidote to intravenous extravasation, promoting rapid resorption of necrotic drugs when intravenous lines have slipped. Similarly, rHuPH20 hyaluronidase might reduce injection site reactions for molecules that are locally toxic and improve patient compliance.

In summary, several factors make rHuPH20 attractive as an interstitial drug dispersion agent: First, the rapid turnover of its substrate in the interstitium makes hyaluronan an exceptional target for matrix modification; second, the ability of conduits generated by rHuPH20 in the interstitial matrix to promote dispersion of molecules up to 200 nm in diameter supports a wide range of therapeutics from small molecules and therapeutic antibodies to even gene therapy vectors [38,39] and nanoparticles [40]; and finally the nanogram doses of rHuPH20 needed to elicit an effective response, the lack of toxicity over a multi-log range of doses in primates, and the high degree of purity and human DNA origin combined with the years of clinical experience from the slaughterhouse extracts makes rHuPH20 a unique technology for use as a drug delivery adjuvant that may allow for subcutaneous administration of drugs that previously could only be given by intravascular or intramuscular injection.

Acknowledgements

We would like to extend our thanks to David Looper and David Kang for their technical skills for the adenovirus animal work, Cynthia Gribskov for chromatographic support, and Dan Vaughn and Ryan English for analytical support. We would also like to thank Torvard Laurent for his helpful discussions.

References

[1] A. Supersaxo, W.R. Hein, H. Steffen, Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration, Pharm. Res. 7 (2) (1990) 167–179.
[2] T.D. Day, The permeability of interstitial connective tissue and the nature of the interfibrillary substance, J. Physiol. 17 (1952) 1–8.
[3] A.S. Craig, E.F. Eikenberry, D.A.D. Parry, Ultrastructural organization of skin: classification on the basis of mechanical role, Connect. Tissue Res. 16 (1987) 213–223.
[4] N. Verzijl, J. DeGroot, S.R. Thorpe, R.A. Bank, J.N. Shaw, T.J. Lyons, J.W. Bijlsma, F.P. Lafeber, J.W. Baynes, J.M. TeKopelle, Effect of collagen turnover on the accumulation of advanced glycation end products, J. Biol. Chem. 275 (50) (2000) 39027–39031.
[5] U.B.G. Laurent, L.B. Dahl, R.K. Reed, Catabolism of hyaluronan in rabbit skin takes place locally, in lymph nodes and liver, Exp. Physiol. 76 (5) (1991) 695–703.
[6] J.R, Fraser, T.C. Laurent, Ciba Found Symp. 143 (1989) 42–59; 281–285.
[7] F. Duran Reynals, Exaltation de l'activité du virus vaccinal par les extraits de certains organes, Comptes Rendus Séances Soc. Biol. Fil. 99 (1928) 6–7.
[8] E.S. Duthie, E.A. Chain, A mucolytic enzyme in testes extracts, Nature 144 (1939) 977–978.
[9] K. Meyer, in: P.D. Boyer (Ed.), Hyaluronidases, vol. 5, Academic Press, New York, 1971, pp. 307–320.
[10] Y. Lin, K. Mahan, W.F. Lathrop, D.G. Myles, P. Primakoff, A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg, J. Cell Biol. 125 (5) (1994) 1157–1163.
[11] R.G. Williams, The effects of continuous local injection of hyaluronidase on skin and subcutaneous tissue in rats, Anat. Rec. 122 (3) (1955) 349–361.
[12] K. Pillwein, R. Fuiko, I. Slavc, T. Czech, G. Hawliczek, G. Bernhardt, G. Nirnberger, U. Koller, Hyaluronidase additional to standard chemotherapy improves outcome for children with malignant brain tumors, Cancer Lett. 131 (1) (1998) 101–108.
[13] A.H. Eberhart, C.R. Weiler, J.C. Erie, Angioedema related to the use of hyaluronidase in cataract surgery, Am. J. Ophthalmol. 138 (1) (2004) 142–143.
[14] G.I. Frost, R. Stern, A microtiter-based assay for hyaluronidase activity not requiring specialized reagents, Anal. Biochem. 251 (2) (1997) 263–269.
[15] A. Dorfman, M.L. Ott, A turbidimetric method for the assay of hyaluronidase, J. Biol. Chem. 172 (1948) 367–375.
[16] H. Min, M.K. Cowman, Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution, Anal. Biochem. 155 (2) (1986) 275–285.
[17] B.G. Huyghe, X. Liu, S. Sutjipto, B.J. Sugarman, M.T. Horn, H.M. Shepard, C.J. Scandella, P. Shabram, Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography, Hum. Gene. Ther. 6 (11) (1995) 1403–1416.
[18] A.A. Miles, E.M. Miles, Vascular reactions to histamine, histamine liberators or leukotoxins in the skin of the guinea pig, J. Physiol. 118 (2) (1952) 228–257.
[19] R.A. Harrison, S.J. Gaunt, Multiple forms of ram and bull sperm hyaluronidase revealed by using monoclonal antibodies, J. Reprod. Fertil. 82 (2) (1988) 777–785.
[20] C.L. Borders, M.A. Raftery, Purification and partial characterization of testicular hyaluronidase, J. Biol. Chem. 243 (13) (1968) 37556–37562.
[21] R.A. Harrison, Hyaluronidase in ram semen. Quantitative determination, and isolation of multiple forms, Biochem. J. 252 (3) (1988) 865–874.
[22] C.M. McKee, M.B. Penno, M. Cowman, M.D. Burdick, R.M. Strieter, C. Bao, P.W. Noble, Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44, J. Clin. Invest. 98 (10) (1996) 2403–2413.
[23] K.R. Taylor, J.M. Trowbridge, J.A. Rudisill, C.C. Termeer, J.C. Simon, R. L. Gallo, Hyaluronan fragments stimulate endothelial recognition of injury through TLR4, J. Biol. Chem. 279 (17) (2004) 17079–17084.
[24] M.C. Filion, N.C. Phillips, Pro-inflammatory activity of contaminating DNA in hyaluronic acid preparations, J. Pharm. Pharmacol. 53 (4) (2001) 555–561.
[25] H. Storch, T. Dellas, H. Bellmann, Experimental studies on immunogenicity, humoral response and danger of anaphylaxis in parenteral administration of hyaluronidase, Z. Exp. Chir. 11 (2) (1978) 128–133.
[26] G. Szabo, S. Magyar, Effect of hyaluronidase on capillary permeability, lymph flow and passage of dye-labelled protein from plasma to lymph, Nature 182 (4632) (1958) 377–379.
[27] T.F. Linsenmayer, in: E.D. Hay (Ed.), Collagen Cell Biology of Extracellular Matrix, Plenum, New York, 1981, pp. 5–37.
[28] H.D. Keiser, V.B. Hatcher, The effect of contaminant proteases in testicular hyaluronidase preparations on the immunological properties of bovine nasal cartilage proteoglycan, Connect. Tissue Res. 6 (4) (1979) 229–233.
[29] V.M. Doctor, H. Spence, E.Y. Carroll, Isolation and properties of a new anticoagulant protein from commercial bovine testicular hyaluronidase, Thromb. Res. 30 (6) (1883) 565–571.
[30] M. Hyuga, R. Kodama, G. Eguchi, Basic fibroblast growth factor as one of the essential factors regulating lens transdifferentiation of pigmented epithelial cells, Int. J. Dev. Biol. 37 (2) (1993) 319–326.
[31] M. Oettl, J. Hoechstetter, I. Asen, G. Bernhardt, A. Buschauer, Comparative characterization of bovine testicular hyaluronidase and a hyaluronate lyase from Streptococcus agalactiae in pharmaceutical preparations, Eur. J. Pharm. Sci. 18 (3–4) (2003) 267–277.
[32] J. Schwartzman, M. Levbarg, Hyaluronidase; further evaluation in pediatrics, J. Pediatr. 36 (1) (1950) 79–86.
[33] J.H. Fessler, Mode of action of testicular hyaluronidase, Biochem. J. 76 (1960) 132–135.
[34] S.J. Lai-Fook, N.L. Rochester, L.V. Brown, Effects of albumin, dextran, and hyaluronidase on pulmonary interstitial conductivity, J. Appl. Physiol. 67 (2) (1989) 606–613.
[35] Y. Boucher, C. Brekken, P.A. Netti, L.T. Baxter, R.K. Jain, Intratumoral infusion of fluid: estimation of infusion rates and implications for the delivery of therapeutic agents, Br. J. Cancer 78 (11) (1998) 1442–1448.

[36] J.H. Fessler, A structural function of mucopolysaccharide in connective tissue, Biochem. J. 76 (1960) 124–132.
[37] L.C. Burket, P. Gyorgy, Clinical observations on the use of hyaluronidase, Ann. N. Y. Acad. Sci. 52 (7) (1950) 1171–1179.
[38] D. Oupický, C. Konak, K. Ulbrich, M.A. Wolfert, L.W. Seymour, DNA delivery systems based on complexes of DNA with synthetic polycations and their copolymers, J. Control. Release 65 (2000) 149–171.
[39] M.G. Rots, D.T. Curiel, W.R. Gerritsen, H.J. Haisma, Targeted cancer gene therapy: the flexibility of adenoviral gene therapy vectors, J. Control. Release 87 (2003) 159–165.
[40] S.T. Reddy, A. Rehor, H.G. Schmoekel, J.A. Hubbell, M.A. Swartz, In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles, J. Control. Release 112 (1) (2006) 26–34.