Transport of circulating IGF-I and LR³IGF-I from blood to extracellular wound fluid sites in rats

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Abstract

Significant levels of IGF-I are found in wound fluid. The contribution that systemic IGF-I makes to the total IGF-I pool in wounds and the influence of IGF binding proteins (IGFBPs) on the delivery of systemic IGF-I to these wound sites has not been established. In the present series of experiments, we have shown that IGF-I flux across a model endothelial cell barrier is decreased in the presence of IGFBPs, whereas flux of Long R³IGF-I (LR³IGF-I, an IGF-I analogue with low affinity for IGFBPs) is unaffected. On the basis of these findings, the transport of IGF-I and LR³IGF-I from blood to extracellular wound fluid was assessed. Wound chambers were implanted subcutaneously in the backs of adult male rats and left in place for 14 days. A single i.v. bolus of either ¹²⁵I-IGF-I or ¹²⁵I-LR³IGF-I (10×10^6 c.p.m.) was administered via a jugular catheter and wound fluid and plasma samples taken at sequential time points between 5 and 240 min. ¹²⁵I-LR³IGF-I was removed from the circulation more rapidly than ¹²⁵I-IGF-I in both sham control and chamber implanted rats. Although implantation of the chambers did

not alter the pharmacokinetic parameters of ¹²⁵I-IGF-I, significant increases in the steady state volume of distribution, clearance rate and half-life were recorded for ¹²⁵I-LR³IGF-I. In addition, significantly more intact ¹²⁵I-LR³IGF-I was recovered in wound fluid than ¹²⁵I-IGF-I at each time point, although only 0.08% of administered ¹²⁵I-LR³IGF-I was recovered per ml of wound fluid at 240 min. Compared with plasma, a greater proportion of wound fluid IGF-I radioactivity had distributed to the lower molecular weight IGFBPs or existed as free peptide. However, a small amount of wound fluid ¹²⁵I-IGF-I was detected in the 150 kDa region 30 min after injection. A greater proportion of ¹²⁵I-LR³IGF-I was associated with the lower molecular weight IGFBPs or existed as free peptide in both wound fluid and plasma. These data point to the importance of IGFBPs in determining the pharmacokinetic parameters of IGF-I in an extracellular fluid-expanded state. They also suggest only a minor role for endocrine IGF-I in surface wound repair.

Journal of Endocrinology (2000) **164**, 77–86

Introduction

The dual effector theory first suggested by Green *et al.* (1985) proposed that insulin-like growth factor-I (IGF-I) has both paracrine/autocrine and endocrine functions. Since IGF activity has been detected in tissue extracts and media conditioned by many cell types, investigations have focused principally on the possible paracrine/autocrine nature of IGF-I. However, evidence has also been accumulating to suggest that IGF-I of endocrine origin promotes growth of kidney, lung, thymus and spleen (Schoenle *et al.* 1982, Zapf *et al.* 1985, Guler *et al.* 1988, Glasscock *et al.* 1991). Additionally, IGF-I acting in the classical endocrine fashion promotes formation and repair of bone (Ebeling *et al.* 1993), stimulates body protein and glucose metabolism (Jacob *et al.* 1989) and increases glomerular filtration rate and renal plasma flow (Guler *et al.* 1989). For IGF-I to exert its action in an endocrine manner, it must first leave the blood by traversing the endothelial cell barrier. IGF-I is able to cross intact

endothelium and capillaries (Bar *et al.* 1990a,c, Boes *et al.* 1992, Taylor *et al.* 1993, Bastian *et al.* 1997) and it has been demonstrated that IGF-I transfers passively across a human umbilical vein endothelial (HUVE) cell monolayer by a non-receptor mediated pathway (Bastian *et al.* 1997). Additionally, several *in vivo* studies have shown that both IGF-I and its analogue LR³IGF-I are able to leave the vascular compartment and distribute to many different tissues (Cascieri *et al.* 1988, Ballard *et al.* 1991, Bastian *et al.* 1993, Hill *et al.* 1997).

In vivo, IGF-I circulates in the blood complexed to multiple specific binding proteins. The role of IGFBPs in the transfer of IGF-I from blood to tissues is still not well understood. Association of IGF-I with the lower molecular weight IGFBPs is thought to permit targeting of specific tissues (Sara & Hall 1990). In support of this, IGF binding proteins IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 have all displayed the ability to traverse the capillary barrier in the perfused isolated beating rat heart (Bar *et al.* 1990b,c, Boes *et al.* 1992). More recently, radiolabeled

recombinant human IGFBP-3 not associated with the ternary complex has been shown to rapidly cross capillary endothelia from blood to extracellular sites including kidney, liver and gastrointestinal tissues, supporting a role for IGFBP-3 in the delivery of IGF-I to peripheral tissues (Arany *et al.* 1993).

The majority of IGF-I is present in the circulation associated with IGFBP-3, which together with an acid-labile subunit forms a ternary complex of 150 kDa (Baxter 1988). The discovery of increased protease activity for circulating IGFBP-3 occurring in pregnancy, post-surgery, cancer, critical illness, renal failure and HIV infection has led to the proposal that the ternary complex may serve as a readily releasable pool of IGFs in blood available during stress (Davenport *et al.* 1990, 1992, Cwyfan Hughes *et al.* 1992, Frost *et al.* 1993, 1996, Lee *et al.* 1994, Timmins *et al.* 1996). That is, an increase in enzymatically processed IGFBP-3 with weaker affinity for IGF-I than intact IGFBP-3 could increase the ease of release of IGF-I from the complex and in turn enhance the tissue bioavailability of IGF-I (Holly *et al.* 1993, Lamson *et al.* 1993).

As a first step in understanding how IGFBPs may affect the translocation of IGFs from plasma to extracellular wound fluid, we used an *in vitro* model of an endothelial cell barrier and examined the effects of the addition of exogenous IGFBPs on the transport of IGF-I across the endothelial cell monolayer. The Hunt–Schilling chamber was originally designed to study respiratory gas tensions in healing wounds (Hunt *et al.* 1967). This model offers a site of readily accessible extracellular wound fluid, free of blood contamination. The current experiments aimed to examine the delivery of IGF-I from the vasculature to extracellular wound fluid *in vivo* using the Hunt–Schilling chambers. The use of LR³IGF-I in parallel experiments, permitted investigation of the effects of IGFBPs on this delivery.

Materials and Methods

Materials

IGFBPs used in these studies were generously supplied by the following: human IGFBP-1, Diagnostic Systems Laboratories (Webster, TX, USA); human IGFBP-2, Dr Eiji Kuto (Basel, Switzerland); human IGFBP-3 and IGFBP-6, Dr Robert Baxter (Sydney, Australia) and human IGFBP-4, IGFBP-5 and truncated IGFBP-5 (tIGFBP-5), Dr Dennis Andress (Seattle, WA, USA). Recombinant human IGF-I and recombinant LR³IGF-I were purchased from GroPep Pty. Ltd (Adelaide, Australia). Both peptides were iodinated with carrier free Na¹²⁵I from Amersham (Buckinghamshire, UK) to a specific activity of 85 µCi/µg using a modification of the chloramine T method (Gargosky *et al.* 1990). [³H]Inulin was also obtained from Amersham. Heparin and BSA were

purchased from Sigma (St Louis, MO, USA). Trichloroacetic acid (TCA) was from BDH Chemicals (Victoria, Australia). Methohexitone sodium was from Eli Lilly (West Ryde, NSW, Australia) and pentobarbitone sodium was from Boehringer Ingelheim Pty Ltd (Smithfield, NSW, Australia).

Cell culture and transmigration studies

Human umbilical vein endothelial (HUVE) cells were isolated from the vein of 12–24 h old umbilical cords and used to perform transmigration studies as described in Bastian *et al.* (1997). Prior to addition of ¹²⁵I-IGF-I or ¹²⁵I-LR³IGF-I to the luminal chamber at time zero, each labeled IGF was incubated for 2 h at 37 °C with a 1:1 molar amount of either hIGFBP-1, -2, -3, -4, -5, -6 or truncated hIGFBP-5. At 5 min following the addition of the labeled IGF-IGFBP mix to the luminal chamber, a sub-sample (100 µl) was taken from the abluminal chamber which was immediately replenished with the same amount of fresh media. Further sub-samples were collected at 30, 60 and 120 min. Transmigrated ¹²⁵I-IGF-I and ¹²⁵I-LR³IGF-I were measured as TCA-precipitable radioactivity. [³H]inulin is impermeable to cell membranes and was used as a marker of intercellular leakage.

Animals and implantation of Hunt–Schilling wound chambers

Twenty male Sprague–Dawley rats with a mean weight $\pm$ S.E.M of 472.6 ± 11.1 g were obtained from the Central Animal House, University of Adelaide. All experimental protocols were approved by both the University of Adelaide Animal Ethics Committee and The Women's and Children's Hospital Ethics Committee.

Stainless steel Hunt–Schilling wound chambers 3 cm long by 1 cm in diameter (Schilling *et al.* 1959, Hunt *et al.* 1967) were constructed from no. 40 mesh and the ends plugged with silicon polymer. Chambers were washed in ethanol, rinsed several times with distilled water and autoclaved. Rats were anesthetized with isoflurane and an oxygen–nitrous oxide mix. Four 1 cm abaxial incisions were made, one either side of the midline at a point just cranial to the sacrum and one either side of the midline at the sacrum, permitting creation of four subcutaneous pockets by blunt dissection for implantation of chambers. Each chamber was introduced using a modified 5 ml syringe cranial to each incision and finally located in a dorso-lateral orientation. The incisions were sutured with non-absorbable material. Implantations were performed using aseptic technique. Eight of the 20 rats acted as sham controls and were not implanted with wound chambers. The animals were kept warm until conscious and allowed to recover with free access to food and water for 14 days prior to experimentation. Preliminary experiments have shown that, by day 14, the chambers are encapsulated by vascularized granulation tissue that comes into close contact with the wound fluid.

Experimental procedures

Two days prior to experimentation, all animals were anesthetized by an i.p. injection of a 9:1 solution of methohexitone sodium and pentobarbitone sodium (3.75 ml/kg of body weight) and a catheter inserted into the jugular vein. The animals were kept warm until conscious, and allowed free access to food and water and left to recover for two days. On the experimental day, rats had a mean weight $\pm$ s.e.m. of 481.0 ± 9.5 g. Prior to infusion of radiolabeled IGFs, a 200 μ l blood sample was withdrawn from the catheter of each rat. Following each blood sampling, the equivalent volume of saline containing 10 IU heparin was injected back into the catheter line. Blood samples were collected into tubes heparinized to a final concentration of 10 IU/ml and centrifuged for 2 min at 10 000 *g* at room temperature. Plasma was removed, frozen in liquid nitrogen and stored at -20°C for Western ligand blot analysis. Rats were randomly chosen for infusion with either ^{125}I -IGF-I or ^{125}I -LR³IGF-I. At time zero, each rat received a bolus dose (100 μ l) of 10×10^6 c.p.m. of ^{125}I -IGF-I or ^{125}I -LR³IGF-I in physiological saline plus 0.01% BSA via the jugular catheter, and the catheter line was flushed with 300 μ l of saline. Blood samples of 150 μ l were withdrawn at 1, 15, 60 and 180 min for TCA precipitation analyses, while 300 μ l blood samples were collected at 5, 30, 120 and 240 min for TCA precipitation analyses and FPLC. At 5, 30, 120 and 240 min, one of the four wound chambers was aspirated of its fluid contents by percutaneous insertion of a 19 gauge needle through the silicon plug. Care was taken to avoid damaging the granulation tissue on the inner surface of the chamber to prevent contamination of wound fluid with blood. Wound fluid from each chamber was collected into separate tubes, heparinized to a final concentration of 10 IU/ml and centrifuged at 10 000 *g* for 2 min at room temperature. Supernatant was aliquotted into tubes, frozen in liquid nitrogen and stored at -20°C .

Trichloroacetic acid precipitation of ^{125}I -IGF-I and ^{125}I -LR³IGF-I in plasma and wound fluid samples

Duplicate portions of plasma (25 μ l) and wound fluid (50 μ l) from each rat and wound chamber were counted in a γ -counter for 10 min. Plasma and wound fluid samples were then mixed with 475 μ l and 450 μ l of ice-cold 10% (v/v) TCA in PBS plus 0.1% (w/v) BSA respectively. Samples were incubated on ice for 2 h then centrifuged at 4°C for 20 min at 10 000 *g*. The supernatant was removed and the radioactivity in both the TCA-soluble and TCA-insoluble portions measured in a γ -counter for 10 min.

Neutral gel permeation chromatography

Pools of time 5, 30, 120 and 240 min plasma (200 μ l) and wound fluid (150 μ l) samples from chamber-implanted

rats infused with ^{125}I -IGF-I or ^{125}I -LR³IGF-I, and plasma samples (200 μ l) from sham controls infused with ^{125}I -IGF-I or ^{125}I -LR³IGF-I, were prepared for neutral gel permeation chromatography. Wound fluid was first centrifuged at 4°C at 10 000 *g* for 5 min to remove solids. Lipids were removed from both the plasma and wound fluid samples by extraction with Freon (1,1,2-trichloro-1,2,2-trifluoroethane) then chromatographed on a Superose-12 column (HR 10/30), as previously described in Bastian *et al.* (1993).

Western ligand blot analysis

To examine the plasma IGFBP profiles of rats implanted with chambers compared with those of the sham control animals, 10 μ l samples of blood collected from each rat before radiolabeled IGF infusion were mixed with 190 μ l of SDS-loading buffer and incubated for 15 min at 65°C . Rat plasma samples (1 μ l equivalence) were subjected to SDS-PAGE, proteins were transferred to nitrocellulose sheets using a Multiphor II NovaBlot electrophoretic unit and IGFBPs detected as described in Bastian *et al.* (1993). Nitrocellulose sheets were probed with either 5×10^5 c.p.m. of ^{125}I -IGF-I or ^{125}I -LR³IGF-I and exposed to X-ray film for 4 days.

Pharmacokinetic analysis

Pharmacokinetic parameters for each rat were calculated by fitting the data to a bi-exponential equation that describes a two-compartment model with utilization of a non-linear curve-fitting program (Tablecurve Windows V1.0; Jandel Scientific, Corte Madera, CA, USA) in which $C = Ae^{-\alpha t} + Be^{-\beta t}$, where C equals the plasma concentration of TCA-precipitable radioactivity per milliliter; A and B correspond to the concentrations of TCA-precipitable radioactivity in each compartment at time 0; α and β are the decay rate constants for each compartment and t is time. The area under the curve (AUC) method was used to determine total plasma clearance (CL) using the relationship $\text{CL} = \text{dose}/\text{AUC}$, where the dose equals IGF radioactivity administered. Initial volume of distribution (V_c) was calculated as $V_c = \text{dose}/(A+B)$. The volume of distribution at steady state (V_{ss}) was calculated as $V_{ss} = (A/\alpha^2 + B/\beta^2)/\text{AUC}$.

Statistical analyses

All statistical analyses were performed using SigmaStat for Windows statistical software from Jandel Scientific (San Rafael, CA, USA). Transmigration data and appearance of TCA-precipitable IGFs in extracellular fluid were analyzed by one-way analysis of variance with all pair-wise multiple comparisons using Student-Newman-Keuls method. Pharmacokinetic data was analyzed by two-way analysis of variance with replications on two main factors:

growth factor and chamber status. $P < 0.05$ was considered significant.

Results

Effect of IGFBPs on transport of ^{125}I -IGF-I and ^{125}I -LR³IGF-I across HUVE cell monolayers

To examine whether IGFBPs have an effect on IGF-I transport across endothelial cell barriers, the transport of ^{125}I -IGF-I (8 ng) and ^{125}I -LR³IGF-I (8 ng) was examined in the presence of IGFBPs. These experiments were carried out at a 1:1 molar ratio of ^{125}I -IGF-I:IGFBP. With the exception of truncated hIGFBP-5, which has a greatly reduced affinity for IGF-I (Andress & Birnbaum 1992), each of the IGFBPs significantly inhibited the transport of ^{125}I -IGF-I from the luminal to abluminal chamber (Fig. 1a). In contrast, when ^{125}I -LR³IGF-I was the labeled ligand under examination (Fig. 1b), there was no inhibition of transport of this peptide, confirming that the IGFBPs inhibit transport by binding to free IGF-I. No difference in transport between inulin and IGF-I was seen as determined in earlier studies (Bastian *et al.* 1997). However, LR³IGF-I transport was significantly lower than that of inulin.

Comparison of IGFBP profiles in plasma of wound chamber-implanted and sham control rats

To examine whether the implantation of chambers in rats caused a perturbation of the plasma IGFBP profile which could potentially alter the transport of IGFs from blood to extracellular fluid sites, Western ligand blot analysis of implanted and sham control pooled rat plasma was performed. In both implanted and sham control pooled rat plasma, a doublet of bands at approximately 46–50 kDa (IGFBP-3), along with two fainter lower molecular weight bands of similar intensity at 31 and 24 kDa were observed when ^{125}I -IGF-I was the radiolabeled ligand (Fig. 2). Laser scanning densitometry showed no significant difference between the intensity of the IGFBP-3 band in the chamber-implanted (103 ± 8.4 OD units; mean $\pm$ s.d., $n=6$) and sham control rats (105 ± 10 OD units; mean $\pm$ s.d., $n=4$). When ^{125}I -LR³IGF-I was the radiolabeled probe, a faint band at 46 kDa was seen in both wound chamber-implanted and sham control rat plasma pools, confirming the weaker affinity of the IGF-I analogue for rat plasma IGFBPs.

Pharmacokinetic parameters of ^{125}I -IGF-I and ^{125}I -LR³IGF-I in wound chamber-implanted and sham control rats

Clearance studies were performed to determine whether the implantation of wound chambers affected the clearance

Figure 1 Effects of IGFBPs on the transport of ^{125}I -IGF-I and ^{125}I -LR³IGF-I across HUVE cell monolayers. ^{125}I -IGF-I (a) and ^{125}I -LR³IGF-I (b) transport is shown as a percentage of total intact labeled IGF added to the luminal chamber at time zero, appearing in the abluminal chamber at 2 h. Labeled IGF transport across HUVE cell monolayers was measured in the presence of a 1:1 molar ratio of either hIGFBP-1, -2, -3, -4, -5, -6 or truncated hIGFBP-5. Data represents the pooled means $\pm$ s.e.m. of triplicate transwells from three experiments. *Significant differences in transport with respect to either ^{125}I -IGF-I or ^{125}I -LR³IGF-I across HUVE cell monolayers ($P < 0.05$).

values of IGFs in rats. The mean TCA-precipitable radioactivity in plasma over time is shown in Fig. 3. In all cases an early rapid and subsequent slower decline in plasma concentration of the labeled growth factors was observed. The area under the curves defining the rapid (a) and slower

Figure 2 Western ligand blots of day 14 plasma from sham control and chamber-implanted rats. Plasma (1 μ l equivalence) from representative sham control (sc) and chamber-implanted (ci) rats prior to infusion of labeled IGF-I or LR³IGF-I were added to SDS-sample buffer and electrophoresed on 12.5% SDS-PAGE gels under non-reducing conditions. The proteins were transferred to nitrocellulose sheets and incubated with 5×10^5 c.p.m. of either ¹²⁵I-IGF-I or ¹²⁵I-LR³IGF-I, and exposed to X-ray film for 4 days. The positions of molecular mass markers (in kDa) are indicated.

(β) components are shown in Fig. 3. The clearance, half-lives of the rapid and slower components plus initial and steady state volumes of distribution are presented in Table 1. Half-lives for the fast component of both ¹²⁵I-IGF-I and ¹²⁵I-LR³IGF-I were significantly greater for chamber-implanted animals compared with controls. The area under the curve for the fast component of ¹²⁵I-IGF-I infused chamber-implanted animals was significantly decreased relative to sham control IGF-I infused and chamber-implanted LR³IGF-I infused rats. For the slower phase, the area under the curve was significantly greater for ¹²⁵I-IGF-I than ¹²⁵I-LR³IGF-I in both sham control and chamber-implanted rats. This indicates that LR³IGF-I radioactivity is cleared more rapidly and that the amount of LR³IGF-I radioactivity in the slow pool is reduced. This is reflected in the metabolic clearance rate of ¹²⁵I-IGF-I, which was significantly reduced, being 2.3-fold and 2.5-fold lower than ¹²⁵I-LR³IGF-I in sham control and chamber-implanted rats, respectively. Additionally, the half-lives of IGF-I were significantly greater than LR³IGF-I for the slow component. For LR³IGF-I in chamber-implanted rats, the area under the curve and the half-life of the slow component were significantly higher than that obtained in sham controls. However, the steady state volume of distribution for LR³IGF-I was much higher in chamber-implanted rats which is reflected in a higher metabolic clearance rate. Steady state volumes of distribution were larger for LR³IGF-I than IGF-I, which indicates that more IGF-I is confined to the circulation, as

Figure 3 Clearance of TCA-insoluble radioactivity from plasma of (a) sham control and (b) wound chamber-implanted rats following injection of either ¹²⁵I-IGF-I (circles and solid lines) or ¹²⁵I-LR³IGF-I (squares and dashed lines) at time zero. Values are means $\pm$ S.E.M. for six animals in chamber-implanted groups and four animals in sham control groups. Decay curves were calculated as described in the Materials and Methods.

expected on the basis of the differences between these two peptides in binding to plasma IGFBPs. These results showed that ¹²⁵I-LR³IGF-I was removed from the circulation more rapidly than ¹²⁵I-IGF-I in both control and chamber-implanted animals. ¹²⁵I-IGF-I in sham control and chamber-implanted rats is cleared with similar kinetics. In contrast, ¹²⁵I-LR³IGF-I is cleared more rapidly in chamber-implanted rats, reflecting the larger volume of distribution for this peptide in these animals.

Appearance and TCA-precipitability of ¹²⁵I-IGF-I and ¹²⁵I-LR³IGF-I in wound fluid following infusion via a jugular catheter

To determine whether IGFBPs affect the transfer of IGF-I from blood to extracellular fluid sites, the appearance of

Table 1 Pharmacokinetic parameters for labeled IGF-I and LR³IGF-I administered to wound chamber-implanted (ci) and sham control (sc) rats. Values are means ± S.E.M. for four animals in each sham control group and six animals in each wound chamber-implanted group. V_c and V_{ss} are the initial and steady state volumes of distribution, respectively. $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the half-lives for the rapid and slow component respectively

	Clearance (ml/min per kg)	Volume of distribution (ml per kg)		Half-lives (min)	
		V_c	V_{ss}	$t_{1/2\alpha}$	$t_{1/2\beta}$
IGF-I (sc)	0.3 ± 0.02	69.7 ± 4.5	84.9 ± 2.9	3.2 ± 0.1	203.1 ± 10.2
IGF-I (ci)	0.4 ± 0.01	62.4 ± 3.2	101.4 ± 5.6	4.9 ± 0.02*	185.4 ± 4.7
LR ³ IGF-I (sc)	0.7 ± 0.02*	70.8 ± 0.6	109.1 ± 4.6*	2.8 ± 0.1	111.1 ± 3.9*
LR ³ IGF-I (ci)	1.0 ± 0.03‡**	78.7 ± 7.1	197.2 ± 6.9‡**	4.6 ± 0.5‡	140.2 ± 2.5‡**

* $P < 0.05$ compared with IGF-I sham control.
** $P < 0.05$ compared with IGF-I chamber-implanted rats.
‡ $P < 0.05$ compared with LR³IGF-I sham control.

both ¹²⁵I-IGF-I and ¹²⁵I-LR³IGF-I in wound fluid was examined following systemic infusion of these peptides into rats implanted with wound chambers. Five minutes following infusion of ¹²⁵I-IGF-I into rats, 0.003 ± 0.001% (mean ± S.E.M, $n = 6$) of total infused, intact ¹²⁵I-IGF-I had appeared per milliliter of wound fluid. By 240 min, this figure had increased up to 0.05 ± 0.004% (Fig. 4). This intact radioactivity represented 35.7 ± 1.7% (mean ± S.E.M: $n = 6$), 32.8 ± 0.5%, 21.3 ± 0.6% and 20.0 ± 0.9% of total radioactivity in wound fluid at 5, 30, 120 and 240 min respectively. In contrast, significantly more

¹²⁵I-LR³IGF-I than ¹²⁵I-IGF-I was recovered per milliliter of wound fluid at each time point (Fig. 4). At 5 min 0.01 ± 0.001% (mean ± S.E.M, $n = 6$, $P < 0.001$) of total infused intact ¹²⁵I-LR³IGF-I had appeared per milliliter of wound fluid. By 240 min the amount had increased to 0.08 ± 0.01% ($n = 6$, $P < 0.01$). These data indicate that the egress of ¹²⁵I-IGF-I from blood to extracellular wound fluid sites is impeded relative to ¹²⁵I-LR³IGF-I. The total amount of radioactivity in wound fluid of rats infused with ¹²⁵I-LR³IGF-I was 40.3 ± 2.0%, 31.8 ± 2.1%, 21.7 ± 1.3% and 18.4 ± 1.3% TCA precipitable at 5, 30, 120 and 240 min respectively.

Figure 4 Appearance of TCA-precipitable (intact) ¹²⁵I-IGF-I or ¹²⁵I-LR³IGF-I in day 14 wound fluid as a percentage of total infused ¹²⁵I-IGF-I or ¹²⁵I-LR³IGF-I. Wound fluid from one chamber from each rat was collected at 5, 30, 120 and 240 min following infusion of labeled growth factors. Values represent the mean ± S.E.M. of samples from one chamber per rat from six rats at each time point. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ represent significant differences in appearance of ¹²⁵I-LR³IGF-I in wound fluid compared with that of ¹²⁵I-IGF-I.

Profiles of IGF radioactivity in plasma and wound fluid

Pooled plasma samples from six animals sampled 5 min after infusion with ¹²⁵I-IGF-I chromatographed as a broad region of radioactivity at 150 kDa, a second at approximately 50 kDa and a third minor peak at the position corresponding to the molecular mass of free IGF-I (Fig. 5). By 30 min the radioactivity of each region had slightly decreased. At 120 min the radioactivity of the 150, 50 and 7.5 kDa regions has decreased by approximately 50%, with a further reduction in the high molecular mass region evident by 240 min. At each time point a preferential decrease of IGF radioactivity associated with low molecular weight IGF-BPs was observed.

The chromatograms of pooled plasma samples after administration of ¹²⁵I-LR³IGF-I are shown in Fig. 5. Unlike IGF-I radioactivity in plasma, less total counts were observed and approximately 40% of the radioactivity in these samples was detected as free ¹²⁵I-LR³IGF-I with much lower amounts appearing in the 150 kDa and 50 kDa regions. Radioactivity collected in fractions corresponding to free IGF peptide was cleared more quickly than radioactivity in higher molecular weight peaks. By 30 min the radioactivity in the 7.5 kDa region had decreased by 50%, while the radioactivity in the 150 kDa and 50 kDa regions evident at 5 min remained

Figure 5 Neutral gel permeation chromatography of plasma from rats 5, 30, 120 and 240 min after an intravenous bolus of ^{125}I -IGF-I (solid lines) or ^{125}I -LR³IGF-I (dashed lines). Pools of plasma from six rats (200 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml/min with the same buffer. The column was calibrated with γ -globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 43 kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I -IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ -counter. Values per microliter of plasma are shown.

approximately the same. At 120 and 240 min the radioactivity in each of the three regions continued to decrease at a relatively similar rate.

In contrast to plasma, less radioactivity was contained within wound fluid following infusion of labeled IGFs. It is important to note the difference in scale of the y -axis for the plasma chromatography profiles (Fig. 5), compared with the scale of the y -axis for the wound fluid chromatography profiles (Fig. 6). Pooled wound fluid samples from six animals sampled at 5 min following infusion of ^{125}I -IGF-I chromatographed as a broad region at approximately 50 kDa and a peak at the position corresponding to free IGF-I (Fig. 6). By 30 min the radioactivity in the 7.5 kDa region had decreased slightly, however a third

Figure 6 Neutral gel permeation chromatography of wound fluid from rats 5, 30, 120 and 240 min after an intravenous bolus of ^{125}I -IGF-I (solid lines) or ^{125}I -LR³IGF-I (dashed lines). Pools of wound fluid from six rats (150 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml/min with the same buffer. The column was calibrated as described in the legend for Fig. 5. Fractions (0.5 ml) were collected and radioactivity counted on a γ -counter.

peak in the 150 kDa region had become apparent. By 120 and 240 min there were no peaks of radioactivity in the 150 kDa region and IGF-I-specific radioactivity had become mainly associated with lower molecular weight IGF-BPs.

When wound fluid samples taken from rats 5 min after infusion of ^{125}I -LR³IGF-I were chromatographed, a similar 5 min profile was obtained to that of ^{125}I -IGF-I containing wound fluid with a broad region at 50 kDa and a peak at 7.5 kDa (Fig. 6). However, by 30 min the profile differed from that of ^{125}I -IGF-I containing wound fluid, in that two large distinct peaks were observed at 50 kDa and 7.5 kDa and no activity occurred in the 150 kDa region. By 120 min the peaks of radioactivity in the 7.5 kDa region had decreased, but the radioactivity in the 50 kDa region had increased by about 25%. A similar profile was observed for the 240 min samples, but a 25% decrease in

Figure 7 Mean percent degradation of ^{125}I -IGF-I and ^{125}I -LR³IGF-I in plasma and wound fluid. TCA-soluble radioactivity is expressed as a percentage of total radioactivity at each time point. Data points are means $\pm$ S.E.M. for six animals in each group. IGF-I (circles) and LR³IGF-I (squares) from plasma are represented by closed symbols and from wound fluid by open symbols.

radioactivity in the 50 kDa region had occurred. In the 120 and 240 min samples more ^{125}I -LR³IGF-I total radioactivity was found relative to ^{125}I -IGF-I.

Degradation of ^{125}I -IGF-I and ^{125}I -LR³IGF-I in wound fluid and plasma

The degradation of growth factors as a function of time after infusion was calculated as a percentage of total plasma or wound fluid radioactivity accounted for by the TCA-soluble fraction (Fig. 7). In plasma, ^{125}I -LR³IGF-I was degraded more rapidly and to a greater extent than ^{125}I -IGF-I. In contrast, in wound fluid ^{125}I -LR³IGF-I and ^{125}I -IGF-I were degraded to the same extent. These data suggest that degradation of IGF-I in plasma, or after uptake and release by cells, is inversely related to the binding of IGF-I to plasma IGFBPs. However, the ability of IGF-I to bind IGFBPs does not afford it protection from degradation in wound fluid.

Discussion

Several observations would suggest that IGF-I is able to leave the vascular compartment and distribute to various tissues (Ballard *et al.* 1991, Hodgkinson *et al.* 1991, Prosser *et al.* 1991, 1992, Bastian *et al.* 1993, Hill *et al.* 1997). It has also been demonstrated that IGFBPs have the ability to increase the circulating half-lives of IGFs (Zapf *et al.* 1986,

Cascieri *et al.* 1988). Results such as these have led to the proposal that a potential role of the IGFBPs is to control rates of IGF transport from the circulation to extravascular sites (Bastian *et al.* 1993). Pharmacokinetic studies of IGF-I variant molecules which display reduced association with IGFBPs have been shown to have increased clearance rates, including des(1–3)IGF-I (Ballard *et al.* 1991), LR³IGF-I (Bastian *et al.* 1993) and [Gln³,Ala⁴,Tyr¹⁵,Leu¹²]-IGF-I (Cascieri *et al.* 1988).

Examination of day 14 plasma by Western ligand blotting did not reveal any differences in circulating IGFBP profiles between rats implanted with chambers compared with sham control animals. However, this technique does not exclude the possible presence of increased amounts of wound-fluid derived cleaved IGFBP-3 in the chamber-implanted animals. The accelerated clearance of LR³IGF-I in the plasma of both sham control and chamber-implanted rats is in agreement with previous studies, and may be associated with increased rates of degradation, either in the blood or by tissues where it is degraded and returned to the circulation (Bastian *et al.* 1993). However, the clearance rates for both peptides in both groups were somewhat lower than in the earlier investigation (Bastian *et al.* 1993), a difference possibly associated with the use of larger animals in the present study. The key finding was that in rats with increased extracellular volume, clearance rates of IGF-I did not significantly differ to that of sham controls. However, $t_{1/2\alpha}$ was significantly increased, reflecting an alteration in the nature of the central compartment to which IGF-I distributes. In contrast, the clearance rate, volume of distribution and $t_{1/2\beta}$ of LR³IGF-I in wound chamber-implanted animals was significantly greater than in sham control animals, which could be attributed to the weaker affinity of LR³IGF-I for IGFBPs, rendering it less confined to plasma. Significantly more LR³IGF-I specific radioactivity was recovered in wound fluid compared with radiolabeled IGF-I, although no difference in the rate of degradation between IGF-I and LR³IGF-I in wound fluid was observed.

These results support the conclusion that transfer of IGF-I peptides from blood to extracellular fluid is inversely related to the ability of the peptides to associate with binding proteins. The results of this comparison are supported by the *in vitro* studies, which showed that the presence of IGFBPs in experimental medium inhibited transport of IGF-I across an endothelial cell monolayer model of the blood vessel wall, but did not affect transport of LR³IGF-I. Our earlier studies indicate that the movement of free IGF-I across HUVE cell monolayers occurs by a passive non-receptor mediated pathway (Bastian *et al.* 1997). Current thought acknowledges a number of determinants of passive barrier transport, including intercellular junctions, cell–cell contact, endothelial glycocalyx and the extracellular matrix. Binding of IGF-I to the IGFBPs may add another level to this regulation. It is of interest to note

that the basal transport of IGF-I alone was significantly greater than that of LR³IGF-I. This may be because LR³IGF-I is a larger molecule than IGF-I or could arise due to charge differences between the two peptides.

While this study was not designed to calculate the proportional contribution intravascular IGF-I makes to wound fluid IGF-I, it is worth noting that over the 4 h time course of the study, only some 0.05% of the administered IGF-I was recovered per milliliter of wound fluid. Other sources of IGF-I during the wound healing process include platelets (Karey & Sirbasku 1989), macrophages (Rappolee *et al.* 1988) and the cells of the wound repair response themselves, such as injured endothelial cells (Taylor & Alexander 1993). Moreover, systemic administration of IGF-I alone, while exerting profound anabolic effects, does not promote repair of incisional (Robertson *et al.* 1997) or burn wounds (Meyer *et al.* 1996). A recent study by our group has shown that reversal of steroid-induced catabolism by IGF-I is not accompanied by improvement of excisional wound repair (J G Robertson, unpublished observations). These data would suggest that the surface wound is not an endocrine target of IGF-I.

The IGFBP band pattern of wound fluid and plasma were similar, except that the intensities of the band at approximately 31 kDa and the band at a molecular mass corresponding to IGFBP-3 were reduced in wound fluid compared with plasma (Robertson *et al.* 1996). A similar IGFBP profile has been described in human skin interstitial fluid (Xu *et al.* 1995) and human peritoneal dialysate (Kale *et al.* 1996). Administration of ¹²⁵I-IGF-I to rats implanted with wound chambers followed by neutral gel filtration chromatography of wound fluid samples, revealed that ¹²⁵I-IGF-I was distributed to the lower molecular weight IGFBPs, with smaller amounts associated with the 150 kDa complex and 7.5 kDa region. A similar profile is seen after *in vitro* incubation of ¹²⁵I-IGF-I with day 14 wound fluid (Robertson *et al.* 1996). These data, taken together with several reports describing the presence of IGFBP-3 protease activity in extracellular fluids (Xu *et al.* 1995, Kale *et al.* 1996, Robertson *et al.* 1996), imply that extravascular IGF-I exists in a form that is more readily available for interacting with specific IGF tissue receptors. The fact that more LR³IGF-I was found in a free form in wound fluid relative to IGF-I may mean that it would have even greater accessibility to interact with tissue IGF receptors than IGF-I.

In summary, we have shown that systemically administered IGF-I peptides are transferred from blood to extravascular wound fluid sites, where they exist in forms that may be more readily available to interact with tissue IGF receptors. The pharmacokinetic parameters of LR³IGF-I, but not IGF-I, are affected by changes in the extracellular fluid volume and the rate of transfer of LR³IGF-I from blood to extracellular fluid is dramatically increased over that of IGF-I. The decreased association of LR³IGF-I with the 150 kDa complex, and the increased

rate of delivery of this peptide from the systemic circulation to extracellular fluid pools suggest that this IGF-I analogue may be more active at the wound site.

Acknowledgements

We are very grateful to Drs Robert Baxter, Eiji Kuto and Dennis Andress plus Diagnostic Systems Laboratories for their kind gifts of IGFBPs. We would also like to thank Ingrid Liepe for help with the isolation and maintenance of HUVE cells and Kerry Penning, Kaylene Pickering, Xenia Georgiou and Gray Robertson for help with the *in vivo* studies.

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Received 22 February 1999

Revised manuscript received 16 August 1999

Accepted 31 August 1999