Clearance of IGFs and insulin from wounds: effect of IGF-binding protein interactions

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Robertson, J. Gray, David A. Belford, and F. John Ballard. Clearance of IGFs and insulin from wounds: effect of IGF-binding protein interactions. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E663–E671, 1999.—We have examined the role binding proteins have in regulating the clearance of exogenous growth factors from wounds. Hunt-Schilling chambers were subcutaneously implanted in rats, and the clearance of insulin-like growth factor (IGF) I from the chamber wound fluid was compared with IGF-II, LR3-IGF-I, which binds poorly to IGF-binding proteins (IGFBP), or insulin. Elimination rate constants of the slow phase of the decay curves did not differ between IGF-I and IGF-II. However, LR3-IGF-I and insulin were cleared more rapidly from wound fluid than IGF-I so that the half-lives for IGF-I, IGF-II, LR3-IGF-I, and insulin were 872, 861, 563, and 324 min, respectively. In wound fluid, minimal degradation of the IGFs occurred, whereas insulin was degraded considerably. The increased clearance of LR3-IGF-I and insulin equated with a reduced association with wound fluid IGFBPs, and increased amounts of radioactivity of these peptides were detected in the circulation and urine. These results show that this model of wound repair may be of use in examining the kinetics of growth factors and other bioactive molecules in extravascular spaces and support the hypothesis that IGFBPs can be significant regulators of IGF bioavailability in vivo.

insulin-like growth factor I analog; extravascular wound fluid; growth factors; wound healing


exist, and consequently, detailed knowledge of the fate of growth factors after application to wounds and their interactions with components of the wound is limited.

The insulin-like growth factors (IGFs) are regulators of tissue growth and differentiation and are normally found associated with six high-affinity IGF-binding proteins (IGFBPs), such that very little unbound peptide exists (for review see Ref. 20). IGF-I is expressed in granulation tissue (14), whereas several cell types involved in the wound repair response are known to express and produce IGFBPs in vitro (3, 40). Although several studies have demonstrated an enhanced effect of IGF in wounds when coadministered with IGFBPs, the mechanism by which each IGFBP acts is unknown. Thus IGFBPs may serve to enhance IGF-I bioactivity by maintaining a pool of IGF-I at the site of action and preventing clearance, by protecting IGF-I from degradation, or by targeting IGF-I to cell membranes or receptors. Experiments have shown that IGFBP-I may enhance IGF actions (21, 42) by binding the α5β1-integrin through an Arg-Gly-Asp sequence (13). IGFBP-3 normally inhibits IGF-I actions, but in the wound this binding protein is proteolyzed (34), a process known to occur in many pathophysiological states (8, 9). The proteolytically modified form of IGFBP-3 has a reduced affinity for IGF-I, a property that may serve to enhance IGF-I actions (6).

Several earlier studies have attempted to measure the transfer of growth factors to tissues in vivo, with mixed success (18, 27). However, most of the data concerning the regulation of the movement of IGFs between extracellular compartments have been derived from plasma pharmacokinetic experiments that identify target organs or tissues but do not provide data on the extracellular kinetics of the IGFs. Analogous of IGF-I that exhibit poor binding characteristics to IGFBPs are more potent in vitro compared with native IGF-I (12), a potency that is attributed to reduced interactions with IGFBPs secreted into the media by cells in culture. However, these analogs are cleared from the circulation more quickly and are degraded to a greater extent than IGF-I (2), suggesting that IGFBPs may regulate the bioavailability of IGFs. The purpose of this study was to determine whether interactions with IGFBPs had any effect on the clearance of IGFs from wound sites. We have compared the rates of elimination and breakdown of IGF-I and IGF-II with an IGF analog that binds IGFBPs poorly, LR3-IGF-I (12), and insulin. We hypothesize that interactions with IGFBPs significantly decrease the rate of clearance of IGFs from wound sites.
MATERIALS AND METHODS

Reagents. Thiobutabarbital (Inactin) was purchased from Research Biochemicals International (Natik, MA). Sodium iodide (Na125I) was obtained from Amersham Australia (Castle Hill, Australia). Heparin, Evans Blue dye, recombinant human insulin, and the size exclusion molecular-mass markers were obtained from Sigma (St. Louis, MO). Recombinant human IGF-I, IGF-II, and LR3-IGF-I were obtained from GroPep (Adelaide, Australia). Each peptide was 125I-labeled with the chloramine-T method (1) to a specific activity of 75–85 μCi/μg and stored at −20°C. The biological activity of these peptides has been described previously (11, 12, 26).

Animal surgery. All animal procedures were approved by the animal ethics committee of the Adelaide Women’s and Children’s Hospital, following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Male Sprague-Dawley rats (175–200 g) were housed under constant temperature and humidity with 12:12-h light-dark cycles. Each rat was individually caged, and ad libitum access to food and water was allowed. Hunt-Schilling chambers were constructed from stainless steel mesh as previously described (34). One chamber was implanted subcutaneously per rat and left in situ for 14 days. This study was performed 14 days after the implantation of the chambers for a number of reasons. First, from the time of implantation there is accumulation of fluid within the lumen of the chamber and an ingrowth of granulation tissue. Second, there was potential for volumetric disturbances caused by injection of tracer or sampling of wound fluid to occur. By conducting the experiment 14 days after implantation when the wound fluid volume was greatest and by limiting injection or sampling volumes to 10% or less of the wound fluid volume, we aimed to minimize the risk of such problems. Finally, the presence of a large and well-perfused vascular structure at this time facilitated the measurement of clearance rates.

Fourteen days after implantation each rat was weighed and anesthetized with Inactin (60 mg/kg ip). Body temperature was maintained with a heating-pad set so that core body temperature was maintained at 37°C. An incision was made through the skin overlying the right jugular vein, and a catheter was aseptically introduced. The catheter was immediately flushed with 100 µl of 0.15 M saline containing 10 IU/ml heparin. To prevent respiratory problems, a tracheotomy tube was inserted to allow aspiration of secretions. The bladder was catheterized to collect urine, and all incisions were sutured after placement of the catheters to prevent loss of body fluids.

The hair overlying the chamber was clipped, and the skin was disinfected with 70% ethanol. A sterile 23-gauge needle was inserted percutaneously so that the tip of the needle was approximately in the center of the lumen of the chamber. A microsyringe was then attached to the needle with a short catheter to allow injection of the radiolabeled analogs and subsampling of the wound fluid within the chamber. After placement of the catheters, the rats were allowed to stabilize for 20–30 min.

Experiment 1: clearance of radiolabeled IGF-I or 125I from wound fluid. An initial experiment was performed to gain an estimate of the rate at which 125I-IGF-I (n = 9 rats) or 125I (n = 3 rats) was eliminated from wound fluid. Preliminary results had indicated that a sampling duration of 6 h was required to observe a reasonable decline in the amount of labeled IGF-I remaining in the chamber. Given the long duration of anesthesia and the potential for hemodynamic changes to occur, the packed cell volumes of the IGF-I injected rats were monitored throughout the experiment. In this experiment, we found that the hematocrit declined marginally from a mean of 42.1% at the start of the experiment to 41.7% at 6 h. The body weights of the rats declined by an average of 4.5 g or 1.3% of initial body weight.

Samples of blood (90 µl) and wound fluid (19 µl) were first withdrawn and heparinized to a final concentration of 10 IU/ml and then were stored on ice, before 0.35 ml of 10 mg/ml Evans Blue dye in 0.15 M NaCl was injected into the jugular catheter. The catheter was washed by several flushings of jugular blood plus a final flush with 100 µl of heparinized saline. Ten minutes after injection of the Evans Blue dye, a second subsample of blood (170 µl) was withdrawn. Additional samples of 40, 170, 40, 200, 140, 140, 140, and 140 µl were similarly withdrawn at 20, 30, 40, 60, 120, 180, 240, 300, and 360 min, respectively. All samples were heparinized (10 IU/ml) and with the exception of the 20- and 40-min samples were treated as follows. Triplicate subsamples of 40 µl were added to tubes containing ice-chilled 10% (wt/vol) TCA, vortexed, and left on ice. The remainder of the blood from the first hour of sampling, including the 20- and 40-min samples, was left at 4°C before measurement of the Evans Blue concentration. Any surplus blood from the 120- to 360-min samples inclusive was returned to the rat. The hematocrit was estimated from the 0-, 60-, 180-, and 360-min blood samples.

Immediately after the injection of the dye into the jugular vein, a bolus of 107 counts/min of 125I-IGF-I (40–70 ng of peptide) or 125I and 25 µl of Evans Blue dye (30 mg/ml in 0.15 M saline) was injected into the lumen of the chamber. To ensure a rapid and even distribution of the radiolabel and marker dye within the chamber, the microsyringe was flushed repetitively with wound fluid. Subsampling of wound fluid occurred at the same intervals as those for blood but with volumes of 27, 19, 27, 19, and 27 µl taken at the 10-, 20-, 30-, 40-, and 60-min times, respectively. Thereafter, 7.5 µl were sampled at each time point. The samples were also heparinized and triplicate subsamples of 2.5 µl were immediately added to 10% (wt/vol) TCA, vortexed, and left on ice. Together with the 20- and 40-min samples, the remaining wound fluid of the samples from the first hour was left at 4°C for measurement of the Evans Blue concentration.

Excretion of the radiolabeled tracer into urine was monitored by collecting urine hourly for the duration of the experiment. An equivalent volume of Hartmann’s saline containing 10% (wt/vol) glucose was injected intravenously to help maintain intravascular volume. Triplicate subsamples (50 µl) of the hourly urine collections were added to 10% (wt/vol) TCA, vortexed, and left on ice. The blood, wound fluid, and urine samples treated with TCA were kept on ice for at least 30 min and centrifuged, and the radioactivity in the TCA-soluble and TCA-insoluble fractions was measured.

At the completion of the experiment, the anesthetized rat was killed and weighed. The volume of wound fluid remaining in the chamber was measured after aspiration and used to calculate the total amount of radioactivity remaining.

Experiment 2: comparison of the rates of clearance of IGF-I, IGF-II, LR3-IGF-I, and insulin from wound fluid. The injection and sampling procedures of experiment 1 were utilized in a second experiment in which the rate of elimination of labeled IGF-I from wound fluid was compared with rates of IGF-II, LR3-IGF-I, or insulin (n = 8 rats/treatment). In addition, after each rat was killed and weighed, the wound fluid was aspirated, and the chamber and tissue immediately surrounding the chamber were removed by dissection and added to 25 ml of 0.5 M NaOH. The rat carcass was...
homogenized in 0.5 M NaOH and digested at room temperature. Radioactivity was measured in the tissue or carcass extracts after digestion.

Measurement of blood and wound fluid volumes. To estimate the total blood or wound fluid volume, a dye marker dilution technique (22) was modified for use with a microplate reader. Samples of plasma or wound fluid collected during the first hour after injection of the respective dye solutions were centrifuged at 10,000 g and 4°C for 10 min to sediment cells and cellular debris. Supernatant samples of 5 µl were diluted in 95 µl of 0.15 M NaCl, the absorbances were measured at 600 nm, and the dye concentrations were obtained by reference to a standard curve. The dye concentration at time 0 (t₀) was extrapolated, and fluid volumes were calculated according to the formula: volume (ml) = (mg of dye injected)/(dye concentration at t₀ in mg/ml).

Neutral-gel chromatography. Subsamples of wound fluid (200 µl) collected from each rat at the completion of experiment 2 were thawed, defatted, and centrifuged, and the supernatant was subjected to size-exclusion chromatography as previously described (34). The column was calibrated with molecular mass standards of 150 kDa (human IgG), 66 kDa (BSA), 30 kDa (carbonic anhydrase), and 12.5 kDa (cytochrome c), as well as the radiolabeled peptides (IGF-I and IGF-II, 7.5 kDa; LR3-IGF-I, 9.1 kDa; and insulin, 5.8 kDa).

Data analysis. All radioactivity measurements were corrected for radioactive decay back to the day the experiment was performed. The amount of TCA-precipitable peptide remaining in the wound fluid at each time point was expressed as a percentage of the initial amount of TCA-precipitable peptide injected into the chamber. The data were then fitted to a biexponential equation (2) with a nonlinear curve-fitting program (TableCurve 2D version 3.0, Jandel Scientific, Corte Madeira, CA). Elimination rate constants (Kd) of the slow phase of the curves were calculated with the 120- to 360-min data. The half-lives of decay for each curve were calculated from the equation t½ = ln2/Kd.

All data are presented as means ± SE. Analysis of the wound fluid decay rate constants and distribution of radioactivity was achieved by one-way ANOVA, whereas post hoc pairwise multiple comparisons were made with Fisher’s protected least square differences procedure (SuperANOVA, 1989, Abacus Concepts, Berkeley, CA). Differences that give P < 0.05 were considered to be statistically significant.

RESULTS

Experiment 1: clearance of radiolabeled IGF-I or 125I from wound fluid. Preliminary data on the decay profiles of IGF-I or 125I in wound fluid were derived from an initial experiment in which tracer was injected into the chamber and the elimination of radioactivity from the wound fluid was monitored. The volume of wound fluid collected from the chamber at the completion of the experiment was added to the volume of wound fluid removed by sampling and was compared with the volume calculated from the Evans Blue dye dilution assay. Because the two procedures gave similar results, subsequent calculations of the amount of tracer remaining in the chamber were made with the volume calculated by the Evans Blue method.

After injection of tracer into the chamber, the radioactivity remaining in the wound fluid, the amount present in the circulation, and the amount of radioactivity accumulating in urine were measured over time and expressed as the percentage of the administered dose. The use of radiolabeled peptides with the TCA-precipitation assay provided a simple and rapid method for measuring total and degraded peptides. Sampling volumes were also minimized. The amount of intact (TCA insoluble) radiolabeled IGF-I present in the chamber decreased over time (Fig. 1A). After 1 h, ~22% of the original amount of peptide had been eliminated. However, from 1 h until the end of the experiment, the

Fig. 1. Clearance of tracer from wound fluid (experiment 1). TCA-insoluble insulin-like growth factor (IGF) I (●; n = 9 rats) or total 125I (○; n = 3 rats) radioactivity was measured in wound fluid (A), blood (B), and urine (C) over 6 h after injection into chamber. Amount of radioactivity remaining was calculated as a percentage of that injected into chamber. All values are means ± SE.
rate of elimination of intact IGF-I from wound fluid was slower, with a decline of ~20% occurring in this time. In contrast to labeled IGF-I, $^{125}$I was rapidly eliminated from the chamber (Fig. 1A), such that by 1 h after injection only 45% of the original material was present. Furthermore, the rapid rate of elimination of $^{125}$I continued after 1 h, with <5% of the administered dose remaining after 6 h.

The amount of intact IGF-I peptide in the circulation was at all times very low compared with the amount injected into the chamber (Fig. 1B). Thus 10 min after injection, <0.1% of that injected was present in blood, and by 6 h this had risen to only 0.6%. By comparison, $^{125}$I radioactivity in the circulation had risen to ~5% of the administered dose at 1 h, but by the end of the experiment this had fallen to 3.5%. Similarly, the accumulation of IGF-I radioactivity in urine over the duration of the experiment was <3% of the total radioactivity injected (Fig. 1C), whereas ~16% of the $^{125}$I radioactivity accumulated in the urine by 6 h.

Experiment 2: comparison of the rates of clearance of IGF-I, IGF-II, LR3-IGF-I, and insulin from wound fluid. After establishment of the decay profile of IGF-I in wound fluid, a second experiment was performed to compare the rate of elimination of IGF-I with rates of IGF-II and LR3-IGF-I, an analog that interacts poorly with IGFBPs (12). The experiment also included insulin, which is of a similar molecular weight to the IGFs but does not interact with the IGFBPs, to establish whether the rate of elimination from the wound fluid was a function of molecular size. The decline of the total amount of radioactivity present in wound fluid was biphasic for each peptide (Fig. 2A). In the first hour after injection, ~20% of the radioactivity of labeled IGF-I or IGF-II was eliminated in contrast to ~30% of the LR3-IGF-I or insulin radioactivity. A slow phase of elimination then occurred from 1 h until the end of the experiment, by which time approximately a further 15% of the radioactivity of the IGF peptides had been eliminated from the wound fluid. Total insulin radioactivity declined by ~28% from 1 to 6 h (Fig. 2A).

The amount of intact IGF-I present in wound fluid declined in a biphasic manner (Fig. 2B). TCA-insoluble radioactivity for IGF-II and LR3-IGF-I (Fig. 2B) showed a similar biphasic pattern of decay to IGF-I. However, the amount of intact LR3-IGF-I remaining after 1 h was 70% of the injected dose compared with 82% for IGF-I (Fig. 2B). By 6 h, this difference was greater, with 55% of intact LR3-IGF-I remaining compared with 68% of intact IGF-I. Insulin showed the fastest decline of TCA-insoluble radioactivity remaining in the chamber, declining rapidly in the first hour to 70% of that injected. Only 35% remained at 6 h (Fig. 2B).

The slopes of the slow phases of the TCA-insoluble curves were used to calculate the elimination rate constant ($K_d$) and half-lives for each peptide (Table 1). The $K_d$ for IGF-I did not significantly differ from that of IGF-II (P > 0.05). This was in contrast to LR3-IGF-I or insulin, which had significantly greater rates of elimination than IGF-I (P < 0.05 and P < 0.001, respectively).

Degradation of the labeled peptides in wound fluid was evaluated by measuring the proportion of intact material present over time (Fig. 3). Each of the IGF peptides remained >89% intact in the wound fluid throughout the experiment, whereas insulin, being 94% TCA insoluble at the beginning of the experiment, declined to be only 73% intact by 6 h. In accordance with injection, <0.1% of that injected was present in blood, and by 6 h this had risen to only 0.6%. By comparison, $^{125}$I radioactivity in the circulation had risen to ~5% of the administered dose at 1 h, but by the end of the experiment this had fallen to 3.5%. Similarly, the accumulation of IGF-I radioactivity in urine over the duration of the experiment was <3% of the total radioactivity injected (Fig. 1C), whereas ~16% of the $^{125}$I radioactivity accumulated in the urine by 6 h.

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![Fig. 2. Clearance of $^{125}$I-labeled proteins from wound fluid (experiment 2). Radiolabeled IGF-I (■), IGF-II (○), LR3-IGF-I [an IGF analog that binds poorly to IGF-binding proteins (IGFBPs); ●], or insulin (●) was injected into chambers, and total radioactivity (A) or TCA-insoluble radioactivity (B) was measured over a 6-h period. Radioactivity is expressed as a percentage of that injected. Values are means ± SE; n = 8 rats/treatment (see Table 1 for statistical summary). cpm, Counts/min.](http://ajpendo.physiology.org/)

![Table 1. Elimination rate constants of intact peptides in wound fluid](http://ajpendo.physiology.org/)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Elimination Rate Constant, $K_d$</th>
<th>Half-life, min</th>
</tr>
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<tbody>
<tr>
<td>IGF-I</td>
<td>0.049 ± 0.003</td>
<td>872</td>
</tr>
<tr>
<td>IGF-II</td>
<td>0.053 ± 0.009</td>
<td>861</td>
</tr>
<tr>
<td>LR3-IGF-I</td>
<td>0.076 ± 0.006*</td>
<td>563</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.141 ± 0.013†</td>
<td>324</td>
</tr>
</tbody>
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Data are means ± SE for 8 rats/treatment. Amount of intact peptide remaining in chamber wound fluid (Fig. 2B) was expressed as a percentage of that injected. Slopes of slow phase of curves were used to calculate elimination rate constants and half-lives as described. IGF, insulin-like growth factor; LR3-IGF-I, IGF analog that poorly binds IGF-binding proteins. *P < 0.05; †P < 0.001 vs. IGF-I by one-way ANOVA.
with this observation, there was an increased rate of elimination of the TCA-insoluble radioactivity in wound fluid (Fig. 2B) compared with the rate of elimination of the total radioactivity (Fig. 2A). In contrast, the rates of elimination of the TCA-insoluble material and total radioactivity for LR3-IGF-I were more similar (Fig. 2, A and B). We recognize that the TCA-precipitation assay may underestimate tracer degradation because some peptide fragments may be precipitable yet functionally inactive (10). This assay was chosen in preference to other techniques, such as radioimmunoassay, which not only may not distinguish between active and inactive fragments, but also would be incapable of distinguishing added from endogenous peptides and may suffer from interference by IGFBPs.

Samples of wound fluid collected from each rat at the end of the experiment were chromatographed under neutral conditions to evaluate the capacity of the respective labeled peptides to bind IGFBPs. IGF-I eluted in three regions corresponding to 150, 30–50, and 7.5 kDa, respectively (Fig. 4A). This profile is similar to that seen after incubating wound fluid with 125I-IGF-I in vitro at 4°C (34). IGF-II showed a similar pattern of elution to IGF-I, although the relative amount of radioactivity recovered in the 150-kDa region was substantially greater, and there was a corresponding reduction in the 30- to 50- and 7.5-kDa peaks. Chromatography of insulin or LR3-IGF-I revealed two small peaks of radioactivity at ~50–70 kDa (Fig. 4, C and D), but the greatest amounts, representing unbound peptide, were recovered in each case as a single peak at ~7 kDa. Although the IGFBP- and receptor-binding abilities of the tracers after incubation in wound fluid were not assessed, the binding properties of similarly prepared labeled peptides have been described previously (11, 12, 26). The neutral-gel filtration chromatography profiles also suggest that the endogenous IGF-binding capacity of wound fluid was not exceeded by the amount of tracer added, which was calculated to be <10% of the total IGF-I present (34, 43).
Radioactivity in blood and urine was measured to determine whether the increased rates of clearance from the wound fluid were reflected in the amount of each appearing in these fluids (Figs. 5 and 6). LR3-IGF-I or insulin showed similar and increasing amounts of intact peptide in blood throughout the experiment (Fig. 5). This was in contrast to the IGF-I or IGF-II radioactivity, which was lower and constant from 120 min onward. For IGF-I, 45% of the total radioactivity present in the blood throughout the experiment was TCA insoluble. Approximately similar values were recorded for LR3-IGF-I, IGF-II, and insulin. Accumulation of radioactivity in urine (Fig. 6) showed similar patterns of appearance as for blood in that by the end of the trial more LR3-IGF-I or insulin radioactivity accumulated than that observed with IGF-I or IGF-II. However, for the duration of the experiment, <5% of the total radioactivity of each peptide injected into the chamber was excreted into the urine (Fig. 6).

At completion of the experiment, radioactivity present in the wound fluid, chamber and associated tissue, and rat carcass was measured (Table 2). Radioactivity present in urine or that removed by blood or wound fluid sampling was also measured to complete the mass balance. For each of the peptide treatments, >92% of the administered dose was recovered with no significant difference occurring between treatments (P > 0.05). Additionally, no significant differences in the tracer distribution were found between IGF-I- and IGF-II-injected rats. Compared with the IGF-I group, there was significantly more radioactivity recovered in the carcasses of LR3-IGF-I- or insulin-treated rats (P < 0.0001). In contrast, the amount of radioactivity recovered from tissue associated with the chambers of insulin-treated rats was approximately one-half of that recovered for the other treatments (P < 0.0001). At 6 h, as inferred from the rates of elimination of tracer from wound fluid, significantly more radioactivity was present in the wound fluid of rats injected with IGF-I than in that injected with LR3-IGF-I or insulin (P < 0.0001).

DISCUSSION

Few studies have investigated the role of IGFBPs in regulating the actions of IGFs in the extravascular environment, and as such, the behavior of IGF peptides is mostly derived from in vitro or circulatory studies. In the present study, we have adapted the Hunt-Schilling chamber model of wound repair (19) to allow repeated sampling of extravascular wound fluid from the same chamber, with minimal risk of contamination with plasma. The results showed that interactions with IGFBPs retarded the elimination from the wound environment of IGF-I and IGF-II, both of which significantly interact with IGFBPs, compared with an analog of IGF-I, LR3-IGF-I, which has a greatly reduced affinity.

All the peptides tested were eliminated from the chamber in a biphasic manner with an initial rapid phase in the first hour followed by a slower phase for the remainder of the experiment. Although few studies have attempted to measure clearance of growth factors from extravascular sites, the patterns of elimination in this study are consistent with those observations. Thus
radiolabeled insulin injected into the wound fluid of Hunt-Schilling chambers that had been implanted in diabetic rats was cleared in a similar manner to the peptides of this study (15). It was also reported that 125I-labeled epidermal growth factor rapidly disappeared from implanted sponges, with only 10% of the radioactivity remaining after 4 h (7). In another study, radiolabeled transforming growth factor-β, platelet-derived growth factor, or basic fibroblast growth factor was emulsified in collagen, and the clearance of each from the chambers over 10 days was examined (37). A biphasic clearance of the growth factors was observed, with ~30–35% of the peptides remaining after 6 h. However, the initial phase of decay lasted up to 24 h, and some radioactivity was recoverable after 10 days. The long duration of that study may be due to the chambers being filled with collagen, which could have impeded clearance of the growth factors, and as such the results may not be directly comparable with ours.

Interaction and binding to components of the extracellular matrix, which may occur with growth factors, would be one mechanism by which the clearance of growth factors could be regulated. However, the results of Sprugel et al. (37), derived with growth factors that are capable of binding to matrix, suggest that such interactions may not be sufficient to increase the tissue half-lives of exogenous growth factors. In contrast, our experiments suggest the presence of a pool of specific carrier proteins, in the form of the IGFBPs in wound fluid, is sufficient to alter the kinetics of an exogenous IGF provided it is capable of interacting with the IGFBPs. We evaluated the clearance of LR3-IGF-I, an analog of IGF-I with a 13-amino acid NH2-terminal extension and a substitution of Arg for the Glu at position 3 (12). Its affinity for IGFBPs, compared with IGF-I, is greatly reduced, a property that increases its potency in vitro and in vivo (12, 41). In an earlier study, LR3-IGF-I was shown to be cleared from plasma more rapidly than IGF-I (2), a finding consistent with the present results. We also demonstrated that, in contrast to IGF-I and IGF-II, LR3-IGF-I was not able to significantly interact with IGFBPs in wound fluid, as chromatographed samples of wound fluid containing LR3-IGF-I eluted as a single peak in the region expected for unbound peptide. Given the in vitro IGFBP-binding characteristics of LR3-IGF-I, it may also be expected that this peptide would also interact to a lesser extent with tissue- or matrix-associated IGFBPs than IGF-I or IGF-II. However, the amount of radioactivity present in the chamber tissue at the end of the experiment did not differ between the IGF peptides, whereas the amount of radioactivity found in the carcass was greater for LR3-IGF-I, consistent with a more rapid transfer of this peptide from the chamber wound fluid to other tissues compared with IGF-I and IGF-II. Although the actual sites of binding were not determined in this study, the data suggest that despite LR3-IGF-I being more rapidly cleared from the chamber, this peptide was still able to interact with wound tissue to the same extent as IGF-I and IGF-II.

It has been reported that IGF-I present in the 150-kDa complex of IGFBP-3 is cleared more slowly from plasma than that present in the 30- to 50-kDa form (25). Additionally, IGFBP-3 in wound fluid is proteolyzed, after which it exhibits a reduced ability to bind radiolabeled IGF-I (6, 34), leading to a greater amount of the labeled IGF-I associating with lower-molecular-weight binding proteins. As an extension of this finding, it might be expected that IGF-II would exhibit a lower rate of elimination from wound fluid than IGF-I because a greater proportion of IGF-II was present in the 150-kDa complex compared with IGF-I. Our finding is consistent with the preferential binding of IGF-II to unoccupied, proteolytically nicked IGFBP-3 to form a ternary complex (24). Why no difference was observed is not obvious from the data, but may, in part, be due to the increased permeability of the capillary barrier in granulation tissue. Nevertheless, we were not able to demonstrate any significant difference between the clearances of IGF-I and IGF-II.

Insulin, which is of a similar molecular weight to LR3-IGF-I and does not associate with binding proteins, was eliminated more rapidly from wound fluid. However, the process of insulin elimination was complex due to degradation of the hormone in wound fluid. In other experiments (15), the half-life of intact radiolabeled insulin in 14-day-old wounds of diabetic rats was calculated as 150 min. The smaller estimate, compared with our data, is reasonable given the well-described disturbances to healing that occur in diabetic rats (15). The degradation of insulin in wound fluid has been attributed to the action of a specific extracellular protease which, in accordance with our own results,
was also shown not to proteolyze either IGF-I or IGF-II (36). However, another study showed that the insulin-degrading enzyme degraded IGF-I slightly in vitro, as assessed by either TCA precipitation or by the loss of ability to bind type-1 IGF receptors (35). Furthermore, Roth et al. (35) observed that IGF-II was more susceptible than IGF-I to proteolysis of the IGFs by the human insulin-degrading enzyme. It is plausible that some degradation of the IGF peptides may have occurred in our experiments, but no breakdown products accumulated because of rapid clearance of such products.

Although rates of elimination of the degraded (TCA soluble) fractions could not be calculated in our experiments, $^{129}$I was rapidly cleared from the chamber such that by 6 h, <5% of the original material remained. The labeled IGFs were 88–92% intact before injection into the chambers, with the range of values attributed to varying specific activities. Over time the TCA insolubility of the IGF peptides in wound fluid increased. A potential bias may exist here because radioactivity could reenter the wound chamber from the circulation. No allowance for this possibility was made in this model. However, in other work in our laboratory, <0.1% of an intravenously administered bolus of IGF-I was detected in wound fluid after 4 h (Bastian, S. E., P. E. Walton, D. L. Andress, and D. A. Belford, unpublished observations). This result, together with the observation that Na$^{129}$I is rapidly cleared from the chamber, supports the view that reentry contributes little to the total chamber radioactivity. Against the view that IGFBPs protect IGFs from proteolytic action, LR1-IGF-I did not appear to be significantly degraded in wound fluid. Interestingly, the greater breakdown of insulin in wound fluid was not reflected in an increase in the proportion of TCA-soluble radioactivity in blood. Presumably, this is due to the rapid removal of these products from the blood, which was reflected in the higher accumulation of insulin radioactivity in urine.

Constitutive expression of IGFs and most IGFBPs by cells of the repair response strongly suggests a role for IGFs in wound repair (14, 38, 40). Furthermore, the potent in vitro stimulatory activity of IGF analogs with low IGFBP affinity on cells that are principal components of the repair process enhances the prospect of IGFs as agents that may have therapeutic value in wound healing. Yet, positive responses to exogenous IGF-I have been equivocal and generally limited to those studies where an IGFBP was administered concomitantly (16, 21, 42), perturbations of the growth hormone-IGF-IGFBP system exist (39, 42), or where the IGF is present at persistently increased levels (5, 7, 39). The roles of the IGFBPs that are present during wound repair and the parts they play in regulating IGF bioavailability are unclear. Nevertheless, some insight has been gained from observations of the effects of application of IGFBPs with IGF-I to wounds. Thus IGFBP-1 in the phosphorylated form has been shown to enhance wound epithelialization and granulation tissue formation when administered with IGF-I (21, 42), an effect that has been attributed to the ability of this IGFBP to associate with tissue integrins (13). Also, proteolytic fragments of IGFBP-3 have a reduced affinity for IGF-I and are thought to enhance IGF-I bioactivity by localizing IGF-I to the cell membrane (23, 32). This process has yet to be confirmed in vivo, although IGFBP-3 is proteolyzed in wound fluid (34), suggesting that such a mechanism does exist. A role for other IGFBPs in dermal wound repair is also postulated on the basis of their expression by components of normal skin (3) and the presence of glycosaminoglycan-binding domains in IGFBP sequences (17). However, not all IGFBPs are expressed in skin, and the expression of each IGFBP is localized to different components of the integument (3), indicating that the activity of IGFs may be regulated according to the target cells involved.

In conclusion, our observations that IGF analogs with reduced affinity for the IGFBPs are cleared from wound fluid at a greater rate than native IGF-I or IGF-II emphasize the importance of IGFBPs in maintaining a pool of IGF at the wound site. Furthermore, the clearance experiments may serve as a general model for measuring the regulation of the bioactivity and transfer of growth factors and other bioactive molecules from one extravascular compartment to another. Nevertheless, whether manipulation of IGF/IGFBP associations can be used as a means to alter the healing processes in wounds has yet to be determined.

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REFERENCES


