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Erythrocyte insulin-like growth factor-I binding in younger and older males

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Summary

OBJECTIVE Insulin-like growth factor-I (IGF-I) levels are lower in older compared with younger subjects. We tested the hypothesis that the reduction in circulating IGF-I would be accompanied by upregulation in tissue IGF-I binding in at least some tissues. We tested erythrocyte IGF-I binding since blood is an accessible tissue in humans, and there is growing evidence to suggest that erythrocyte IGF-I binding is influenced by circulating IGF-I.

DESIGN AND PATIENTS We compared 9 healthy older males (61–68 years old) with 9 healthy younger males (15–19 years old).

MEASUREMENTS Standard techniques were used to assay circulating IGF-I and IGF binding proteins 1–5 (IGFBPs 1–5). Erythrocyte IGF-I binding was first measured by studies in which native [125I]-IGF-I was displaced with unlabelled native IGF-I. In order to determine a possible role for IGF binding proteins (IGFBP), native [125I]-IGF-I was displaced with des-(1-3)-IGF-I, which binds with IGF receptors but not IGFBPs.

RESULTS As expected, circulating IGF-I was significantly lower in the older compared with the younger subjects. In addition, IGFBP-3 and 5 were significantly lower, and IGFBP-4 higher, in older compared with younger subjects. When native [125I]-IGF-I was displaced with unlabelled native IGF-I, the number of IGF-I binding sites per erythrocyte was higher in the older subjects (43 ± 5 vs. 18 ± 2, older vs. younger, respectively; P < 0.05). In contrast, when native [125I]-IGF-I was displaced with des-(1-3), IGF-I binding capacity was not different between the two age groups.

CONCLUSIONS Erythrocyte IGF binding was increased in older compared with younger subjects. Surprisingly, the mechanism of the increase may not be a simple up regulation of IGF-I receptors in response to reduced circulating IGF-I, but possibly by an increase in the levels of as yet unidentified erythrocyte membrane-associated IGF binding proteins.

It is becoming increasingly clear that insulin-like growth factor-I (IGF-I) exerts its tissue anabolic effects through a complex interaction of ambient IGF-I levels, binding proteins, and cellular receptors (LeRoith et al., 1991). While circulating levels of IGF-I in response to a variety of stimuli are relatively easy to measure, the assessment of cellular IGF-I binding usually necessitates invasive tissue sampling. Thus, there has been a growing interest in erythrocyte IGF-I binding, particularly in human studies, as a relatively accessible reflection of tissue responses to changes in levels of circulating IGF-I. In addition, erythrocyte IGF-I receptors are well characterized (Polychronakos et al., 1983; Catanese et al., 1986). Although circulating erythrocytes have no nuclei and are incapable of multiplication, their binding sites most likely represent the binding characteristics of competent precursor cells found in the bone marrow.

In this study we focused on the physiological observation that circulating IGF-I levels differ between two healthy human populations, namely younger and older men in whom IGF-I levels are relatively high and low, respectively. The lower IGF-I levels in the elderly is currently felt to reflect diminished pituitary GH secretion (Corpas & Harman, 1993) and, consequently, reduced hepatic production of circulating IGF-I. We hypothesized that erythrocyte IGF-I binding would be greater in elderly subjects, reflecting the well-described phenomenon of ligand-mediated receptor upregulation.
It is becoming increasingly apparent that IGF-I tissue binding need not reflect only IGF-I receptor function. IGF binding proteins (IGFBPs) can bind to IGF-I with affinities equal to or greater than those exhibited by the IGF receptors; moreover, IGFBP-1 and -2 possess cell membrane integrin-binding domains (RGD sequence) that allow them to associate strongly with the surface of cells. There is also evidence that membrane-associated IGFBPs-3 and -5 play a role in the bioactivity of IGF-I (Conover, 1992; Mohan et al., 1995b). Recent studies have demonstrated that binding due to IGF receptors can be distinguished from IGFBPs by using competitive binding studies in which both native IGF-I and desaminated IGF-I [des-(1-3)IGF-I] are used to displace [125I]-IGF-I (Hsu & Olefsky, 1992). Native IGF-I will bind to both IGFBPs and IGF receptors while des-(1-3)IGF-I binds only to the receptors; thus, the difference in binding parameters using the two forms of IGF-I can be used to quantify the role of IGFBPs in tissue IGF binding responses. We present here the results of such studies from a group of 15–19-year-old men and 61–68-year-old men.

Materials and methods

Subjects

We studied 9 healthy adolescent males 15–19 years of age (all Tanner V), and 9 healthy adult males 65–68 years of age. Two additional subjects aged 21 and 71 years old were recruited only for the affinity cross-linking study. The subjects were not taking any chronic medications for serious heart, lung or metabolic diseases. All subjects and parents of subjects less than 18 years of age signed an informed consent form before participation. The study was approved by the Human Subjects Committee of Harbor–UCLA Research and Education Institute and the University of Connecticut Health Center.

Hormones and chemicals

Human recombinant insulin was obtained from SIGMA (St Louis, MO, USA) and human recombinant IGF-I from Bachem (Torrance, CA). Labelled [125I]-IGF-I with specific activity 2000 Ci/mmol was purchased from Amersham (Arlington Heights, IL). Des-(1-3)IGF-I was obtained from GroPep (Adelaide, Australia).

Separation of erythrocytes

Specimens were obtained between 0700 h and 1100 h in the non-fasting state in heparinized tubes. Haemoglobin, haematocrit and reticulocyte and erythrocyte counts were determined by standard techniques. The blood was centrifuged for 10 minutes at 400 g at 22°C and the plasma layer was discarded. The cells were mixed with 2 volumes of phosphate buffered saline (PBS), pH 7.4, applied to a Ficoll-Hypaque gradient and centrifuged for 30 minutes at 400 g at 22°C. Then the erythrocytes were washed at 4°C twice with 0.01 m PBS and once with Gambhir buffer (Gambhir et al., 1978), pH 8.0.

Binding studies

A total of 400 μl of erythrocytes were incubated at 4°C for 18–24 h with 50 μl of [125I]-IGF-I containing 30–50 kcpm/tube with the addition of 50 μl unlabelled IGF-I at varying concentrations (0–1000 μg/l). This procedure was also performed using varying concentrations of unlabelled des-(1-3)IGF-I. After incubation, 200 μl aliquots of the red cell suspension were transferred to chilled microfuge tubes containing 200 μl of dibutyl-phthalate and 100 μl of Gambhir’s buffer. After microcentrifugation at 8000 g for 3 minutes the supernatant was removed and discarded. Radioactivity of the pellets was measured.

Data from competition experiments were analysed using LIGAND (Monson & Rodbard, 1980; Monson, 1992), an iterative curve-fitting set of algorithms developed by P. Monson (NIH, Bethesda, MD, USA) which estimates the affinity constant (Kd), its inverse (Ko), and binding capacity. The LIGAND package provides a statistically validated, appropriately weighted, least-squares curve fitting algorithm with objective measurement of goodness of fit to estimate the characteristic binding parameters. The model has the advantage over graphical solutions to ligand-receptor problems by actually fitting exact equations that describe the ligand-receptor interaction. The number of binding sites per erythrocyte was then calculated using the binding capacity and the erythrocyte count.

RIA for IGF-I and IGFBPs 1–5

RIA s are currently used in our laboratory for human IGF-I with antibodies provided by the NIH and standards purchased from Bachem. The intra-assay coefficient of variation is 3.3% and inter-assay coefficient is 5.4%. We used the acid–ethanol extraction method (Daughaday et al., 1987) to extract IGF-I from binding proteins.

IGFBPs 1–3 were measured using commercially available kits (Diagnostic System Laboratories Inc., Webster, TX, USA). IGFBP-4 and -5 were measured in our co-author’s laboratory (SM), as described recently (Mohan et al., 1995a; Honda et al., 1996). For IGFBP-1, inter-assay coefficient of variation was 1.7–6.7% and intra assay coefficient of variation was 2.4–4.5%. Assay sensitivity is 0.11 μg/l. For IGFBP-2, inter-assay coefficient of variation was 6.4% and intra-assay coefficient of variation was 6.5%. Assay sensitivity is <0.6 μg/l. For
IGFBP-3, inter-assay coefficient of variation was 0.6–1.9% and intra-assay coefficient of variation was 1.8–3.9%. Assay sensitivity is 0.5 μg/l. For IGFBP-4 inter-assay coefficient of variation was <8.1% and intra-assay coefficient of variation was <5%. Assay sensitivity is <0.5 μg/l. For IGFBP-5 inter-assay coefficient of variation was <8% and intra-assay coefficient of variation was <4%. Assay sensitivity is <5 μg/l.

**IGFBP-3 proteolysis**

IGFBP-3 protease activity was measured as described by Lamson (Lamson et al., 1991).

**Affinity cross-linking**

We adapted the methodology described for fibroblasts by Siebler et al. (Siebler et al., 1995). Erythrocytes were isolated from whole blood by centrifugation, washing and resuspension in phosphate-buffered solution (PBS). The blood suspension was layered onto histopaque-1077 (Sigma; St Louis, USA), centrifuged at 3000 g for 30 minutes, and the erythrocytes isolated. The cells were suspended in PBS containing 1% Triton X-100, 0.1% bovine serum albumin (BSA), 2 mM sodium benzoyl-l-arginine ethyl ester, and 0.1 mM phenylmethylsulphonyl fluoride. Protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL, USA). [125I]IGF-I (60 000 cpm: Amersham, Arlington Heights, IL, USA) was incubated with the cells (125 000 cpm: Amersham, Arlington Heights, IL, USA) was incubated with the cells (125 μg) in 100 μl of the same buffer in the absence or presence of unlabeled IGF-I (500 ng/ml) for 16 h at 4°C. The receptor and IGF-I were cross-linked by the addition of disuccinimidylsuberate (Pierce) in dimethylsulphoxide to a final concentration of 10 mM. After 60 minutes at room temperature, the reaction was terminated by the addition of reducing Laemmli sample buffer and boiling. Electrophoresis was performed using 4–15% gradient gels. Human gingival fibroblasts, kindly provided by Dr Quiang Zhu, were used as a positive control as described by Siebler et al. (1995).

**Statistical analysis**

Differences between the two groups were analysed using an independent t-test. Within each group, differences between the native IGF-I and des-(1-3)IGF-I experiments were analysed using a paired t-test. Standard techniques of linear regression were used to determine correlation coefficients between certain variables. Results are expressed as mean ± SE. A P-value less than 0.05 was considered significant.

**Results**

**Hematocrit and erythrocyte counts**

There was no difference between the younger and older men with respect to haematocrit (0.43 ± 0.03 vs. 0.44 ± 0.03, respectively) and erythrocyte counts (5.0 ± 0.1 × 10⁹/mm³ vs 4.8 ± 0.1 × 10⁹/mm³, respectively). The corrected reticulocyte count was increased in the older men (2.2 ± 0.3%) compared to the younger men (1.4 ± 0.2%, P < 0.01). In a previous study by Morris et al. (1989), there was no correlation between specific binding and reticulocyte count in the range of 0.3–3.0%.

**Serum IGF-I and IGFBPs 1–5**

Serum levels of IGF-I were significantly higher in the younger (355 ± 24 μg/l) compared with the older men (173 ± 13 μg/l, P < 0.001). There were no significant differences between younger and older subjects for IGFBP-1 (8.1 ± 2.6 μg/l and 8.3 ± 2.3 μg/l, respectively) or IGFBP-2 (109 ± 13 μg/l and 120 ± 1 μg/l, respectively). Serum levels of IGFBP-3 were significantly higher in the younger men (5220 ± 170 μg/l vs. 3800 ± 220 μg/l, P < 0.001). IGFBP-4 was significantly lower in the younger subjects (199 ± 15 μg/l) compared with older subjects (308 ± 31 μg/l, P < 0.009), while IGFBP-5 was significantly higher in the younger (357 ± 15 μg/l) compared with the older subjects (291 ± 14 μg/l, P < 0.006). Finally, although substantial IGFBP-3 proteolysis was noted in pregnant female control serum, there was no evidence of IGFBP-3 proteolytic fragments in any of the younger or older subjects.

**Table 1** Erythrocyte binding affinity (K_d) and binding capacity in younger and older men

<table>
<thead>
<tr>
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<th>K_d (l/mol)</th>
<th>Binding capacity (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Younger subjects</td>
<td>Older subjects</td>
</tr>
<tr>
<td>Native IGF-I</td>
<td>2.1 ± 0.3 × 10⁸</td>
<td>1.4 ± 0.4 × 10⁸*</td>
</tr>
<tr>
<td>des-(1-3)IGF-I</td>
<td>1.5 ± 0.2 × 10⁸</td>
<td>1.7 ± 0.2 × 10⁸*</td>
</tr>
</tbody>
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* P < 0.05 compared to younger subjects. † P < 0.05 compared to native IGF-I experiment.

Binding experiments

Binding capacity (Table 1) and binding sites per erythrocyte were inversely correlated with serum IGF-I levels (in mg/l) \( y = -0.097x + 56.0; r = -0.62, P < 0.0001 \). Correlations were not significant within the younger and older subjects considered as separate groups.

Affinity cross-linking experiments

The IGF-I receptor \( \alpha \)-subunit (135 kD) was present and unlabelled IGF-I competed for binding sites (Fig. 4). Both the younger and older subject had the subunit present on their erythrocytes. No other obvious cross-linked moiety was
observed. Densitometric analysis revealed that the cross-linked IGF-I receptor $\alpha$-subunit signal was about 20% less intense in the older man compared with the younger man.

Discussion

We found a substantial increase in erythrocyte IGF-I binding in the older compared with younger males. This increase in erythrocyte binding was observed along with the expected reduction in circulating IGF-I. In addition, haematocrit and erythrocyte counts were the same in the older and younger men despite the reduced circulating IGF-I in the older subjects.

It is unlikely that erythrocyte IGF-I binding plays a physiological role in the non-nucleated circulating red cell. However, erythrocyte IGF-I binding may indicate receptor-ligand interactions in erythrocyte precursor cells. For example, an inverse correlation between circulating IGF-I and erythrocyte binding has been observed in very different clinical situations. Eshet et al. (1991, 1993) noted increased erythrocyte IGF-I binding in Laron dwarfism, a syndrome characterized by low circulating IGF-I, but when circulating IGF-I is increased (by treatment with GH), Mandel et al., (1995) observed reduced erythrocyte binding. Such observations have usually been interpreted as resulting from ligand-mediated regulation of erythrocyte precursor IGF-I receptors.

It is important to note, however, that not all studies of erythrocyte IGF-I receptors are consistent. Acquafredda et al. (1988), for example, showed a strong positive correlation between specific binding and age in a group of 13 children aged 8 months–11 years. The authors noted that the apparent increase in erythrocyte IGF-I binding occurred at a time when circulating IGF-I was also increasing, and this observation is not consistent with ligand-mediated autoregulation. In contrast, Morris et al. (1989) found an increase in binding affinity in prepubertal children (aged 3–10 years old) compared with adults (aged 20–47 years old), but there was no difference in receptor concentration between the two groups.

Discrepancies also exist in the number of erythrocyte IGF-I binding sites reported by different workers. Our values (ranging from about 20–40 per erythrocyte) are in the same range as Hizuka et al. (1985); slightly lower than those observed by Morris et al. (1989) (about 50–70 per erythrocyte); and higher than those found by Eshet et al. (1991, 1993) (about 2–10). These discrepancies are not readily explainable, but probably relate to differences in analysis of binding data (e.g. Scatchard analysis vs. iterative curve-fitting), as well as to unspecified differences in technique among the various laboratories.

The increased erythrocyte IGF-I binding we observed in the older subjects could be interpreted as a manifestation of ligand-mediated autoregulation of tissue binding. There is also evidence that the increased binding might be physiologically important. Recombinant human IGF-I stimulates erythropoiesis in adult mice (Bechensteen et al., 1994), and Anttila et al. (1994) noted that IGF-I may be involved in the rise of haemoglobin during puberty, a developmental stage marked by large increases in circulating IGF-I. However, in our study there was no apparent haematopoietic effect of the reduced circulating IGF-I in older men whose haematocrit and number of erythrocytes were not different from the younger subjects.

Although our data might be explained by an increase in receptor number, the data comparing displacement of $[^{125}\text{I}]$-IGF-I with native vs. des-(1-3)$[^{125}\text{I}]$IGF-I suggest an alternative
erythrocyte IGF-I receptor. In fact, Kleinman et al. (1995), using \(^{125}\text{I}\)-des-(1-3)IGF-I (Fig. 1). A similar discrepancy was not observed in younger individuals. This suggests that the increased binding observed in the older subjects results from IGFBPs rather than from an upregulation of type 1 IGF-I receptors. In fact, Kleinman et al. (1995), using \(^{125}\text{I}\)-des-(1-3)IGF-I as tracer, recently demonstrated that IGFBPs and not IGF receptors accounted for 95\% of the binding of IGF-I to Ishikawa endometrial cancer cells grown in culture. In our studies, IGFBPs in the older subjects may have accounted for approximately 50\% of erythrocyte IGF-I binding.

It was for this reason that we measured IGFBPs 1–5 in the serum to determine whether or not any of these circulating factors might be responsible for increased erythrocyte IGF-I binding. As noted, IGFBPs 1 and 2 were not affected by ageing. IGFBP-3 was significantly reduced in the older subjects, most probably reflecting reduced GH secretory activity (Sara & Hall, 1990). Moreover, there was no detectable IGFBP-3 proteolytic activity precluding the possibility that IGFBP proteolysis might have played a role in red cell binding. IGFBP-4 was elevated, confirming recent observations about the relationship between IGFBP-4 and age (Honda et al., 1996), but cell culture experiments indicate that IGFBP-4 may actually inhibit the anabolic functions of IGF-I (Honda et al., 1996). Finally, IGFBP-5 does potentiate physiological effects of IGF-I (Mohan et al., 1995a), but we found that IGFBP-5 was reduced in the older subjects. This latter observation also confirms recent investigations of IGFBP-5 in subjects of various ages (Mohan et al., 1995a). In summary, our data confirm that circulating IGFBPs 3–5 are influenced by ageing, but the data do not point to specific role for these BPs as an explanation for the increased erythrocyte IGF binding in the elderly subjects.

Kleinman et al. (1995) in their in vitro studies reported that non-specific binding and other technical difficulties precluded identification and quantification of those IGFBPs that actually accounted for the increased binding. Similarly, we were unable to delineate a specific IGFBP in human erythrocyte samples using standard Western ligand techniques that might account for the increased binding in the older subjects. Thus, which of the IGFBPs may play a role in erythrocyte IGF binding could not be determined.

Although we studied only two subjects, the affinity cross-linking experiments added additional insight. First, we demonstrated the feasibility of cross-linking studies of the IGF-I receptor \(\alpha\)-subunit on human erythrocytes. The position of the erythrocyte IGF-I receptor \(\alpha\)-subunit at 135 kDa corresponded well with previous studies performed on fibroblasts (Siebler et al., 1995). Interestingly, the signal intensity of the 135 kDa moiety was about 20\% less in the older compared with the younger subject, supporting the idea that the increased binding in the older subjects does not necessarily result from upregulation of IGF-I type 1 receptors. However, the cross-linking experiments failed to identify another specific moiety that could account for the increased binding as determined by the competitive inhibition experiments.

The binding affinities \(K_{\text{aff}}\) are, by definition, constants (determined by the equilibrium concentrations of binding sites and bound and unbound ligand) and are characteristic of the interaction between a particular ligand and its receptor or binding protein. However, we found significantly different \(K_{\text{aff}}\)s between young and old subjects and, in the older subjects, between native and des-(1-3)IGF-I (see Table 1). These differences are consistent with the idea that in younger subjects, IGF-I binds primarily to IGF-I receptors on erythrocytes while in older subjects IGF-I binds to both receptors and IGFBPs. The implication of the statistically significant differences in \(K_{\text{aff}}\) between younger and older subjects is that there may be some effect of ageing on the structure of the IGF-I receptor. Whether or not this statistical difference is biologically important has yet to be determined.

Our data, along with previous studies, suggest that changes in erythrocyte IGF-I binding occur at different stages throughout life. As noted above, Morris et al. (1989) found an increase in binding affinity in prepubertal children compared with adults, but there was no difference in binding site concentration between the two groups. Like prepubertal children, the older adults in our study had lower levels of circulating IGF-I than did the comparison group (young adults, roughly, in both studies). It appears that despite reduced pituitary GH secretion in older adults certain tissues, in this case erythrocytes, maintain the ability to adapt to an environment in which circulating IGF-I is reduced. Interestingly, this adaptation may be mediated by IGFBPs rather than by IGF receptors.

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