Expression of Growth Hormone Receptor, Insulin-Like Growth Factor 1 (IGF-1) and IGF-1 Receptor mRNA and Proteins in Human Skin

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A cDNA corresponding to the membrane receptor for growth hormone (GH) was amplified by polymerase chain reaction (PCR) directly from human skin. The cDNA was cloned and found to have complete sequence homology to the extracellular domain of human liver GH receptor (GH-R). Northern analysis, using the cloned GH-R as probe, revealed relatively higher levels of GH-R transcripts in cultured human dermal fibroblasts compared to cultured keratinocytes. Semi-quantitative PCR analysis indicated that the level of GH-R mRNA in cultured melanocytes was similar to that in fibroblasts. The receptor protein encoded by GH-R mRNA in fibroblasts was shown by affinity cross-linking to have an apparent M, of 115-120 kDa, similar to that of 3T3-F442A fibroblasts used as a control. mRNA transcripts for the major mediator of GH actions, insulin-like growth factor 1 (IGF-1), were detected by PCR in fibroblasts, melanocytes, and keratocyte biopsies, but not in keratinocytes. In contrast, IGF-1 receptor mRNA were abundant in cultured keratinocytes and skin biopsies, as determined by Northern analysis. IGF-1 but not GH (5-50 ng/ml) promoted clonal proliferation of cultured keratinocytes. In contrast, GH (10 ng/ml) after 5 d markedly increased fibroblast cell numbers (70%, p < 0.009) over 0.2% serum control. These data indicate that human skin cells possess the molecular elements necessary to respond to GH and raise the possibility that GH may influence skin growth in vivo.

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Keratinocyte Cultures and Growth Assays Primary cultures of human KC were derived from keratome biopsies and subcultured in MCDM 153 medium modified for high-density growth [16] supplemented with 0.15-mM Ca++, hydrocortisone (HC, 0.5 μg/ml), bovine pituitary extract (BPE, 0.4%), insulin from bovine pancreas (5 μg/ml), and epidermal growth factor (EGF, 10 ng/ml) from murine salivary gland. KC cultures were passaged when 60% confluent, approximately 7 × 10⁸ cells/cm², and used between passages 2 and 5. Cell replacement strategies were conducted using the clonal growth assay [17]. Cells growing in the above medium were trypsinized and seeded at 2 × 10⁸ cells/cm² in 35-mm dishes and allowed to attach and grow for 48 h. At this time the medium was replaced and cells were trypsinized and counted in a hemocytometer. FB were allowed to grow for 3 or 5 d at which time they were trypsinized and counted in a hemocytometer.

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been shown to hybridize to human IGF-1 sequence [21]. The GH-R cDNA probe was a PCR fragment amplified in this work directly from epidermal biopsies. This fragment was gel purified using the Geneclean II kit (BIO 101, La Jolla, CA), cleaved using BamHI and ligated using T4 DNA ligase [22] into the BamHI-digested pGEM3Z plasmid (Promega, Madison, WI). The β-globin probe used for DNA hybridization was also a PCR product. DNA sequence analysis and comparison of cloned genes were carried out as previously described [22].

Affinity Cross-Linking Studies in Fibroblasts and Keratinocytes FB were cultured as above whereas 3T3-F442A cells were grown in 100-mm dishes as described previously [20]. Cells were washed, switched, and maintained in DMEM containing 0.1% BSA for 12–18 h prior to cross-linking experiments. KC were grown as above to 60–80% confluency and then switched to modified MCDB medium lacking HC, BPE, insulin, and EGF for 48 h. Cross-linking experiments were performed as previously described [20]. Briefly, after rinsing cells in KRP buffer (0.12 M NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 10 mM NaPO₄, pH 7.4), binding was carried out at 4°C for 6 h in the same buffer containing 1% BSA and 30 ng/ml [¹²⁵I]-human GH (NEN-DuPont, Wilmington, DE) (for FB and KC) and 10 ng/ml (3T3-F442A) cells. These conditions were chosen because lower concentrations of iodinated GH did not produce reproducible results in FB. After washing with cold KRP plus 1% BSA, cross-linking was performed for 15 min at 4°C in KRP buffer to which was added 1 mM diisuccinimidyl suberate (DSS, Pierce Chemical Co., Rockford, IL), freshly prepared in DMSO. Cell lysates were prepared by detaching monolayers in the presence of a solution containing protease inhibitors, 25 mM HEPES buffer, 0.1% Triton X-100, and 0.5 mM DTT, and electrophoresed in 8% polyacrylamide gels with a tris-glycine discontinuous buffer system [20].

RESULTS

Expression of GH-R mRNA and Protein in Human Skin A 720–base pair (bp) DNA fragment was obtained from human skin RNA by reverse transcription using GH-R primers 1 and 3. This DNA fragment was re-amplified with a nested (internal) 5'-primer (GH-R primer 2, nucleotides 270–289) and primer 3 yielding the expected fragment of 523 bp, which also included the 20 bp BamHI-adaptor sequence (results not shown). The PCR fragment was cloned into pGEM3Z and digested with BamHI resulting in the release from the plasmid vector of a 503-bp insert whereas BamHI/EcoRV and BamHI/AvaII digests each yielded the predicted subfragments (data not shown). The skin-derived PCR product was partially sequenced and exhibited 100% homology to the nucleotide sequence of human liver GH-R [5] (results not shown).

PCR analysis of RNA isolated from cultured cells identified FB, melanocytes (MC), and KC as three sources of GH-R RNA transcripts in skin (Fig 1A). Liver is the positive control. Cultures of KC, however, were often found to contain 2–4% MC that might have contributed to the GH-R mRNA signal detected in these cells by PCR. Therefore, Northern analysis and semi-quantitative PCR were performed to determine the relative abundance of GH-R mRNA in the cultured cells and keratome biopsies. A single GH-R mRNA of 5.3–5.5 kb was detected in poly (A)⁺ RNA that was most abundant in FB, but present only at very low levels in KC or skin biopsies (Fig 1B). No mRNA for GHBP, which is present in rat and mouse liver is the positive control. Cultures of KC, FB, and skin, or total RNA (40 µg) from skin. The absence of a signal in the lane containing total RNA indicates that the signal was not due to cross-hybridization of GH-R cDNA to 28S ribosomal RNA (B). The same RNA blot was probed with cyclophilin cDNA probe, as an internal control for gel loading. Positions of 28S and 18S ribosomal RNA markers are shown. C) Semi-quantitative PCR determination (average of three measurements) of GH-R mRNA in FB, MC, and KC. Results are normalized to exogenous input β-globin mRNA as described in Materials and Methods.

Figure 1. GH-R gene expression in human skin. A) GH-R mRNA was detected by PCR in cultured fibroblasts (FB), melanocytes (MC), keratinocytes (KC), skin, and liver (control). B) Northern hybridization of [³²P]-labeled GH-R probe to poly (A)⁺ RNA (5 µg) from KC, FB, and skin, or total RNA (40 µg) from skin. The absence of a signal in the lane containing total RNA indicates that the signal was not due to cross-hybridization of GH-R cDNA to 28S ribosomal RNA (B). The same RNA blot was probed with cyclophilin cDNA probe, as an internal control for gel loading. Positions of 28S and 18S ribosomal RNA markers are shown. C) Semi-quantitative PCR determination (average of three measurements) of GH-R mRNA in FB, MC, and KC. Results are normalized to exogenous input β-globin mRNA as described in Materials and Methods.
mRNA were detected in KC by this method (Fig 1C), consistent with the Northern analysis (Fig 1B).

We next examined by affinity cross-linking the presence of GH-R protein in cultured human KC and FB. In intact FB monolayers and control 3T3-F442A cells, known to express the membrane GH-R [20], [125I]-GH (10 or 30 ng/ml) formed a complex with GH-R of M, 135–140 kDa (lane 1 in Fig 2A,B) that was specifically reduced by a hundredfold (1 or 3 μg) excess unlabeled human GH (lane 2 in Fig 2A,B). This indicated a protein of about M, 115–120 kDa for GH-R in both cell types if the M, of iodinated GH was subtracted. In contrast, no evidence for expression of GH-R protein was observed in cultured KC (lanes 1 and 2, Fig 2C), even under conditions where IGF-IR was readily cross-linked (data not shown). The cross-linking results were compatible with measurements of GH-R mRNA levels, indicating that FB and MC, but not KC, expressed GH-R. This also suggests that FB and MC could potentially respond to GH by IGF-1 production.

**Pattern of IGF-1 mRNA Expression in Skin** Expression of transcripts for IGF-1, the major mediator of GH effects, was next examined in skin cells. Using exon-specific primers, IGF-1 mRNA was detected by PCR in human dermal FB, skin (n = 3) and liver but not in KC cultures from three individuals (Fig 3A). Cultured KC were also negative for IGF-1 mRNA expression by Northern blotting (data not shown), which further substantiated the PCR data. In a second PCR experiment depicted in Fig 3B, IGF-1 mRNA expression was demonstrated in FB from a different individual, MC, skin, and liver. The integrity of KC RNA samples shown in Fig 3A,B was confirmed by PCR amplification of cyclophilin [29], as shown. The presence of GH mRNA transcripts in skin was investigated by two degenerate PCR primers. However, no mRNA for GH was found in normal human skin, although it was detected in bovine pituitary RNA (Fig 3C). Because the results indicated that IGF-1 was produced in skin cells, we next examined expression of receptors for IGF-1 in FB, KC, and skin biopsies.

**IGF-1 Receptor mRNA Expression** IGF-1R mRNA was detected by PCR in FB, KC, and skin (Fig 4A). Liver expressed very low levels of IGF-1R transcripts, consistent with a previous report [4]. Because the physiologic role of IGF-1 is in part determined by the distribution and concentration of its receptors on target cells [4],
Figure 4. IGF-1R gene expression in human epidermal cells. A) IGF-1R mRNA was identified by PCR in FB, KC, and skin, and in lesser amounts in liver RNA. B) Relative abundance and size of IGF-1R transcripts were determined by Northern analysis of 50 μg of total RNA per lane. Multiple transcripts of approximately 3 kb, 7 kb, and 11 kb were detected in FB, KC, and skin (n = 3). Abbreviations as in Fig 1.

Figure 5. Effects of GH on proliferation of cultured human FB and KC. A) FB were maintained in DMEM plus 0.2% serum for 48 h prior to the indicated treatments on day 1. Cells were counted on day 3 (open bars) and day 5 (solid bars). Cell numbers are expressed as percentage increase over serum control (solid bars). After 5 d, FB cell numbers increased by about 70% (p < 0.009) over serum control (Fig 5A, solid bars). GH at higher doses (50 ng/ml) had little or no effects on FB growth beyond that achieved by 0.2% serum alone (Fig 5A). Recombinant GH at 5–50 ng/ml (data for 20 ng/ml shown) had no effect in stimulating clonal growth of epidermal KC. In contrast, either insulin at 50–5000 ng/ml or IGF-1 at 0.5–50 ng/ml, used as control (Fig 5B, data for 5 ng/ml IGF-1 and 500 ng/ml insulin shown), promoted clonal growth of KC cultures, consistent with previously published data [31]. GH in combination with either insulin or IGF-1 also did not produce any significant effect beyond that seen with either insulin or IGF-1 alone as shown (Fig 5B), or at several other concentrations used (data not shown).

DISCUSSION

GH receptor gene expression has been previously demonstrated in epiphyseal chondrocytes and osteoblast-like cells in vitro [12,13] and various rat tissues in vivo [6], but not in skin. Thus this report constitutes the first direct evidence for expression of GH-R in human skin in vivo and cultured human MC and dermal FB in vitro.

FB and MC cultures used in these studies were essentially free of other cell types as the few KC initially present in the primary cultures differentiate on subculturing in the media used to propagate FB and MC and thus are subsequently eliminated. KC cultures,

Effects of GH and IGF-1 on Epidermal Cell Growth The ability of GH to stimulate growth of dermal FB shown to express GH-R was next examined in vitro. In the absence of serum, GH at several concentrations had no effects on cell numbers or thymidine incorporation into DNA (data not shown), in agreement with previous reports [30]. However, addition of GH at 10 ng/ml in the presence of 0.2% serum stimulated cell replication after 3 d by about 21% over serum control (Fig 5A, open bars). After 5 d, FB cell numbers increased by about 70% (p < 0.009) over serum control (Fig 5A, solid bars). GH at higher doses (50 ng/ml) had little or no effects on FB growth beyond that achieved by 0.2% serum alone (Fig 5A).
however, may contain 2–4% MC, whose RNA may significantly contribute to the GH-R signal detected by PCR and Northern analysis in cultured KC. Judged by the low GH-R mRNA levels in KC cultures and epidermal biopsies, which are themselves composed of 95% KC, we conclude that human KC express probably little if any GH-R transcripts. The size of the receptor mRNA in FB and skin samples was similar to that reported for the membrane-bound GH-R mRNA species (4.8–5.1 kb), which mediates GH action in liver [5]. Other investigators have also observed a 5.2-kb GH-R transcript in human skin (Dr. M.J. Waters, University of Queensland, Queensland, Australia, personal communication). We have shown that both GH-R mRNA and protein are expressed in FB consistent with the expression of membrane-anchored GH-R previously reported in other cell types [5,20]. In addition, we have directly demonstrated for the first time that the size of membrane-bound GH receptor is approximately 120 kDa in human dermal FB.

GH-R/GHBP was previously immunolocalized in mammalian skin [14]. However, the antibodies utilized in that study recognized both the binding protein and membrane-bound receptor [32], making it impossible to determine the exact nature of the GH-R in skin [14]. On the other hand, based on the specificity of the PCR primers, the size of the single GH-R mRNA in Northern blots and the size of cross-linked receptor on SDS gels, we conclude that human skin expresses the membrane-bound GH-R, which presumably is involved in mediating direct and indirect effects of GH. Previous work [30] has shown that GH in vitro is mitogenic within its physiologic plasma concentration (10–30 ng/ml) [1,8]. We also found that GH at 50 ng/ml did not stimulate FB proliferation in vitro. This refractoriness to higher doses of GH has been attributed to the increased rate of GH-R internalization, which is believed to attenuate GH responsiveness in target cells [28].

GH effects are mediated primarily by IGF-1 production [30,33] and the ability of GH to induce IGF-1 production in human FB has been previously demonstrated [30], although this issue was not directly addressed in the present study. In addition to FB, we found IGF-1 mRNA transcripts in MC, distinct pigment forming dendritic cells in human skin. KC comprise close to 95% of the cells in human skin and the ability to proliferate is intrinsic to their basal population, which lie immediately adjacent to FB and MC. Because both FB and MC express GH-R and have the ability to express IGF-1 mRNA, one can speculate that resident MC and dermal FB, as shown here, or Langerhans cells [34], by virtue of their potential for IGF-1 production, have the capacity to influence growth of epidermal KC in a paracrine fashion. In support of our findings, immunoreactive IGF-1 has been recently demonstrated in Langerhans cells in UV-irradiated mouse epidermis [34].

In agreement with a previous report [31], we found IGF-1 to be a potent stimulator of KC growth. Our data also suggest that in KC in vitro, the effects of IGF-1 are mediated mainly through its own receptors rather than through insulin receptors. This conclusion is compatible with the extremely low-level binding obtained when iodinated insulin has been used in affinity cross-linking studies in epidermal KC [31]. Further, the fact that on a molar basis insulin is nearly a hundredfold less active in stimulating KC growth than IGF-1 [21,31] lends support to our notion. This is also consistent with the recent immuno-localization of IGF-1R to basal layers in human epidermis [21,35].

It is evident that regulation of KC proliferation in skin is under the control of a vast array of intrinsic and extrinsic factors produced locally with short-distance paracrine or autocrine effects [36]. Given the pivotal location of FB and MC in skin, their ability to produce IGF-1 may therefore have important implications in regulation of epidermal growth, inflammatory conditions, and cutaneous wound healing. Although systemic IGF-1 may play a critical role in the proposed Ahprome, our results provide circumstantial evidence that GH may regulate skin growth through local production of IGF-1. The fact that GH is produced and secreted by immuno-regulatory cells [37] emphasizes a potential role for GH/IGF-1 axis in tissues other than classical endocrine organs. The extent to which the molecular elements identified here actually contribute to normal and abnormal cutaneous growth in vivo is currently under investigation.

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