Extra-pituitary growth hormone in peripheral tissues of early chick embryos

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Abstract

Early embryonic growth is independent of pituitary growth hormone (GH), since it occurs prior to the differentiation of pituitary somatotrophs. Embryogenesis is therefore thought to be regulated by local growth factors. As GH is now known to be produced in many extra-pituitary sites, in which it acts in an autocrine or paracrine manner, the possibility that extra-pituitary GH may participate in embryogenesis and organogenesis was assessed by determining the immunocytochemical presence and location of GH- and GH-receptor (GHR)-like proteins in the peripheral tissues of chick embryos during their 21-day incubation period.

Immunoreactive (IR)-GH, detectable by a monoclonal and two polyclonal antibodies for chicken GH, was specifically and ubiquitously present in tissues of 3-day-old embryos. At embryonic day (ED) 5, IR-GH was widespread in ectodermal, mesodermal and endodermal tissues,

but it was not present in every cell of each tissue. IR-GH was particularly abundant in the neural tube, notochord, limb bud, somites, heart, stomach, liver, kidney, Wolffian duct and the amnion. By ED8, IR-GH was still widespread and was now present in limb bud cartilage, although the heart and liver were no longer GH immunoreactive. GH receptor immunoreactivity was also present in most tissues and cells of ED3-ED8 embryos.

These results demonstrate that extrapituitary GH is abundantly present during early embryogenesis, prior to the differentiation of pituitary somatotrophs (at ED12). Since GH- and GHR-like proteins are present in most tissues of the chick embryo, it is proposed that extra-pituitary GH may act as a local growth factor during embryonic development.

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Introduction

Growth hormone (GH) is obligatory for postnatal growth (Scanes & Daughaday 1995), but embryonic or fetal growth is thought to reflect a 'growth-without-GH' syndrome, independent of pituitary GH (Geffner 1996). The late ontogeny of pituitary somatotrophs and pituitary GH secretion during incubation and gestation (Hill 1992, Thommes & Woods 1993, Porter 1997) and the inability of exogenous GH to promote embryonic or fetal growth (Hill 1992, Thommes *et al.* 1992), supports this view. It is, therefore, thought that early embryonic or fetal growth is regulated by local growth factors rather than by endocrine secretions (Geffner 1996).

The anterior pituitary gland is the predominant site of GH secretion postnatally, although numerous extra-pituitary target sites (e.g. neural, immune and reproductive tissues) are also sites of GH production (Harvey & Hull 1997). Extrapituitary GH is not released into the systemic circulation, but acts locally in autocrine or paracrine ways (Harvey & Hull 1997). It is therefore possible that extrapituitary GH may act as a local growth factor in

early embryonic or fetal development. This possibility is supported by the recent demonstration of GH mRNA and GH immunoreactivity in murine blastocysts prior to implantation and cellular differentiation (Pantaleon *et al.* 1997). It is, however, not known if GH is present in early embryonic tissues during embryogenesis or organogenesis. This possibility has, therefore, been addressed in the present study, in which GH immunoreactivity has been determined in chick embryos, in which the ontogeny of organogenesis is well described.

Materials and Methods

Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were incubated at 37.5 °C in humidified air (Hamburger & Hamilton 1951). The eggs were turned one quarter of a revolution each day during incubation. Whole chick embryos at embryonic day (ED) 3, 4, 6, 7, and 8 were dissected into phosphate-buffered saline (PBS, pH 7.4). These stages were selected since they cover the

Figure 1 GH immunoreactivity in the adult chicken anterior pituitary gland. Transverse section showing the distribution of immunoreactive somatotrophs in the caudal (Ca) and cephalic (Ce) lobes of the anterior pituitary gland. Bar = 1 mm. Staining was carried out using α cGH1 as the primary antibody.

periods of gastrulation and organogenesis during the 21-day incubation period (Romanoff 1960) and occur well before the ontogenic differentiation of pituitary somatotrophs (at ED12; Jozsa *et al.* 1979, Malamed *et al.* 1993, Porter *et al.* 1995).

Immunocytochemistry

Tissues were fixed in freshly prepared paraformaldehyde (4% w/v) (Sigma, Mississauga, Ontario, Canada) or Bouin's fixative, overnight, at 4 °C. Tissues were then dehydrated in a graded series of alcohol (50%, 15–30 min; 70%, 30–60 min; 95%, 30–120 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24–48 h at 60 °C, under normal atmospheric pressure. Serial transverse (4–8 μ m) sections were taken using a microtome and mounted on treated slides (Fisher Scientific). For consistency, most of the sections were taken from the mid region of the embryos.

Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlingame, CA, USA; Sigma) using the avidin-biotin-peroxidase (ABC) (Hsu *et al.* 1981) or the alkaline phosphatase, anti-alkaline phosphatase (APAAP) (Sternberger 1979) method. Sections were incubated with specific polyclonal antisera raised in rabbits against native chicken (c) GH (α cGH1: Harvey & Scanes 1977) or recombinant cGH (α cGH2: Porter *et al.* 1995). Both of these antibodies were diluted 1:4000 in PBS or in 1–5% normal goat serum (NGS) overnight, at room temperature. A mouse mono-

clonal antiserum raised against glycosylated chicken growth hormone (IH7; Berghman *et al.* 1987) was also used at 1:1000, diluted in NGS or PBS. After incubation, the slides were washed 3 times for 15 min in PBS. Sections were then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (IgG) (Sigma, 1:500) or in a biotinylated anti-mouse IgG (Sigma, 1:500). The slides were washed in PBS and incubated in ABC reagent for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (DAB) (Sigma), which resulted in a brown coloration. Nickel chloride (Sigma) was sometimes added to the DAB, resulting in a black precipitate. The specificity of staining was determined by preabsorbing the GH antisera with recombinant cGH (Amgen, Thousand Oaks, CA, USA; 1 mg/ml) for 1 h prior to section incubation. Antibody specificity was also shown by cross-reactivity with chicken somatotrophs (Fig. 1). Non-specific staining was determined by replacing GH antisera with pre-immune rabbit serum. Other controls included the omission of the secondary antibody and the replacement of the primary antibody with PBS.

The APAAP technique involved the use of a secondary antibody conjugated with alkaline-phosphatase (Sigma). After incubation with the secondary antibody, the sections were washed in PBS and incubated with 10% APAAP, for 1 h at room temperature. The sections were then washed, and Fast Red TR/Napthol As-MX (Sigma) was applied to tissue sections until optimum color development.

GH receptor (GHR) immunoreactivity was detected using a polyclonal antibody (at a concentration of 1:400)

raised in rabbits against recombinant chicken GH binding protein (rcGHBP) (Huang *et al.* 1993, Hull *et al.* 1996), using the ABC technique, as detailed above. The specificity of staining was demonstrated by replacement of the primary antibody with non-immune rabbit serum (NRS) or PBS. In previous studies (Hull *et al.* 1996), the specificity of staining was also established by the ability of excess rcGHBP or homogenates of COS cells expressing cGHBP to completely block its immunostaining. Since cGHBP is the extracellular domain of the cGHR, the primary antibody recognizes GHR/GHBP immunoreactivity.

Polymerase chain reaction

The presence of GH mRNA was assessed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA in pooled tissue of whole ED2 embryos and in the headless bodies of ED6 and ED8 embryos was reversed transcribed in the presence of 10 pmol of the oligodeoxythymine GH primer, khu 14, 5'-GACTCAGTC GACATCGTTT TTTT TTTT TTTT-3' (Nucleotide Synthesis Laboratory, University of Alberta), 5 × RT buffer (Promega, Madison, WI, USA), excess deoxynucleotides (10 nM each of deoxy-ATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Quebec, Canada) and 10 mM dithiothreitol. The mixture was incubated at 42 °C (1 h) and the generated cDNA was diluted in 480 µl double-distilled water. For comparative purposes, reverse transcribed RNA from adult chicken pituitary glands was used as a positive control. Tissue RNA not reverse transcribed (in the absence of Superscript) served as a negative control. The cDNA was amplified in the presence of oligonucleotide primers GH 3' (antisense) and GH 5' (sense) (GH 3', 5'-GCCTCAGATGGTGCAGTTG CTCTCTCCGAA-3', GH 5', 5'-CGTTCAAGCAA CACCTGAGCAACTCTCCCCG-3', Nucleotide Synthesis Laboratory, University of Alberta), excess deoxynucleotides (1.25 mM of each), 10 × PCR buffer, 25 mM MgCl₂ and *Thermus aquaticus* (TAQ) DNA polymerase (5 U, Promega). The primers were designed to generate a 689 base pair (bp) fragment spanning the entire coding region of the cGH gene (Render *et al.* 1995). The reaction mixture was overlaid with mineral oil, denatured and subjected to 30 cycles of denaturation (92 °C for 1 min), annealing (60 °C for 1 min) and extension (72 °C for 2 min) with a final extension at 72 °C for 10 min, in a thermal cycler (MJ Research, Watertown, MA, Canada). The amplified cDNA was then electrophoresed in an ethidium bromide-stained agarose gel (1.2% wt/vol) and viewed under ultra-violet light (UV).

Results

The distributions of GH and GHR/GHBP immunoreactivity in the tissues of ED3-ED8 embryos are summarized in Tables 1 and 2 respectively.

Table 1 GH immunoreactivity in tissues of early chick embryos

Tissue	Age (embryonic day)				
	3	5	6	7	8
Ectoderm	+++	+++	+++	+++	
Neural tube	+++	+++	+++	+++	
Notochord	+++	+++	+	+	
Mesoderm	+++	+++	+++	+++	
Limb bud					
Mesoderm	—	++	+++	+++	++
Cartilage				+++	++
Somites					
Dermo-myotome	+++	+++	+++	+++	
Sclerotome	+++	+++	+	+	
Heart	+++	+++	+++	++	—
Lung				+++	
Stomach	+++	++	++	++	
Liver	+++	+++	++	++	—
Kidney					
Mesonephros	+++	++	++	++	
Gonadal					
Wolffian duct		+++	+++	++	
Müllerian duct		—	—	+++	
Amnion					
Ectodermal layer	+++	+++	+++	+++	
Mesodermal syncytium					
Cytoplasm		—	—	—	
Nuclei		++	+++	++	

—, no immunoreactivity; +, faint immunoreactivity; ++, moderate immunoreactivity; +++, intense immunoreactivity.

Table 2 GHR/GHBP immunoreactivity in tissues of early chick embryos

Tissue	Age (embryonic day)			
	2	3	4	8
Ectoderm	+++	+++	+++	
Neural tube	+++	+++	+++	
Notochord	+++	+++	+++	
Mesoderm	+++	+++	+++	
Limb bud				
Mesoderm		+++	+++	++
Cartilage			+	+
Somites				
Dermo-myotome	+++	+++	+++	
Sclerotome	+++	+++	+++	
Heart		+++	+++	
Stomach		+++	+++	
Liver		+++	+++	
Kidney				
Mesonephros		+++	+++	
Amnion				
Ectodermal layer	+++	+++	+++	
Mesodermal syncytium	+++	+++	+++	

—, no immunoreactivity; +, faint immunoreactivity; ++, moderate immunoreactivity; +++, intense immunoreactivity.

Figure 3 GH immunoreactivity in the developing kidney and reproductive system of ED5 embryos. (A) Transverse section through the Wolffian duct (Wd). Staining is present in most cells lining the duct (arrowheads). Lighter staining is also seen in scattered cells of the kidney tubules (tu). (B) Preabsorption of α cGH1 with recombinant cGH completely abolished staining. (C and D) Higher magnification of A showing strong staining in the epithelial cells of the Wolffian duct (arrowheads). Note that not all cells lining the duct are immunoreactive for GH. Bars = 100 μ m. e, epidermis; m, mesenchyme. The ABC technique was used with α cGH1 as the primary antibody.

At ED3, GH immunoreactivity was ubiquitous in transverse tissue sections taken through the trunk (Fig. 2A and C) or through the developing heart (Fig. 2E) of the embryo. Incubation with α cGH1 (Fig. 2A, G and H), α cGH2 (Fig. 2C) or α IH7 (Fig. 2E) intensely labeled all tissues at all levels of the ED3 embryos.

However, while most cells in the neural tube, notochord and mesonephric duct were immunoreactive, many mesenchymal cells in the sclerotome had no GH immunoreactivity (Fig. 2G and H). Control sections incubated with NGS or PBS were unstained (data not shown), as were sections incubated with primary

Figure 2 Widespread GH immunoreactivity in the ED3 embryo. GH immunoreactive cells were detected using 2 polyclonal antibodies (α cGH1 and α cGH2) raised against cGH, and a mouse monoclonal antibody (α IH7) raised against glycosylated cGH. These cells were visualized by a biotinylated antibody, avidin-peroxidase complex and DAB (Fig. 2A, E-G) or DAB with nickel ions which produces a black substrate (Fig. 2C, D). (A) Transverse section through the trunk at the level of the mesonephros, using α cGH1 (Harvey & Scanes 1977). (B) Preabsorption of polyclonal α cGH1 with recombinant cGH abolished staining in an adjacent section to A. (C) Transverse section through the trunk at the level of the mesonephros and dorsal aorta using α cGH2 (Porter *et al.* 1995). (D) Preabsorption of α cGH2 with recombinant cGH reduced staining in a similar section to C. (E) Transverse section through the trunk stained with mouse monoclonal α IH7 (Berghman *et al.* 1987). (F) Preabsorption of α IH7 with recombinant cGH abolished most staining. (G) Higher power illustration of immunoreactivity in the neural tube, somite and mesonephric duct, showing widespread labeling. (H) High power illustration of the somitic sclerotome, showing that not all cells of this mesenchymal tissue are immunoreactive. a, amnion; at, atrium; e, ectoderm; en, endoderm; m, mesonephric duct; n, notochord; nt, neural tube; pcv, posterior cardinal vein; s, somite; sc, sclerotome; so, somatopleure; sp, splanchnopleure; st, stomach. Bars = 80 μ m in A, B, C and D; 100 μ m in E and F; 80 μ m in G; 10 μ m in H.

Figure 4 GH immunoreactivity in the ED6 embryo. (A) Transverse section at the level of the wing buds. GH immunoreactivity (α IH7) was widespread throughout the embryo, although absent from the notochord (n). Strong immunoreactivity was seen in the amnion (a), myotome (my), neural tube (nt), limb bud mesenchyme (L), mesonephric tubules (me) and throughout the mesoderm (m) of the body. (B) Preabsorption of α IH7 with recombinant cGH abolished staining. (C) Higher magnification of the mesoderm in section A. Many cells show dense staining for GH although there are cells (arrowheads) which are unstained. (D) Pre-incubation of α IH7 with recombinant cGH did not result in staining of the mesoderm. pcv, posterior cardinal vein; d, dorsal aorta. Bars = 250 μ m (A and B) or 20 μ m (C and D).

antisera preabsorbed with recombinant cGH (Fig. 2B, D and F).

At ED5, GH immunoreactivity was observed in the developing kidney and reproductive system (Fig. 3). The Wolffian duct was more intensely stained than adjacent

tissues (kidney tubules and somatic mesoderm) (Fig. 3A and C), and a single layer of densely stained cells was seen in the Wolffian duct (Fig. 3D). GH immunoreactivity was not, however, present in all of these cells. Preabsorption of α cGH1 with recombinant cGH completely abolished this

Figure 5 GH immunoreactivity in the ED6 extra-embryonic membrane determined using α 1H7 as the primary antibody. (A) The ectodermal layers (e) of the amnion are strongly immunoreactive. (B) Preabsorption of α 1H7 with cGH reduced staining. (C) The mesodermal (m) layer of the amnion is beginning to differentiate from a syncytium into musculature, and the nuclei (n) of these cells are intensely GH immunoreactive. (D) Preabsorption of the α 1H7 with recombinant cGH reduced nuclear staining, but shows that some staining in the ectoderm (e) may be non-specific. (E) Section through the trunk, showing immunoreactivity in the liver (L), ventricle (V), stomach (S) and limb buds (I). (F) Preabsorption abolishes reactivity in a section similar to E. Bars=50 μ m.

Figure 6 GH immunoreactivity in the ED7 embryo. (A) The amnion of the 7-day embryo was intensely GH immunoreactive. Bar = 20 μ m. (B) The ectoderm (e) and myotome (s) were also strongly immunoreactive, although GH immunoreactivity in the mesoderm (m) was restricted to certain cells scattered within the mesodermal tissue (Fig. 6C). (C) The endothelium (en) of blood vessels showed intense GH immunoreactivity. (D) Preabsorption of α CGH1 completely abolished staining in a section similar to C. Bars = 50 μ m (B-D). b, blood cells; nt, neural tube.

staining (Fig. 3B). Similar results were also observed with α CGH2 as the primary antibody (data not shown).

Cells immunoreactive with α IH7 were also widespread in the 6-day embryo (Fig. 4A) with the exception of the notochord. Strong staining was present in the cells of the neural tube, myotome, limb bud mesenchyme, amnion,

developing kidney, and in mesoderm throughout the body. The staining was not, however, in every cell, as indicated by the sparse GH immunoreactivity in embryonic mesoderm (Fig. 4C). Although the irregularly shaped mesodermal cells appear to be homogeneous, some cells had little staining or were unstained (arrows). A similar

Figure 7 GH and GHR/GHBP immunoreactivity in the ED8 limb bud. (A) GH immunoreactivity (determined using α cGH1 as the primary antibody) in cells from the hypertrophic zone of the limb bud cartilage. Some cells show strong cytoplasmic staining (c), while others show strong nuclear staining (n) and others do not appear to be immunoreactive (arrowhead). (B) GH immunoreactivity in the cytoplasm of cells undergoing mesenchymal condensation from the zone of flattened cells. (C) GHR immunoreactivity in cells from the hypertrophic zone. Note the strong nuclear staining and less dense cytoplasmic staining. Some cells do not appear to be immunoreactive (arrowhead). (D) Preincubation of α cGH1 with recombinant cGH completely abolished GH staining in cartilage cells. An adjacent section to A is shown for illustration. Bars=10 μ m (A-D). (E) Low power illustration of a cross-section through the limb bud, showing the distribution of GH immunoreactive chondrogenic cells. Bar=100 μ m.

Figure 8 GHR/GHBP immunoreactivity in the ED3 and ED4 embryo. GHR/GHBP immunoreactivity is widespread at ED3 (A) and ED4 (C) and is particularly prevalent in nuclei (C). Replacement of the primary antibody with NGS abolished ED3 (B) and ED4 (D) immunoreactivity. Bar = 250 μm.

Figure 9 GH mRNA in chick embryos. A 689 bp cDNA is amplified by RT-PCR from RNA extracted from whole ED2 (2) embryos and the headless bodies of ED6 (6) and ED8 (8) embryos, in comparison with adult pituitary (P) RNA. M, molecular markers.

distribution of immunoreactivity was observed when α cGH1 or α cGH2 was used as the primary antibody (data not shown). Preabsorption of the monoclonal antibody (α IH7) with recombinant cGH abolished specific staining in ED6 tissues (Fig. 4B and D).

The ED6 amnion or extra-embryonic membrane showed an interesting pattern of GH immunoreactivity. The ectodermal layers of the membrane were strongly immunoreactive (Fig. 5A). This was not due to non-specific staining, since preabsorption of the primary antisera with recombinant cGH greatly reduced staining (Fig. 5B). At this stage the mesodermal layer of the amnion is beginning to differentiate from a syncytium into muscle tissue. Within the syncytium the nuclei of the differentiating cells were strongly immunoreactive, whereas the cytoplasm was unstained (Fig. 5C). Preincubation of primary antisera with recombinant cGH greatly reduced staining (Fig. 5D). In the trunk, intense GH immunoreactivity was present in the liver, ventricle, stomach and limb buds (Fig. 5E). Preabsorption of the primary antibody completely blocked the staining in these tissues (Fig. 5F).

At ED7, GH staining was seen in the amnion (Fig. 6A), myotome (Fig. 6B), epidermis (Fig. 6B) and the endothelial lining of blood vessels and in some circulating blood cells (Fig. 6C). Although scattered mesodermal cells were immunoreactive (Fig. 6C) the mesoderm was not as densely stained as in the ED6 embryos (Fig. 4C). Preincubation of α cGH1 with recombinant cGH completely abolished staining (Fig. 6D). An identical pattern of GH staining was seen when α IH7 was used as the primary antibody (data not shown).

In ED8 embryos the limb bud was strongly GH immunoreactive (Fig. 7A, B and E). Immunoreactivity was seen in the cartilage cells of the zone of hypertrophy (Fig. 7A). Some cells showed strong cytoplasmic staining or nuclear staining, although others did not appear to have any immunoreactivity (Fig. 7A). GH immunoreactivity was similarly observed in many cells in the zone of flattened cells undergoing mesenchymal condensation (Fig. 7B). Most cells had either cytoplasmic or nuclear staining, although some had both nuclear and cytoplasmic staining and others were totally devoid of GH immunoreactivity. Preincubation of α cGH1 with recombinant cGH abolished staining in the cartilage cells (Fig. 7D). GHR/GHBP immunoreactivity was also present in the cytoplasm of these cells, although GHR staining was predominantly in the nuclei (Fig. 7C).

GHR/GHBP immunoreactivity was also seen in ED3 and ED4 embryos (Fig. 8A and C), and in ED6 embryos (data not shown), with a similar distribution to GH immunoreactivity. Staining was again particularly strong in the nuclei of cells in the neural tube (Fig. 8C). Staining was not observed when the antibody was replaced by NGS (Fig. 8B and D) or PBS (data not shown).

The presence of GH mRNA in early chick embryos was demonstrated by RT-PCR (Fig. 9). A 689 bp cDNA moiety, identical in size to that in the adult pituitary gland, was amplified from reverse-transcribed RNA extracted from ED2 embryos and the headless bodies of ED6 and ED8 embryos. These moieties were not generated from RNA in the absence of Superscript (data not shown).

Discussion

These results clearly demonstrate a widespread distribution of GH-like proteins in extra-pituitary tissues of early chick embryos. These proteins were immunoreactive with 3 different anti-chicken GH antibodies and were not detected following the preabsorption of the antibodies with recombinant GH. The specificity of the GH staining was also indicated by its absence in some cells (e.g. blood cells in ED7 embryos) and tissues (e.g. in amniotic mesoderm of the ED6 embryo). This immunoreactivity was also shown to be associated with proteins identical in size to monomer and dimer GH moieties present in the pituitary glands of adult chickens (Johnson 1998).

These GH-like proteins are likely to reflect a widespread expression of the GH gene, as indicated by the presence of GH mRNA in the bodies of early chick embryos (Fig. 9). Moreover, since pituitary somatotrophs do not secrete GH until ED16 (Porter 1997) and as GH is not detectable in plasma until ED17 (Harvey *et al.* 1979), this GH immunoreactivity in embryonic tissues cannot result from the sequestration of pituitary GH from peripheral plasma.

The presence of GH-like proteins in extra-pituitary sites is now well established (Harvey & Hull 1997) but this

is the first study to identify early embryonic tissues that have GH immunoreactivity. Although Pantaleon *et al.* (1997) demonstrated that non-maternal GH mRNA was present in pre-implantation mouse embryos prior to the formation of the morula, they did not determine if GH gene expression persisted during embryonic development, and Yang *et al.* (1999) similarly found GH immunoreactivity and GH mRNA in trout embryos during gastrulation and organogenesis although they did not determine tissue distribution during ontogeny. The present results indicate that all tissues and most cells initially express the GH gene, although this capacity appears to be lost in some cells and some tissues (e.g. the notochord and mesoderm) by the end of the first third of incubation, suggesting an ontogenic and tissue-specific extinction of GH gene expression.

The extra-pituitary expression of the GH gene during embryogenesis suggests roles for GH in early embryonic growth or differentiation, especially as the GHR gene also appears to be ubiquitously expressed at this time. It is therefore possible that GH acts as a local growth factor, as first suggested by Garcia-Aragon *et al.* (1992) following their discovery of GHR in fetal rat tissues, prior to pituitary differentiation. This possibility is also supported by the ability of exogenous GH to induce glucose transport in pre-implantation mouse embryos, in which GH and GHR immunoreactivity and mRNA are found (Pantaleon *et al.* 1997). Local GH actions are also thought to stimulate the development and hatching of mouse pre-implantation embryos (Drakakis *et al.* 1995, Fukaya *et al.* 1998). Paracrine or autocrine actions of GH are similarly thought to be responsible for the growth and differentiation of immune tissues and hematopoietic cells (Auernhammer & Strasburger 1995, Hull & Harvey 1997), and for the growth and differentiation of mammary and uterine tissues (Strowitzki *et al.* 1991, Mol *et al.* 1996), pituitary cells (Gardner *et al.* 1990), fibroblasts (Hauner 1992) and adipocytes (Catalioto *et al.* 1992). The recent demonstration of GH and GHR immunoreactivity in the teeth of perinatal rats is similarly thought to reflect local actions of GH in chondrocyte development (Zhang *et al.* 1997). The demonstration that GH immunoneutralization blocks Wolffian duct differentiation in fetal rats indicates that local GH actions are involved in early development, by a mechanism overcome by exogenous GH (Nguyen *et al.* 1996).

The putative actions of GH during embryonic development could be direct or mediated by other embryonic growth factors, particularly insulin-like growth factors (IGFs) (De Pablo *et al.* 1990). Although pituitary and plasma GH are not correlated with hepatic IGF-I production during chick embryogenesis (Radecki & Scanes 1997), GH may induce local IGF production in extra-hepatic sites. Indeed, the local actions of GH within the mammary gland and the mammalian immune system are known to be at least partly mediated through local IGF

production and IGF-I receptors (see Harvey & Hull 1997 for review). The widespread expression of IGF-I and its receptor in chick embryos from the first day of development (Serrano *et al.* 1990, Scavo *et al.* 1991) supports this possibility.

The detection of GHR immunoreactivity in early chick embryos is a novel discovery, since hepatic GHR mRNA (Burnside & Cogburn 1992, Tanaka *et al.* 1996) and GH binding sites (Vanderpooten *et al.* 1991, 1993) were not thought to be present until late embryogenesis. This may, however, reflect methodological differences in sensitivity. Our findings are, however, consistent with the presence of GHRs in pre-implantation mouse (Pantaleon *et al.* 1997, Fukaya *et al.* 1998) and bovine (Kolle *et al.* 1998) embryos.

The presence of GHR immunoreactivity in early chick embryos suggests they are not GH resistant. The possibility is supported by the ability of exogenous GH to induce DNA, RNA and protein synthesis in early chick embryos (Sobel 1958, Thommes *et al.* 1992) and to increase blood sugar levels (Hsieh *et al.* 1952), to promote angiogenesis (Gould *et al.* 1995), to reduce allantoic sodium concentrations (Murphy *et al.* 1986) and to increase tibial length (Thommes *et al.* 1992). Thus, while exogenous GH may not be able to augment normal growth rate during embryonic development, it appears to have numerous actions not directly related to whole body growth.

The presence of GHRs in the early chicken embryo may also account for the subcellular distribution of GH in many embryonic tissues. Although GH might be expected to be present in the cytoplasm of GH-producing cells, GH and GHR/GHBP immunoreactivity was often associated with the nucleus. It is, however, well established that GH binds to membrane receptors in target cells and is rapidly internalized to nuclear compartments (Lobie *et al.* 1991, Fraser & Harvey 1992). The occurrence of GH immunoreactivity in nuclear compartments has also been demonstrated in other studies (Rezvani *et al.* 1973, Bonifacio *et al.* 1983). A nuclear localization of GH within GH-producing cells may also occur if it escapes packaging in the Golgi following its synthesis, and this is particularly likely in rapidly growing or differentiating embryonic cells, as demonstrated in proliferating or tumorous mammary tissues (Mol *et al.* 1996).

The internalization of GH in its target sites provides an extra-pituitary store of GH in peripheral tissues (Ilondo *et al.* 1992). The amount of GH in extra-pituitary tissues is, however, minimal in comparison with that in the pituitary somatotrophs. For instance, GH immunoreactivity in the chicken brain, thymus, and bursa accounts for <10% of that in the pituitary gland (Render *et al.* 1995). The pituitary is also primarily responsible for plasma GH, since circulatory GH levels are not detectable in hypophysectomized chickens (Harvey & Scanes 1977). Extra-pituitary tissues that produce GH are therefore unlikely to be GH-secreting endocrine glands. The storage of GH

in the pituitary gland thus appears to be developmentally acquired and associated with peri- and postnatal growth. The expression of GH in pituitary tissue is also a phylogenetic development, since GH-like proteins are present in primitive vertebrates and in invertebrates lacking pituitary glands (Wright 1986, Inestrosa *et al.* 1990, Swinnen *et al.* 1990). Moreover, as luteinizing hormone immunoreactivity also occurs in the stomach and lungs of early chick embryos (Shirasawa *et al.* 1996), the extra-pituitary production of pituitary hormones prior to pituitary differentiation may be a general phenomenon.

In summary, these results demonstrate that GH is present in most tissues and organs of the chick embryo during organogenesis. Since GHR immunoreactivity is present in the same tissues, these results suggest local roles for GH in early embryonic growth.

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References

- Auernhammer CJ & Strasburger CJ 1995 Effects of growth hormone and insulin-like growth factor-I on the immune system. *European Journal of Endocrinology* **133** 635–645.
- Berghman LR, Lens P, Decuypere E, Kuhn ER & Vandesande F 1987 Glycosylated chicken growth hormone. *General and Comparative Endocrinology* **68** 408–414.
- Bonifacino JS, Roguin LP & Paladini AC 1983 Formation of complexes between ¹²⁵I-labeled human or bovine somatotropins and binding proteins *in vivo* in rat liver and kidney. *Biochemical Journal* **214** 121–132.
- Burnside J, Liou SS & Cogburn LA 1991 Molecular cloning of the chicken growth hormone receptor complementary deoxyribonucleic acid: mutation of the gene in sex-linked dwarf-chickens. *Endocrinology* **128** 3183–3192.
- Catalioto RM, Gaillard D, Ailhaud G & Negrel R 1992 Terminal differentiation of mouse preadipocyte cells: the mitogenic adipogenic role of GH is mediated by the protein kinase C signaling pathway. *Growth Factors* **6** 255–264.
- Drakakis P, Loutradis D, Milingos S, Michalas S, Kallianidis K, Bletsas R, Aravantinos D & Kiessling AA 1995 A preliminary study of the effect of growth hormone on mouse preimplantation embryo development *in vitro*. *Gynecological and Obstetrical Investigation* **40** 222–226.
- De Pablo F, Scott LA & Roth J 1990 Insulin and insulin-like growth factor-I in early development: peptides, receptors and biological events. *Endocrine Reviews* **11** 558–577.
- Fraser RA & Harvey S 1992 Ubiquitous distribution of growth hormone receptors and/or binding proteins in adenohypophyseal tissue. *Endocrinology* **130** 3593–3600.
- Fukaya T, Yamanaka T, Terada Y, Murakami T & Yajima A 1998 Growth hormone improves mouse embryo development *in vitro*, and the effect is neutralized by growth hormone receptor antibody. *Tohoku Journal of Experimental Medicine* **184** 113–122.
- Garcia-Aragon J, Lobie PE, Muscat GE, Gobius KS, Norstedt G & Waters MJ 1992 Prenatal expression of the growth hormone (GH) receptor/binding protein in the rat: a role for GH in embryonic and fetal development? *Development* **114** 869–876.
- Gardner MJ, Morrison CA, Stevenson LQ & Flint DJ 1990 Production of anti-idiotypic antisera to rat growth hormone antibodies capable of binding to growth hormone receptors and increasing body weight gain in hypophysectomized rats. *Journal of Endocrinology* **125** 53–59.
- Geffner ME 1996 The growth without growth hormone syndrome. *Endocrinology and Metabolism Clinics of North America* **25** 649–663.
- Gould J, Aramburo C, Capdevielle M & Scanes CG 1995 Angiogenic activity of anterior pituitary tissue and growth hormone on the chick embryo chorio-allantoic membrane: a novel action of GH. *Life Sciences* **56** 587–594.
- Hamburger V & Hamilton HL 1951 A series of normal stages in development of chick embryo. *Journal of Morphology* **88** 49–92.
- Harvey S & Scanes CG 1977 Purification and radioimmunoassay of chicken growth hormone. *Journal of Endocrinology* **73** 321–329.
- Harvey S & Hull KL 1997 Pituitary and extra-pituitary growth hormone in birds. In *Advances in Comparative Endocrinology*, pp 899–905. Eds S Kawashima & RS Kikuyama. Bologna: Monduzzi Editore.
- Harvey S, Davison TF & Chadwick A 1979 Ontogeny of growth hormone and prolactin secretion in the domestic fowl, *Gallus domesticus*. *General and Comparative Endocrinology* **59** 270–273.
- Hauner H 1992 Physiology of the fat cell with emphasis on the role of growth hormone. *Acta Paediatrica* (Suppl) **383** 47–51.
- Hill DJ 1992 What is the role of growth hormone and related peptides in implantation and development of the embryo and fetus? *Hormone Research* **38** (Suppl 1) 28–34.
- Hsieh KM, Wang TY & Blumenthal HT 1952 The diabetogenic and growth promoting activities of growth hormone somatotropin in the developing chick embryo. *Endocrinology* **51** 298–301.
- Hsu SM, Raine L & Fanger H 1981 Use of avidin-peroxidase complex ABC in immuno-peroxidase techniques: a comparison between ABC and unlabeled antibody AAP procedures. *Journal of Histochemistry and Cytochemistry* **29** 577–580.
- Huang N, Cogburn LA, Agarwal SK, Marks HL & Burnside J 1993 Over-expression of a truncated growth hormone receptor in the sex-linked dwarf chicken: evidence for a splice mutation. *Molecular Endocrinology* **7** 1391–1398.
- Hull KL & Harvey S 1997 Growth hormone: an immune regulator in vertebrates. In *Advances in Comparative Endocrinology*, pp 565–572. Eds S Kawashima & S Kikuyama. Bologna: Monduzzi Editore.
- Hull KL, Thiagarajah A & Harvey S 1996 Cellular localization of growth hormone receptors/binding proteins in immune tissues. *Cell and Tissue Research* **286** 69–80.
- Ilondo MM, Vanderschueren-Lodeweyck M, Coustoy PJ & De Meyts P 1992 Cellular processing of growth hormone in IM-9 cells: evidence for exocytosis of internalized hormone. *Endocrinology* **130** 2037–2044.
- Inestrosa NC, Labarca R, Perelman A, Campos EO, Arana R, Gonzalez M, Brandan E, Sanchez JP & Gonzalez-Plaza R 1990 Biotechnological aspects in 'loco' larvae. *Archives de Biologia y Medicina Experimentales* **23** 179–186.
- Johnson CD 1998 Growth hormone in chick embryogenesis. *MSc Thesis*. University of Alberta.
- Jozsa R, Scanes CG, Vigh S & Mess B 1979 Functional differentiation of the embryonic chicken pituitary glands studied by immunohistological approach. *General and Comparative Endocrinology* **39** 158–163.
- Kolle S, Sinowatz F, Boie G, Lincoln D, Palma G, Stojkovic M & Wolf E 1998 Topography of growth hormone receptor expression in the bovine embryo. *Histochemical and Cell Biology* **109** 417–419.
- Lobie PE, Barnard R & Waters MJ 1991 The nuclear growth hormone-receptor binding protein. *Journal of Biological Chemistry* **266** 22645–22652.

- Malamed S, Gibney JA, Cain LD, Perez FM & Scanes CG 1993 Immunocytochemical studies of chicken somatotrophs and somatotroph granules before and after hatching. *Cell and Tissue Research* **272** 369–374.
- Mol JA, van Gederen E, Rutteman GR & Rijnberk A 1996 New insights into the molecular mechanisms of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans. *Journal of Steroid Biochemistry and Molecular Biology* **57** 67–71.
- Murphy MJ, Brown PS & Brown SC 1986 Osmoregulatory effects of prolactin and growth hormone in embryonic chicks. *General and Comparative Endocrinology* **62** 485–492.
- Nguyen AP, Chandorkar A & Gupta C 1996 The role of growth hormone in fetal mouse reproductive tract differentiation. *Endocrinology* **113** 225–234.
- Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ & Kaye PL 1997 Functional growth hormone GH receptors and GH are expressed by pre-implantation mouse embryos: a role for GH in early embryogenesis? *PNAS* **94** 5125–5130.
- Porter TE 1997 Regulation of somatotroph differentiation during chicken embryonic development: a review. In *Perspectives in Avian Endocrinology*, pp 47–56. Eds S Harvey & RJ Etches. Bristol: Journal of Endocrinology Ltd.
- Porter TE, Couger GS, Dean CE & Hargis BM 1995 Ontogeny of growth hormone GH-secreting cells during chicken embryonic development: initial somatotrophs are responsive to GH-releasing hormone. *Endocrinology* **136** 1850–1856.
- Radecki SV & Scanes CG 1997 The hypothalamo-pituitary growth hormone-insulin-like growth factor axis: coupling and uncoupling. In *Perspectives in Avian Endocrinology*, pp 119–133. Eds S Harvey & RJ Etches. Bristol: Journal of Endocrinology Ltd.
- Render CL, Hull KL & Harvey S 1995 Neural expression of the pituitary GH gene. *Journal of Endocrinology* **147** 413–422.
- Rezvani I, Maddaiah VT, Collipp PJ, Thomas J & Chen SY 1973 Kinetics of uptake and subcellular distribution of human growth hormone in liver slices. *Biochemical Medicine* **7** 432–440.
- Romanoff AL 1960 *The Avian Embryo: Structural and Functional Development*. New York: The Macmillan Company.
- Scanes CG & Daughaday WH 1995 Growth hormone action: growth. In *Growth Hormone*, pp 351–369. Eds S Harvey, CG Scanes & WH Daughaday. Boca Raton: CRC Press.
- Scavo LM, Serano J, Roth JD & De Pablo F 1991 Genes for the insulin receptor and the insulin-like growth factor-I receptor are expressed in the chicken embryo blastoderm and throughout organogenesis. *Biochemical and Biophysical Research Communications* **176** 1393–1401.
- Serrano J, Sculiner AR, Roberts CJ, Le Roith D & De Pablo F 1990 The insulin-like growth factor-I IGF-I gene is expressed in chicken embryos during early organogenesis. *Endocrinology* **127** 1547–1549.
- Shirasawa N, Shiino M, Shimizu Y, Nogami H & Ishii S 1996 Immunoreactive luteinizing hormone ir-LH cells in the lung and stomach of chick embryos. *Cell and Tissue Research* **283** 19–27.
- Sobel H 1958 Antagonistic effects of cortisone and growth hormone on the developing chick embryo. *Cell and Tissue Research* **283** 19–27.
- Sternberger LA 1979 *Immunocytochemistry*, edn 2. New York: John Wiley.
- Strowitzki T, Wiedemann R & Hepp H 1991 Influence of growth factors EGF, IGF-I and human growth hormone on human endometrial stromal cells *in vitro*. *Annals of the New York Academy of Sciences* **626** 308–311.
- Swinnen K, Broeck JV, Verhaert P & de Loof A 1990 Immunocytochemical localization of human growth hormone- and prolactin-like antigenic determinants in the insects, *Locusta migratoria* and *Sarcophaga bullata*. *Comparative Biochemistry and Physiology* **95** 373–378.
- Tanaka M, Hayashida Y, Sakaguchi K, Okhubo T, Washita M, Hoshino S & Nakashima K 1996 Growth hormone-independent expression of insulin-like growth factor I messenger ribonucleic acid in extra-hepatic tissues of the chicken. *Endocrinology* **137** 30–34.
- Thommes RC & Woods JE 1993 Endocrine regulation of the growth/development of warm-blooded vertebrate embryos/fetuses. In *The Endocrinology of Growth Development and Metabolism in Vertebrates*, pp 495–514. Eds MP Schreibman, CG Scanes & PKT Pang. Toronto: Academic Press.
- Thommes RC, Woods JE, Aramburo C, Buonomo FC & Scanes CG 1992 Effects of surgical decapitation and chicken growth hormone (cGH) replacement therapy on chick embryo growth. *Growth Development and Aging* **56** 167–178.
- Vanderpooten A, Huybrechts LM, Decuypere E & Kuhn ER 1991 Differences in hepatic growth hormone receptor binding during development of normal and dwarf chickens. *Reproduction Nutrition and Development* **31** 47–55.
- Vanderpooten A, Janssens W, Buyse J, Leenstra F, Berghman L, Decuypere E & Kuhn ER 1993 Study of the hepatic growth hormone (GH) receptor at different ages in chickens selected for a good feed conversion (FC) and a fast weight gain (GL). *Domestic Animal Endocrinology* **10** 199–206.
- Wright GM 1986 Immunocytochemical demonstration of growth hormone, prolactin and somatostatin-like immunoreactivities in the brain of larval, young adult and upstream migrant adult sea lamprey, *Petromyzon marinus*. *Cell and Tissue Research* **246** 23–31.
- Yang BY, Greene M & Chen TT 1999 Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Onchorynchus mykiss*. *Molecular Reproduction and Development* **53** 127–134.
- Zhang CZ, Li H, Young WG, Bartold PM, Chen C & Waters MJ 1997 Evidence for local action of growth hormone in embryonic tooth development in the rat. *Growth Factors* **14** 131–143.

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