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 extrapituitary GH may act as a local growth factor during embryonic development.


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, Thrommes & Woods 1993, Porter 1997) and the inability of exogenous GH to promote embryonic or fetal growth (Hill 1992, Thrommes 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 extrapituitary 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
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 µm) 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 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 chromogen 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 oligodeoxynucleotide GH primer, khu 14, 5’-GACATCGTTTTTTTTTTTTTTTT-3’ (Nucleotide Synthesis Laboratory, University of Alberta), 5 × RT buffer (Promega, Madison, WI, USA), excess deoxy-nucleotides (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’-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA-3’, GH 5’, 5’-CGTTCAAGCATT-3’, CACCTGAGCAACTCTCCCCG-3’, Nucleotide Synthesis Laboratory, University of Alberta), excess deoxy-nucleotides (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 (Rendel et al. 1995). The reaction mixture was overlayed with mineral oil, denatured and annealed (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.
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 \( \alpha \) cGH1 (Fig. 2A, G and H), \( \alpha \) cGH2 (Fig. 2C) or \( \alpha \) H7 (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. 2 G and H). Control sections incubated with NGS or PBS were unstained (data not shown), as were sections incubated with primary antibody.

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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 αcGH1 with recombinant cGH completely abolished this

Figure 4  GH immunoreactivity in the ED6 embryo. (A) Transverse section at the level of the wing buds. GH immunoreactivity (αH7) 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 αH7 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 αH7 with recombinant cGH did not result in staining of the mesoderm. pcv, posterior cardinal vein; d, dorsal aorta. Bars=250 μm (A and B) or 20 μm (C and D).
Figure 5  GH immunoreactivity in the ED6 extra-embryonic membrane determined using uH7 as the primary antibody. (A) The ectodermal layers (e) of the amnion are strongly immunoreactive. (B) Preabsorption of uH7 with cGH reduced staining. (C) The mesodermal (m) layer of the amnion is beginning to differentiate from a syncitium into musculature, and the nuclei (n) of these cells are intensely GH immunoreactive. (D) Preabsorption of the uH7 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 (l). (F) Preabsorption abolishes reactivity in a section similar to E. Bars = 50 μm.

**GH: embryonic GH** • S HARVEY and others
staining (Fig. 3B). Similar results were also observed with αcGH2 as the primary antibody (data not shown).

Cells immunoreactive with α1H7 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 6  GH immunoreactivity in the ED7 embryo. (A) The amnion of the 7-day embryo was intensely GH immunoreactive. Bar = 20 µm. (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 αcGH1 completely abolished staining in a section similar to C. Bars= 50 µm (B-D). b, blood cells; nt, neural tube.
Figure 7 GH and GHR/GHBP immunoreactivity in the ED8 limb bud. (A) GH immunoreactivity (determined using αcGH1 as the primary antibody) in cells from the hypertropic 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 hypertropic zone. Note the strong nuclear staining and less dense cytoplasmic staining. Some cells do not appear to be immunoreactive (arrowhead). (D) Preincubation of αcGH1 with recombinant cGH completely abolished GH staining in cartilage cells. An adjacent section to A is shown for illustration. Bars=10 µm (A-D). (E) Low power illustration of a cross-section through the limb bud, showing the distribution of GH immunoreactive chondrogenic cells. Bar=100 µm.
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.
distribution of immunoreactivity was observed when αcGH1 or αcGH2 was used as the primary antibody (data not shown). Preabsorption of the monoclonal antibody (αIH7) 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 syncitium into muscle tissue. Within the syncitium 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 αcGH1 with recombinant cGH completely abolished staining (Fig. 6D). An identical pattern of GH staining was seen when αIH7 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 αcGH1 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 (Vanderpoosten 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 allostatic 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 to be present in the cytoplasm of GH-producing cells, as demonstrated in proliferating or tumorous mammary 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 circulating 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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