Extrapituitary GH in the chicken: underestimation of immunohistochemical staining by Carnoy's fixation

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Abstract

GH has previously been shown to be present in peripheral extrapituitary tissues of chick embryos, but the cellular distribution of GH immunoreactivity is still uncertain because of differing immunohistochemical findings. The possibility that this uncertainty reflects differences in fixation of the embryonic tissues was assessed by comparing GH immunoreactivity in tissues fixed in 4% (w/v) paraformaldehyde or Carnoy's fluid (60% ethanol (v/v); 30% chloroform (v/v); 10% acetic acid (v/v)).

A widespread distribution of GH immunoreactivity was seen in paraformaldehyde-fixed tissues, although it was particularly intense in the spinal cord, dorsal and ventral root ganglia, notochord, myotome, epidermis, crop, heart, lung and humerus. In marked contrast, GH immunoreactivity in embryonic tissues fixed with Carnoy's was more discrete and mainly restricted to marginal and mantle layers of the spinal cord, spinal nerves, the ventral root

ganglia and the extensor nerve of the anterior limb bud. Since these are neural derivatives, Carnoy's fixation appears to preferentially result in neural GH staining, whereas GH staining in neural and non-neural tissues is seen after paraformaldehyde fixation. Carnoy's, because it is a precipitative fixative, may only fix large GH moieties, whereas GH in peripheral tissues includes numerous molecular variants, many of which are of relatively small size. Paraformaldehyde, because it is a cross-linking fixative, preferentially fixes peptides and small proteins, and it may therefore fix more GH moieties than Carnoy's fluid. Carnoy's fixation appears to underestimate GH immunoreactivity in immunohistochemical studies on the cellular distribution of GH-like proteins in embryonic chicks.

Journal of Endocrinology (2003) **177**, 223–234

Introduction

Growth hormone (GH) gene expression primarily occurs in pituitary somatotrophs, although numerous extrapituitary sites of GH expression have been documented. Indeed, in domestic fowl, GH mRNA has been identified in the brain (Render *et al.* 1995a), in the thymus, bursa and spleen (Render *et al.* 1995b), in the testes and vas deferens (Harvey *et al.* 2002, A E Murphy, M Luna, C Arámburo, K L Hull & S Harvey, unpublished observations), in the ovary (Luna *et al.* 2000), in the heart (Takeuchi *et al.* 2001) and in the eye (Takeuchi *et al.* 2001). In mammalian species, GH gene expression has additionally been determined in the placenta (Schwarzler *et al.* 1997), mammary gland (Mol *et al.* 1995a,b), liver (Recher *et al.* 2001), lung (Allen *et al.* 2000), salivary gland (Tresguerres *et al.* 1999), skin (Palmetshofer *et al.* 1995, Slominski *et al.* 2000) and in smooth muscle and endothelial cells of blood vessels (Wu *et al.* 1996, Recher *et al.* 2001). Proteins with GH immunoreactivity are also present in these sites and in other peripheral tissues (e.g. in teeth, kidney and gut; Kyle *et al.* 1981, Costa *et al.* 1993,

Zhang *et al.* 1997). Nevertheless, with the exception of limited studies on the nervous (Lechan *et al.* 1981, Ramesh *et al.* 2000, Takeuchi *et al.* 2001) and immune (Kao *et al.* 1992, Maggiano *et al.* 1994) systems, this immunoreactivity has been determined by radioimmunoassay of tissue extracts and its cellular location is largely unknown. Two immunohistochemical studies on the localization of GH in peripheral tissues of embryonic chicks have, however, been published (Harvey *et al.* 2000, Murphy & Harvey 2001).

In one study, GH immunoreactivity was found to be abundant and widespread in the bodies of embryonic tissues (Harvey *et al.* 2000). This immunoreactivity was thought to be specific, since it was detectable by three different GH antibodies (two polyclonals, one monoclonal) and was completely lost following pretreatment of the primary antibodies with excess recombinant GH. A different tissue distribution of GH immunoreactivity was, however, observed in a more recent study (Murphy & Harvey 2001), in which the cellular distribution of GH was restricted to discrete cells or tissue layers. The main difference between these two studies was the fixative used

for immunohistochemistry: 4% paraformaldehyde (Harvey *et al.* 2000) and Carnoy's fixative (Murphy & Harvey 2001). Since different fixatives can result in quantitative and qualitative changes in protein detection (Chiu *et al.* 1994, Vince *et al.* 1997, Hemmer *et al.* 1998, Giaccone *et al.* 2000), the influence of fixation on the immunocytochemical detection of GH in chick embryos was therefore empirically assessed.

Materials and Methods

Tissues

Fertile White Leghorn chicken eggs from the University of Alberta Poultry Unit were incubated at 37 °C in humidified air. The eggs were turned one quarter of a revolution each day during the incubation period. Whole embryos at embryonic day 7 (ED 7) of the 21-day period of embryogenesis were dissected and decapitated in phosphate-buffered saline (PBS; pH 7.4) prior to fixation. ED 7 embryos were used since pituitary somatotrophs do not differentiate until ED 12 to ED 14 (Porter 1997) and hence tissue GH immunoreactivity could not be due to the sequestration of pituitary GH. Peripheral tissues were similarly used to ensure that GH immunoreactivity was not due to pituitary GH detection.

Fixation

Tissues were collected into freshly prepared paraformaldehyde (4% (w/v), in PBS; Sigma, Mississauga, Ontario, Canada) or Carnoy's fluid (60% ethanol (v/v); 30% chloroform (v/v); 10% acetic acid (v/v)). Paraformaldehyde is a cross-linking fixative that forms links (hydroxyl-methylene bridges) between reactive end-groups of adjacent protein chains, whereas Carnoy's is a precipitant fixative that denatures proteins by destroying the hydrophobic bonds that hold together the tertiary structures of large protein molecules.

Immunocytochemistry

The headless embryos were fixed, overnight, at 4 °C. They were then dehydrated in a graded series of alcohol (50% 15 min; 70% 15 min; 95% 30 min; 100% 2 × 30 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 2 × 30 min. The tissues were then infiltrated with paraffin wax for 24 h at 60 °C under normal atmospheric pressure. Serial transverse (8 µm) sections were then taken using a microtome and mounted onto charged slides (Fisher Scientific). Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlingame, CA, USA; Sigma) using the avidin-biotin-peroxidase (ABC) method

(Hsu *et al.* 1981). Sections were incubated overnight at 4 °C with a specific polyclonal antiserum raised in rabbits against native (pituitary) chicken GH (Harvey *et al.* 2000), diluted 1:1000 in 1% normal goat serum. After incubation, the slides were washed three times for 5 min in PBS. Sections were then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (Sigma; 1:500). The slides were then 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. The specificity of staining was determined by preabsorbing the GH antiserum with recombinant chicken GH (Amgen, Thousand Oaks, CA, USA; 1 mg/ml) for 1 h prior to section incubation. All tissues collected in paraformaldehyde or Carnoy's fluid were processed together under identical conditions with the same reagents. Digital images were collected using a SPOT digital Microscope Camera (Carsen Group, Markham, Ontario, Canada) mounted on an Olympus B microscope.

Results

After Carnoy's fixation, intense immunocytochemical staining for GH was seen in the spinal cord, although restricted to the marginal cells of the white matter, to the ventral horn and mantle, to spinal nerves, and to the ventral root nuclei (Fig. 1A, B and C). The surrounding tissue was, however, only faintly stained or unstained. In contrast, strong staining was seen throughout the spinal cord following paraformaldehyde fixation, including the ependymal cells surrounding the spinal canal (Fig. 1D, E and F). In contrast to the tissues fixed in Carnoy's, the tissues fixed in paraformaldehyde had intense GH immunoreactivity in both the ventral and dorsal root ganglia (Fig. 1D, E and F). Similarly, while GH immunoreactivity was abundant and widespread in the notochord, notochordal sheath and in the myotome of paraformaldehyde-fixed tissues (Fig. 2B and D), GH immunoreactivity was not detectable in these tissues following Carnoy's fixation (Fig. 2A and C).

A single layer of epithelial cells lining the esophagus (crop) had GH immunoreactivity following Carnoy's fixation (Fig. 3A), although intense GH immunoreactivity was seen in numerous cells in the mucosal epithelium, submucosa and muscular layers of this structure following paraformaldehyde fixation (Fig. 3B). Strong GH staining was, likewise, seen in the bronchus after fixation in paraformaldehyde (Fig. 3D), but only faint staining was seen after fixation in Carnoy's (Fig. 3C). Similar differences in GH staining were also seen in the heart, in which intense staining was present in the myocardium of the atria and in atrial cushions (Fig. 4C and D) and ventricles (Fig. 5C and D) fixed in paraformaldehyde, but not in the atria

Figure 1 GH immunoreactivity in the spinal cord of embryonic chicks. Following fixation in Carnoy's fluid (A, B and C), intense staining is present in the marginal layer (ml), spinal nerves (spn) and the ventral root nuclei (vr). Light staining is also present in the ventral horn (vh) and the mantle region (m). Magnification: A $\times 100$; B $\times 400$; C $\times 400$. Following fixation in 4% (w/v) paraformaldehyde, GH staining is present throughout the spinal cord, including the ependymal cells (e) surrounding the spinal canal and the dorsal root nuclei (dr) (D, E and F). GH immunoreactivity is also present in the ventral root nuclei (vr) and the surrounding myotome (my), vertebral arch (arch), notochord (n), notochordal sheath (ns) and the head of a rib (r). Magnification: D $\times 100$; E $\times 400$; F $\times 400$.

Figure 2 GH immunoreactivity in the notochord (n), notochordal sheath (ns; B) and myotome (my; D) of embryonic chicks following fixation in 4% (w/v) paraformaldehyde, in comparison with comparable tissues fixed in Carnoy's fluid (A and C). Magnification: $\times 400$.

(Fig. 4A and B) or ventricles (Fig. 5A and B) fixed in Carnoy's. Strong GH immunoreactivity was also seen in the cells lining the pericardial cavity and in the body wall of embryos fixed in paraformaldehyde (Fig. 4C and D), but not in embryos fixed in Carnoy's (Fig. 4A and B). Although intense staining was present in the myocardial cells of the heart following paraformaldehyde fixation, only faint, diffuse staining was present in blood cells in the atria and ventricles and in the pericardial space (Fig. 4C and D,

Fig. 5C and D). The atrio-pulmonary septum surrounding the ventricles was also only slightly stained following paraformaldehyde fixation (Fig. 5C and D). In marked contrast, there was no staining in myocardial or blood cells of the ventricle following preabsorption of the primary antiserum with excess recombinant GH (Fig. 6).

In the limb bud, strong and discrete GH staining was localized in the extensor nerve following fixation in Carnoy's (Fig. 7A), whereas GH immunoreactivity was

Figure 3 GH immunoreactivity in the esophagus (B) and bronchus (D) of embryonic chicks following fixation in 4% (w/v) paraformaldehyde, in comparison with similar tissues fixed in Carnoy's fluid (A and C): Abbreviations: e, epithelium; s, submucosa; m, esophageal muscularis. Magnification: $\times 400$. Only a single layer of esophageal epithelial cells (arrowed) were GH immunoreactive following Carnoy's fixation, whereas GH immunoreactivity was widespread following paraformaldehyde fixation.

widespread in the limb bud fixed in paraformaldehyde. Following paraformaldehyde fixation, GH immunoreactivity was particularly abundant in chondrocytes of the humerus (Fig. 7F) (as in chondrocytes in the ribs, Fig. 1D) and in a layer of outer epidermal cells (Fig. 7E), which were only faintly stained or unstained after Carnoy's fixation (Fig. 7B and C). Intense GH immunoreactivity

was also present in the dorsal and ventral muscle mass of the wing bud following paraformaldehyde fixation (Fig. 7D), but not after Carnoy's (Fig. 7A).

In all tissues fixed in Carnoy's or paraformaldehyde, the GH staining was specific and completely lost following preabsorption of the primary antibody with excess recombinant GH (data not shown). The data shown are also

Figure 4 GH immunoreactivity in the atria of embryonic chicks following fixation in 4% (w/v) paraformaldehyde (C and D), in comparison with atria fixed in Carnoy's fluid (A and B). Intense GH immunoreactivity is present throughout the myocardium (mc) of the right atria (ra), atrial cushion (ac) and the body wall (bw) following fixation in paraformaldehyde (C and D). Only faint, diffuse staining to blood cells (arrowed) is seen in the atrium and pericardial cavity. Magnification: A and C $\times 100$; B and D $\times 400$. Abbreviations: avc, atrio-ventricular canal; pc, pericardial cavity.

representative of sections taken from at least 20 embryos fixed in Carnoy's and at least 20 embryos fixed in paraformaldehyde.

Discussion

These results clearly showed the presence of GH-like proteins in peripheral tissues of early chick embryos, but demonstrate tissue-specific differences in staining following paraformaldehyde or Carnoy's fixation.

The distribution of GH immunoreactivity in the peripheral tissues of paraformaldehyde-fixed ED 7 chicks was widespread, but particularly striking in the spinal cord, dorsal and ventral root ganglia, notochord, myotome, epidermis, heart and the humerus. These findings extend preliminary observations by Harvey *et al.* (2000) using paraformaldehyde-fixed tissues from ED 6 and ED 7 embryos. The cellular localization of GH staining in the neural tube, dorsal and ventral root ganglia, notochord, heart and limb bud was, however, much clearer in this

Figure 5 GH immunoreactivity in the ventricles of chick embryos following fixation in 4% (w/v) paraformaldehyde (C and D), in comparison with ventricles fixed in Carnoy's fluid (A and B). Intense GH immunoreactivity is present throughout the ventricular myocardium (mc) of tissues fixed in paraformaldehyde, whereas only faint GH staining is present in the aortico-pulmonary septum (aps) or to blood cells (arrowed) in the ventricular lumen. In contrast, GH immunoreactivity is not seen in ventricular tissue fixed in Carnoy's (A and B). Abbreviations: ra, right atrium; avc, atrio-ventricular canal; ivs, intraventricular septum; bw, body wall; pc, pericardial cavity; lv, left ventricle; rv, right ventricle. Magnification: A and C $\times 100$; B and D $\times 400$.

Figure 6 GH immunoreactivity in the ventricles of chick embryos following fixation in 4% (w/v) paraformaldehyde before (A, B and C) or after (D, E and F) the primary antiserum had been preabsorbed with excess recombinant chicken GH (1 mg/ml for 1 h). The arrows indicate faint diffuse staining in blood cells. Magnification: A and D $\times 100$; B and E $\times 200$; C and F $\times 400$.

Figure 7 GH immunoreactivity in the chick embryo wing bud following fixation in Carnoy's fluid (A, B and C) or 4% (w/v) paraformaldehyde (D, E and F). Intense GH staining is present in the extensor nerve (exn, and arrows) following fixation in Carnoy's (A and B), although cells in the dorsal (dm) and ventral (vm) muscle masses, the epidermis (ep) and the humerus (h) were unstained. In contrast, strong GH immunoreactivity is seen throughout the wing bud following paraformaldehyde fixation, but is particularly striking in the extensor nerve (arrows), muscle masses, epidermis and humerus (D, E and F). Magnification: A and D $\times 100$; B, C, E and F $\times 400$. The insets show tracts of extensor nerve at $\times 1000$ magnification.

study and is presented at high magnification for the first time. The staining for GH in these tissues is clearly cellular and within the cytoplasm, whereas it could not be clearly differentiated from background staining in the earlier study (Harvey *et al.* 2000). The presence of GH staining in the esophagus (crop), lung (bronchus), myotome and the anterior extensor nerve following paraformaldehyde staining are novel observations.

A widespread distribution of GH immunoreactivity might be expected, since the headless bodies of ED 6 and ED 8 embryos readily express the GH gene (Harvey *et al.* 2000). A widespread cellular distribution of GH immunoreactivity might also be expected in view of the finding, in mammals, of GH mRNA and immunoassayable GH in extracts of most peripheral tissues (e.g. Kyle *et al.* 1981, Costa *et al.* 1993, Wu *et al.* 1996, Recher *et al.* 2001), particularly in perinatal animals (Costa *et al.* 1993, Recher *et al.* 2001). A widespread distribution of GH immunoreactivity might also be expected, since GH may act as an autocrine or paracrine growth factor during embryonic development (Harvey & Hull 1997), and GH receptors (Harvey *et al.* 2000) and a GH-responsive gene (Harvey *et al.* 2001a) are ubiquitous in embryonic tissues. Extrapituitary GH is thus likely to be involved in normal embryogenesis or fetal development. Extrapituitary GH is, however, likely to be only one of many growth factors that jointly regulate ontogenic development (Adamson 1993, Waters *et al.* 1999) and GH is unlikely to be critically required, since normal or near-normal development occurs in dwarf chickens and mammals with GH receptor dysfunction (Hull & Harvey 1999). Other growth factors are thus likely to compensate for GH under conditions of GH or GH receptor dysfunction. Under normal conditions, extrapituitary GH may act as an autocrine/paracrine factor to stimulate growth by acting as a cell survival factor. Indeed, a recent study has shown that GH reduces apoptosis in bovine embryos during early embryogenesis (Kolle *et al.* 2002).

The distribution of GH immunoreactivity in ED 7 tissues fixed in Carnoy's was noticeably different from that observed following paraformaldehyde fixation. After Carnoy's, the immunohistochemical staining of GH was more discrete (mainly restricted to marginal and mantle layers of the spinal cord, the ventral root ganglia, to spinal nerves and to the anterior extensor nerve). These results are similar to those published by Murphy & Harvey (2001), in which GH staining was also observed in the trigeminal nerve and vagal nerve following Carnoy's fixation. This suggests that Carnoy's fixative preferentially detects GH immunoreactivity in neural tissues. The discrete presence of GH in the extensor nerve is a novel finding of this study, as is the absence of GH staining in chondrocytes and cardiac cells fixed in Carnoy's fluid. Indeed, apart from the neural derivatives, GH immunoreactivity is barely detectable in the peripheral tissues of embryos fixed in Carnoy's. It is therefore of interest that

the GH staining in the heads of ED 7 chicks fixed in Carnoy's (Murphy & Harvey 2001) was also much less than in heads fixed in paraformaldehyde (Harvey *et al.* 2001b). This is particularly evident for Rathke's pouch (a non-neural pharyngeal derivative that differentiates into the pituitary gland), which is almost devoid of GH immunoreactivity following Carnoy's fixation (Murphy & Harvey 2001) but abundantly stained for GH immunoreactivity following paraformaldehyde fixation (Harvey *et al.* 2001b). The controversy in the literature (Porter 1997) on the ontogeny of pituitary somatotrophs (ED 4-5, Thommes *et al.* 1987 (Bouin's fixation); ED 10, Malamed *et al.* 1993 (Zenker's fixative); ED 12, Józsa *et al.* 1979, Allaerts *et al.* 1999 (Zamboni's fluid); ED 12, Porter *et al.* 1995 (B-5; formalin-mercuric chloride)), might therefore partly reflect differences in the fixatives used. Qualitative differences in the distribution of GH staining in other tissues of embryonic chicks may similarly reflect differences in tissue fixation. For instance, while Harvey *et al.* (2001b) observed widespread staining for GH in neural retinas fixed in paraformaldehyde, Takeuchi *et al.* (2001) found GH immunoreactivity only in pigmented retinal epithelial cells following fixation in Bouin's (a picric acid-based fixative).

Paraformaldehyde is a cross-linking fixative and is particularly useful for fixing smaller proteins and peptides (Polak & Van Noorden 1997). Carnoy's, in contrast, is a precipitant fixative and more useful for the fixation of large proteins. Carnoy's fluid is a better fixative than paraformaldehyde in preserving tissue architecture and in facilitating tissue sectioning (Polak & Van Noorden 1997), but it reduces specific staining for GH immunoreactivity. Carnoy's fixative has similarly been found to reduce antigen staining in other immunocytochemical studies, in comparison with formalin or paraformaldehyde (e.g. Cammer *et al.* 1985, Schutte *et al.* 1987, Bos *et al.* 2000). It is therefore possible that Carnoy's and paraformaldehyde fixatives may differentially fix the different GH moieties present in neural and non-neural tissues. It is now well established that pituitary GH comprises a family of size and charge variants that may result from differential gene transcription or post-translational modifications (Arámburo *et al.* 2000, 2001a,b; Martínez-Coria *et al.* 2002). This may include oligomerization (into dimers, trimers, quadramers and pentamers) and proteolytic cleavage (into fragments of 15 kDa and 7 kDa) of the monomer (26 kDa) moiety (Arámburo *et al.* 2001b). Small GH moieties of 14 kDa to 17 kDa have also been identified in pituitary extracts and, interestingly, these are preferentially produced in embryonic chicks (Arámburo *et al.* 2000). GH size heterogeneity has also been identified in the testes of embryonic and adult chickens (Luna *et al.* 2000, M Luna, L Huerta, L Berúmen, H Martínez-Coria, S Harvey & C Arámburo, unpublished observations), in which the submonomer variants are the most abundant moieties, particularly in embryonic tissues. Submonomer

GH variants are also present in the eyes of chick embryos, but not in the eyes of neonatal chicks (Takeuchi *et al.* 2001). Submonomer GH moieties, therefore, appear to be more abundant in peripheral extrapituitary tissues than in the pituitary and more abundant in embryos than in neonatal or adult chickens. It is, therefore, possible that these smaller moieties are not fixed by Carnoy's fluid, underestimating the GH immunoreactivity that is detected by the cross-linking of small and large GH proteins during paraformaldehyde fixation. Fixation with Carnoy's fluid does, however, appear to selectively label GH in neural derivatives, and it may be more useful than paraformaldehyde for immunohistochemical studies on neural GH. This selectivity may, interestingly, reflect the fact that no submonomer GH moieties are present in Western blots of hypothalamic and extrahypothalamic brain tissues (Render *et al.* 1995a), whereas submonomer GH moieties are abundantly present in the other non-neural tissues (Render *et al.* 1995a, Luna *et al.* 2000, Harvey *et al.* 2002, M Luna, L Huerta, L Berumen, H Martínez-Coria, S Harvey & C Arámburo, unpublished observations).

In summary, qualitative and quantitative differences in tissue GH immunoreactivity are evident in immunohistochemical studies employing paraformaldehyde and Carnoy's fixatives. The presence of GH immunoreactivity in the embryonic chick is underestimated by fixation in Carnoy's fluid, and caution should be used in interpreting immunohistochemical data obtained with this fixative.

Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada. A E M is in receipt of a studentship from NSERC, and M-L Baudet is in receipt of a studentship from the Alberta Heritage Foundation for Medical Research.

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Received in final form 15 January 2003

Accepted 16 January 2003