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Immunocytochemical demonstration of the binding of growth-related polypeptide hormones on chick embryonic tissues

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Summary. The presence of endogenous growth-related polypeptide hormones, such as growth hormone (GH), somatomadin-C/insulin-like growth factor-1 (SM-C/IGF-1), prolactin (PRL) and Mullerian inhibiting substance (MIS) on chick embryonic tissues have been detected by electron microscopic (EM) immunocytochemistry. Antiserum against GH, anti-SM-C/IGF-1, anti-PRL and anti-MIS were used respectively as primary antibodies for immunolabeling probes by peroxidase (PO) and avidin-biotin complex (ABC)-gold ligands. Cross-reaction studies by ELISA showed negative or weak antigen-antibody interactions. Chick embryos, gonads, and Mullerian ducts (Mds) of various ages were fixed in 2.5% glutaraldehyde for 30 min. Washes in phosphate buffer were administered between each of the following incubations: (*i*) 2% BSA; (*ii*) primary antibody; (*iii*) biotinylated or PO-conjugated secondary antibody; (*iv*) avidin conjugated with gold particles. SM-C/IGF-1 bindings were negative on 1d embryonic disc, heavily stained on 2d endoderm. However, the GH bindings were found on the embryonic layers of 1d and 2d embryos, and increasing on the luminal epithelial cells of Mds during development. PRL was found in parallel with GH, but in less amount. The 10d Mds were double labeled with anti-SM-C/IGF-1-gold and anti-MIS-PO (MIS-PO), and the results showed SM-C/IGF-I negative, but MIS-PO positive bindings. This study provides the first immunocytochemical evidences for: (*i*) The presence of GH, SM-C/IGF-1, PRL and MIS bindings on chick embryonic tissues, and further supports their potential role as growth mediators during embryonic development. (*ii*) The amount of GH and MIS bindings were found correspondingly to their physiological status of Md growth or regression. (*iii*) MIS is secreted by the embryonic gonads.

Introduction

It has been well established that growth hormone (GH) plays the major role in the growth promotion function during postnatal development (Donner 1983; Gluckman et al. 1983; Kan et al. 1984; Maes et al. 1983a, b; Murphy et al. 1983; Wang and Teng 1988). The GH and its receptors have been identified biochemically and radioimmunologically in various cell types, such as hepatocytes (Donner 1983; Gluckman et al. 1983; Maes et al. 1983a, b), epiphysial cartilage chondrocytes (Kan et al. 1984), fibroblasts (Murphy et al. 1983), and lymphocytes (Lesniak et al. 1974; Wang et al. 1989). However, during the fetal development, the mechanism of growth and programmed cell death is still a mystery.

The "somatomedin (SM) hypothesis" proposed that SM is a GH-dependent intermediate in the skeletal growth (Ernst and Froesch 1988; Isaksson et al. 1985). One of the SMs, somatomadin-C/insulin-like growth factor-1 (SM-C/IGF-1), has been found in diverse tissues of human fetus (Han et al. 1987; Sara and Carlsson-Skwirut 1988), and it's action is mostly under the control of GH (Ooi and Herington 1988). Since the liver is the major site for GH-stimulated SM synthesis (Haselbacher et al. 1980; Maes et al. 1983a), hepatocytes are also considered the main target cells of GH (Donner 1983; Gluckman et al. 1983; Simpson et al. 1983). Blood SM content falls following hepatectomy, and increases during liver regeneration (Vassilopoulou-Sellin and Phillips 1982). Reduction of hepatic binding capacity can act as a mechanism for the reduction of SM caused by fasting (Maes et al. 1983b). However, the liver exhibit a variety of responses to GH during development.

Maes et al. (1983b) have reported that, in rodents, high GH levels at birth does not induce high levels of GH-dependent SM, whereas, at puberty, the SM and GH levels increase parallelly. The GH and SM binding activities during hepatocyte membrane development on their target cells as well as during embryonic development therefore play an important role in the action of GH stimulation. Recently, the presence of SM/IGF and its receptor (IGF-R) were identified in 9–12 d mouse embryos (Smith et al. 1987). Similarly, IGF-R has also been found in chick embryonic tissues (Adams et al. 1983). Insulin binding has been found in morula and blastocyst stages of mouse embryos (Rosenblum et al. 1986). These findings support the potential role of IGFs and insulin as growth or differentiation mediators in early stages of development. It is now evident that administration of GH improves growth performance. The feasibility of applying GH as a growth promoter to the animal industry is currently under investigation. However, the relationship of physiological and biochemical actions of GH and SM remains poorly understood. And, there is very little data available with regard to the relationship of hormone binding and biological interactions in the tissues of fetal animals.

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During chick embryonic development, the embryos are ambisexual at 5–6 d with two pairs of reproductive tracts: Mullerian ducts (Mds) and Wolffian ducts (Wds) (Jost 1953; Teng and Teng 1979). The Mullerian or paramesonephric duct ultimately forms the Fallopian tubes, cervix and part of the vagina in mammalian females. The anlage of the male reproductive duct system, i.e., the Wolffian or mesonephric duct, which ultimately forms the epididymis, vas deferens and seminal vesicles. The Wds in the male embryos developed, whereas, the Mds regressed and disappeared by 13 d of incubation under the influence of Mullerian inhibiting substance (MIS) (Donahoe et al. 1976). In the female chick, the left Md is maintained and developed into the oviduct, uterus (shell gland) and cloaca. On the other hand, the right Md ceased to grow at about 9 d and then begins to regress at 11 d of age (Price et al. 1977, 1979). By grafting a piece of testicular tissue near the developing female gonad of rabbit, Jost (1953) indicated that the Wd hypertrophied, however the Md regressed. If a crystal of testosterone instead of a piece of testicular tissue was used, the Wd hypertrophied, but the Md remained unaffected. Thus, Jost suggested that the fetal testis produced both androgen and MIS. This simple experiment also indicated that Md regression is dependent on gonadal secretions. MIS has been proved to be synthesized in the embryonic and adult gonads (Teng 1987). Recently, Ueno et al. (1989) reported that, MIS was localized in the developing ovary of the rat. The aim of the present study is to demonstrate the bindings of growth-related hormones or factors on embryonic discs, on growing or regressing embryonic Mds and corresponding gonads in parallel to their physiological conditions.

Material and methods

Preparation of embryonic disc, Md and gonads. Fertilized chicken eggs obtained from chicken farm were incubated in an humidified incubater kept at 39° C. 1 d and 2 d chick embryonic discs were isolated under the dissecting microscope and rinsed with Dulbecco's phosphate buffer (D-PBS, pH 7.2), fixed in 2.5% buffered glutaraldehyde (GA) at room temperature (RT) for 30 min. The samples were then rinsed overnight in the same buffer and cut transversely into 0.1 to 0.2-mm-thick tissue slices with razor blades.

The right and left Mds of various ages of chick embryos (7, 10, 12, and 13 d) from at least 12 female or male chick eggs were collected. The Mds were straightened on filter paper and fixed with 2.5% GA in D-PBS for 25 min, or in 4% paraformaldehyde plus 0.5% GA in D-PBS for 3 h at RT. The middle one third of Mds were cut into 0.2-mm-short segments in order to expose the lumen epithelium of the ducts to antibodies and ligands applied later. Segments from left and right Mds obtained from chick embryos at each age were pooled. The samples were washed in D-PBS overnight to remove residual aldehyde remained in the tissue. The male and female Mds of 7 d chick embryos were too small to permit isolation. Therefore, they were fixed together with the mesonephros and gonads. After overnight rinse in D-PBS, the samples were embedded in 1% agar and cut transversely with a Sorvall tissue-sectioner at 40 μm. Frozen sections, 25 μm thick, were also obtained after treatment in 10% dimethyl sulfoxide (DMSO) in D-PBS for 1 h at RT. The sections were then washed several times in D-PBS prior to immunolabeling.

Production of antisera for MIS and GH. Wheat-germ agglutinin (WGA) column-purified MIS and HPLC-purified GH were used as antigens. Two female rabbits were immunized for each group according to the procedures reported previously (Wang and Teng 1987; Wang et al. 1989).

Origin of other antibodies. Anti-SM-C/IGF-1 was purchased from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. Prolactin (PRL) and primary antisera for PRL were obtained from Chemicon, El Segundo, CA, USA. Antibodies were diluted at 1:1000 according to the supplier for ELISA assay and at 1:200–1:500 for EM immunocytochemistry in this study.

Enzyme-linked immunosorbant assay (ELISA). Antigen-antibody reaction concentrations used were previously determined by ELISA tests for immunolabeling experiments as described before (Wang et al. 1989).

Electron microscopy immunocytochemistry. Indirect immunocytochemical labeling techniques were performed according to the previous work (Wang et al. 1989). Briefly, the embryonic discs, gonads and Mds samples were washed overnight in D-PBS and then incubated in 1% BSA for 1 h. The Mds in short segments and gonads in frozen sections (25 μm) were incubated in polyclonal antibody solutions for MIS or GH (1:200–1:500 dilution), in SM-C/IGF-1 (1:200), or in PRL (1:200) for 2 h at RT. The tissues were washed with D-PBS containing 0.1% Tween-20 before secondary blocking in 1% BSA. The antigenic sites were visualized with a secondary antibody of goat anti-rabbit IgG conjugated to PO (KPL, 1:200–1:500). Protein-A gold, or ABC-gold (20 nm in diameter) methods (1:50) were used in some groups. For PO-labeled groups, the samples were treated with diaminobenzidine (DAB) containing 0.012% hydrogen peroxide, and then reacted with osmium tetroxide to produce electron dense antigen-ligand deposits. The samples were dehydrated with graded ethanol and embedded in Spurr. Orientation and reembedding were necessary for the Mds. Unstained thin sections were examined under Philips-410 or Zeiss-109 electron microscopies.

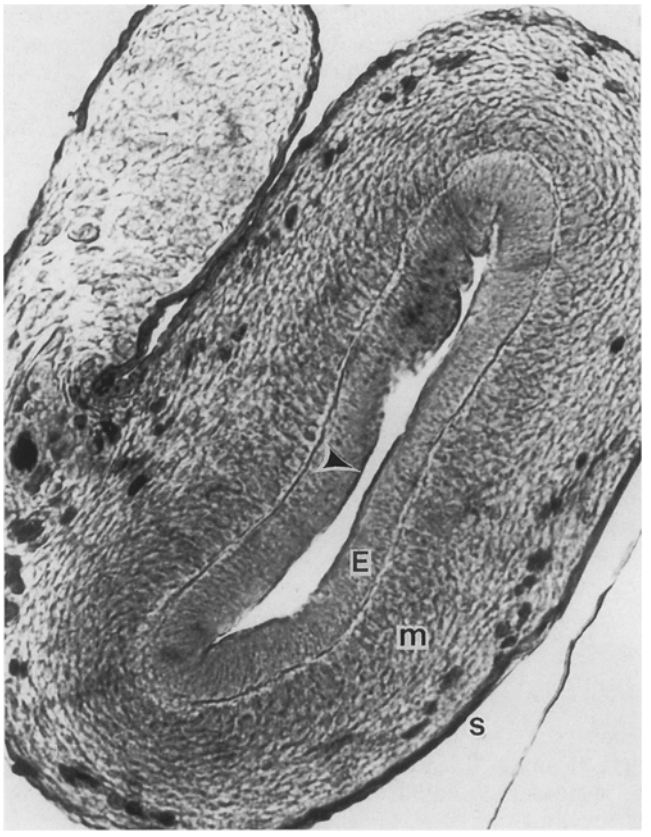


Fig. 1. Histological sections of resin embedded 13 d female left Md stained with toluidine blue O. WGL binding is present on the apical surface (*E*) around the lumen (*arrowhead*). Some of the lectin penetrated into the mesenchyme (*m*) and stained on the surface of serosa (*S*). × 380

Double labeling of SM-C/IGF-1-gold plus MIS-PO, and WGA-PO plus MIS-gold. The 10 d and 13 d Mds were cut into short segments, immunolabeled with SM-C/IGF-1-gold or WGA-PO, then washed thoroughly with D-PBS containing 0.05% BSA. MIS-Ab was labeled secondarily tagged with PO or gold respectively as the first labeling. Color reactions with DAB and osmium staining were performed later.

Quantitative analysis. The homogeneously distributed antigen-ligands on the apical cell surface labeled by the PO-conjugated anti-

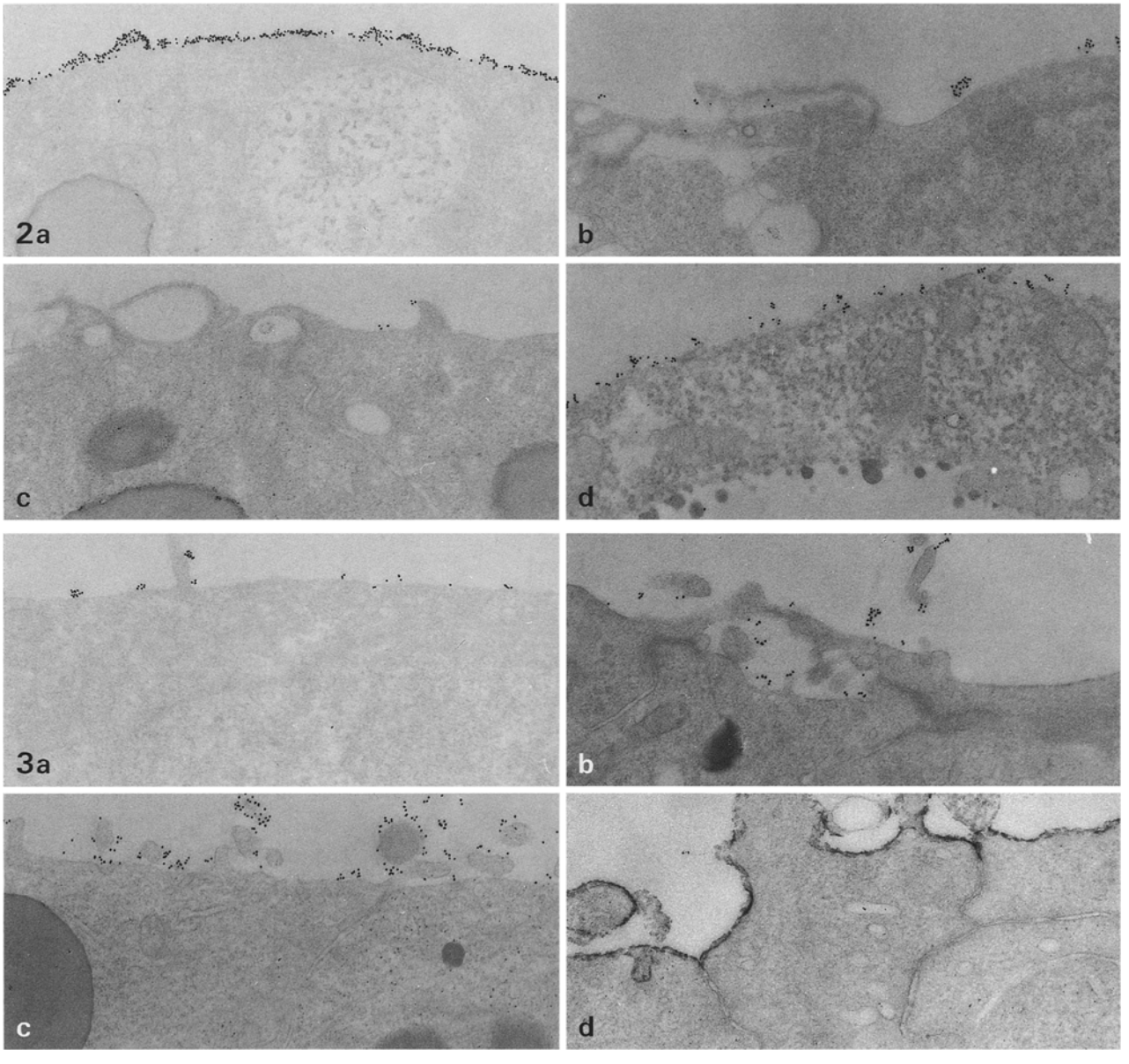
Fig. 2a–d. GH-gold and SM-C/IGF-1-gold binding on chick embryonic discs. **a** GH binding on ectoderm of a 2 d embryo. ×18460. **b** GH binding on endoderm of a 2 d embryo. Note the binding is less than that on the ectoderm. ×18460. **c** SM-C/IGF-1 binding on ectoderm of a 1 d embryo. Note that very little binding occurred. ×18460. **d** SM-C/IGF-1 binding on endoderm of a 2 d embryo. Note the densely bound hormone-gold ligands. ×27000

Fig. 3a–d. PRL and MIS immunolabelings on the embryonic tissues. **a** PRL-gold labeling on a 1 d chick embryonic ectoderm. ×18000. **b** PRL-gold labeling on the apical surface of a 10 d left Md. ×18000. **c** MIS-gold bindings on the 2 d ectoderm of a embryo. ×18500. **d** Double immunolabeling of MIS-PO and then with SM-C/IGF-1-gold on a 10 d left right Md, showed negative SM-C/IGF-1 binding, but positive MIS-PO deposits. ×27000

Table 1. Results of interspecies antibody cross-reactions

Antibodies	Antigens			
	pGH	pPRL	cGH (in situ)	cMIS
Anti-human PRL	−	++	−	−
Anti-porcine GH	++++	−	+++	±
Anti-chicken MIS	±	−	−	++++
Anti-human IGF-1	+	−	−	−

pGH: porcine GH; pPRL: porcine PRL; cGH: chicken GH; cMIS: chicken MIS; "−": negative reaction; "±": weak reaction, "+" to "++++": increasing strong reactions



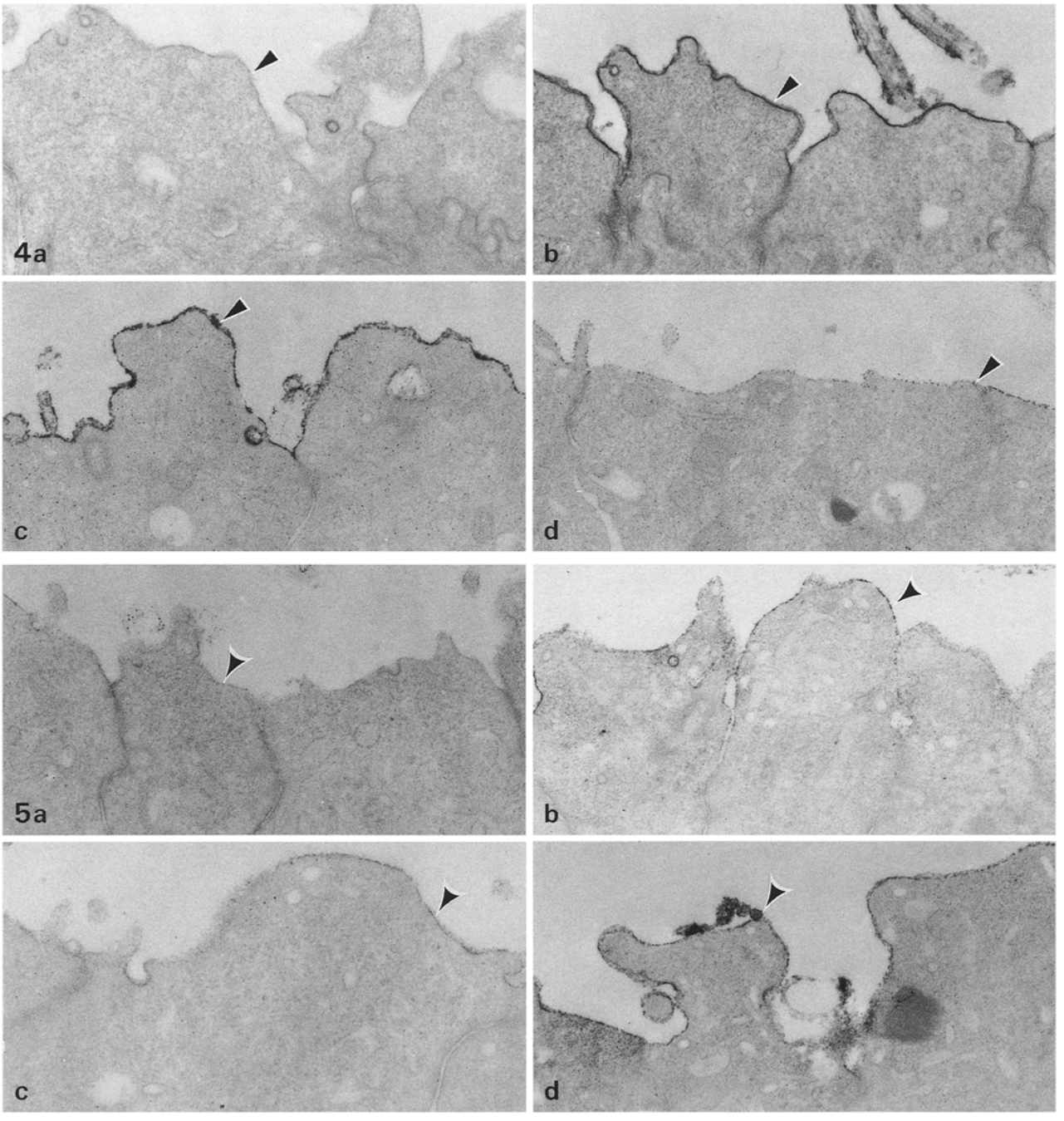


Fig. 4a–d. A comparison of the amount of GH binding on the right and the left female Mds at different ages as revealed by the density of GH-Ab-PO deposits (*arrowheads*) on the apical cell surfaces. **a** Left Md at $7^1/_2$ d of incubation. The average densitometer reading is 0.32 ± 0.03. ×18000. **b** Right Md at $7^1/_2$ d of incubation. The densitometer reading is 3.86 ± 0.03. Note the heavier binding of GH-Ab-PO on the cell surface when compared to the left Md in Fig. 2a. ×18000. **c** Left Md at 12 d with a densitometer reading of 3.45 ± 0.03. ×18460. **d** Right Md of 12 d. The average densitometer reading is 1.65 ± 0.03. Note the density of GH-Ab-PO binding is less than on the left Md in Fig. 1c. ×18460

Fig. 5a–d. A comparison of the amount of MIS binding on the right and the left female Mds at different ages as revealed by the density of the MIS-Ab-PO granules (*arrowheads*) on the apical cell surfaces. **a** Left Md of $7^1/_2$ d. The average densitometer reading is 0.474 ± 0.03. ×18460. **b** Right Md of $7^1/_2$ d. The average reading is 2.286 ± 0.03. Note that the binding is much heavier when compared to Fig. 1a. ×11400. **c** Left Md of 11 d. The average reading is 0.56 ± 0.03. ×18000. **d** Right Md of 11 d. The densitometer reading is 2.90 ± 0.03. Note that the binding is much heavier when compared to Fig. 1c. ×18000

body allowed us to analyze quantitatively with a densitometer (Hoefer's GS 300 scanning densitometer) at OD 580. The transmittance mode directly through the EM negatives was used. The data were analyzed with a modified method (Wang and Teng 1987). The average densitometer reading and the standard deviation for each sample were obtained from 100 to 150 individual readings

from 10 or more EM films taken from 5 different resin blocks of each sample.

Results

The basal light microscopic structures of a 13 d left Mullerian duct (Md) was shown in Fig. 1. Heavy stains of WGA-PO deposits were seen on the serosa epithelium, in the mesenchyme, and in the epithelial cells. Dark staining appeared on the apical surfaces of the epithelium. The results of interspecises cross reactions from ELISA of GH, MIS, PRL and SM-C/IGF-1 bindings were shown in Table 1.

The GH bindings, immunolabeled by colloidal gold, were found on 1 d and 2 d chick embryos both on the ectoderm and endoderm layers in the form of patches or continuous distribution (Fig. 2a and b). SM-C/IGF-1 bindings were rarely found on 1 d embryonic discs (Fig. 2c), but were seen on the ectoderm and endoderm on 2 d chicken embryos (Fig. 2d). The PRL-gold particles were labeled on the 1 d embryonic discs (Fig. 3a), and on the 10 d left Md epithelium (Fig. 3b). MIS molecules were found as early as on 2 d embryonic discs (Fig. 3c). The control groups omitted primary Ab incubation showed negative labeling. The results of double labeling showed negative SM-C/IGF-1-gold but positive MIS-PO bindings on 10d Md (Fig. 3d).

The GH bindings on the left Md (Fig. 4a) is lower than that on the right Md (Fig. 4b). Strong GH binding on the female right Md was seen during early stages of incubation (5–7 d stages) (Fig. 4b). However, at 12 d of incubation, the GH bindings on the left Mds were high (Fig. 4c), that on the right Mds were low (Fig. 4d). GH binding on the male Md (not shown) is as low as that of the female left Md. MIS bindings on the left Md (Fig. 5a) of 7 d female chick embryos is lower than that of the right Md (Fig. 5b). For the 12 d embryos, the MIS bindings were low on the left Mds (Fig. 5c), and those on the right ones (Fig. 5d) were high. The results of double lebeling with lectin-PO and MIS-colloidal gold showed that lectin labeling could not block the recognition of anti-MIS to its antigens on the Mds (Fig. 6a and b). On the right Md (Fig. 6b), MIS-gold particle density was higher than that on the left one (Fig. 6a). Control groups without the primary Ab treatment did not form Ag-ligand complexes (Figs. 6c). The Wds from male or female chicken embryos at different ages, which were processed together with Mds, showed negative MIS binding (Fig. 6d). A granulosa cell in a 10 d female gonad showed MIS-PO positive deposits on the cell surface (Fig. 7). The granules of MIS-PO formed patches on the surface and microvilli as pointed by arrowheads. The quantitative analysis by densitometer reading of MIS and GH bindings on the 7d male and female Mds are summarized in Fig. 8.

The amount of MIS binding on right female Mds, as indicated by both densitometer tracing and the electron micrographs, showed higher binding density than that of the left Mds at corresponding stages of incubation from 7 d to 15 d of incubation and 1 day after hatching with PO labeling (Wang and Teng 1987). The strong MIS binding noticed at 10 d coincide with the initiation of the cranial to caudal regression of the right Md at 11 d. MIS binding on the left Mds (Fig. 5a and c) was consistently lower than that on the right Mds (Fig. 5b and d) at the stage of 12 d of incubation. The results are summarized in Fig. 9 and Table 2.

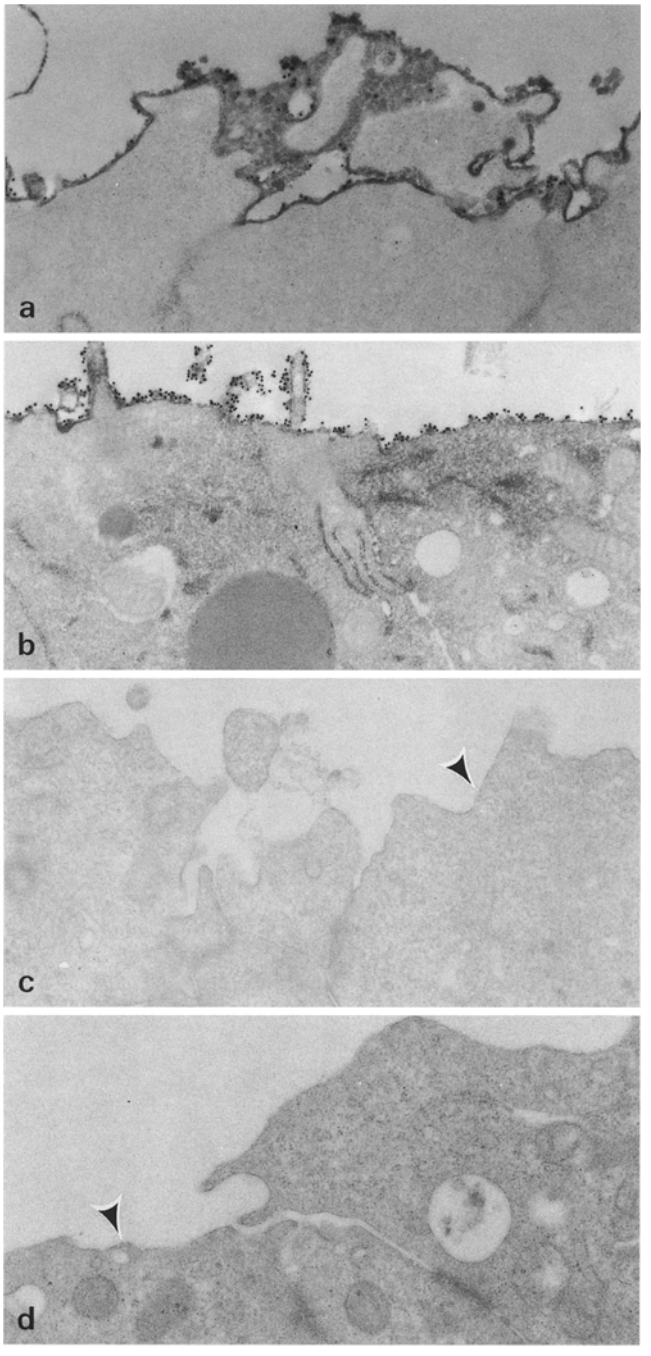


Fig. 6a–b. Double labeling with WGL-PO and MIS-Ab-gold on 13 d Mds. **a** WGL-PO and MIS-ABC-gold labeling on the left Md. The gold particles are seen among the PO labelings. ×11400. **b** WGL-PO and MIS-ABC-gold labeling on the right Md. Note that MIS-gold particles are most numerous on the cells heavily stained with WGL-PO in the cytoplasm. The binding of MS-gold is consistently higher on the right Md than that on the left Md as shown in **a**. ×18460. **c–d** Control groups lack dense granules (*arrowheads*) indicating the absence of polypeptide hormones on their apical surfaces. **c** $7^1/_2$ d female right Md with the primary antibody incubation omitted. The densitometer reading is 0.09 ± 0.01. ×18000. **d** An 11 d Wolffian duct processed together with Mds. The densitometer reading is about 0.09 ± 0.01. ×18000

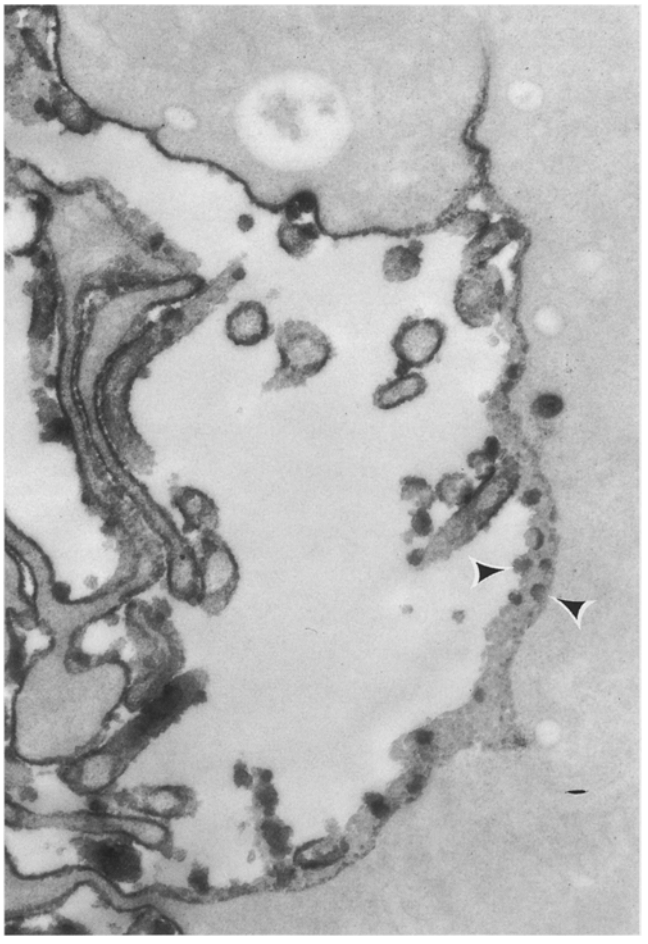


Fig. 7. MIS-PO immunolabeling of a 12 d female gonad after cryosectioned at 25 µm in thickness. Locations of MIS-PO ligands were found on the surface of a granular cell. Dense MIS-PO granules (*arrowheads*) were seen among the homogenous labeling. ×21000

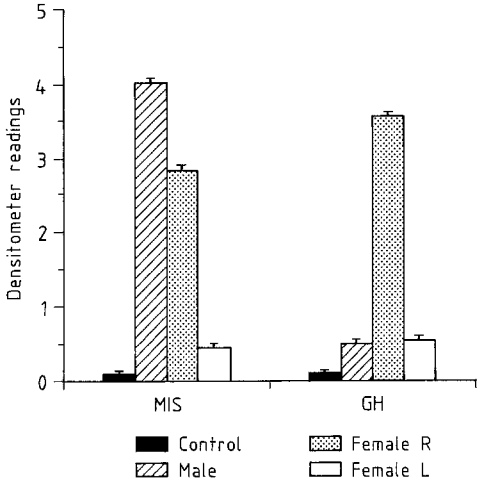


Fig. 8. Quantitative analysis of MIS-PO and GH-PO bindings on the Mds of $7^1/_2$ d chick embryos. The average densitometer readings for control groups are: 0.09±0.01. R, *right* Md; L, *left* Md. *MIS-PO bindings:* male Md, 4,02±0.05; female right Md, 2.85±0.03; female left Md, 0.45±0.01. *GH-PO bindings:* male Md, 0.5±0.09; female right Md, 3.58±0.11; female left, 0.55±0.07

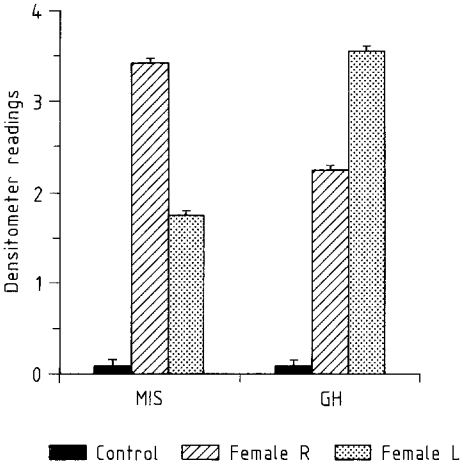


Fig. 9. Quantitative analysis of MIS-PO and GH-PO bindings on Mds of 12 d female chick embryos. The average densitometer readings for control groups are: 0.09±0.01. R, *right* Md; L, *left* Md. *MIS-PO bindings:* right 3.410±0.11, left 2.233±0.06. *GH-PO bindings:* right 1.741±0.07, left 3.542±0.06

Table 2. Summary of results of EM immunocytochemistry

Tissues		Hormones tested					
		PRL	IGF-1		MIS	GH	Control
1 d chick embryo disc	Ecto-	+	±		+	+	−
	Endo-	+	−		+	+	−
2 d chick embryo disc	Ecto-	±	±		++	++	−
	Endo-	±	+++		++	+	−
7 d Md	Epi.	−	−		++	++	−
	Serosa	++	−		+++	++	−
			IGF-1 + MIS				
10 d Md	R	++	−	+++	+++	+	−
	L	++	−	+	++	+++	−
Wolffian duct		/		/	−	/	−

PRL: prolactin; IGF-1: somatomedian-C/insulin-like growth factor-1; GH: growth hormone; MIS: Mullerian inhibiting substance. Ecto-: ectoderm; endo-: endoderm; epi.: epithelium; R: right; L: left "−" means negative reaction; "±" means weak reaction; "+" to "+++" means increasing strong reactions; "/": not detected

Discussion

One of the methods to predict which hormones are important for fetal growth is to measure the hormonal levels in serum. Another way is to measure hormone receptor levels, or more directly, to measure the amount of hormones bound to the receptor on the target tissues. It has been known that polypeptide hormones such as GH and insulin initiate cell responses by binding to the target cell (Gorden et al. 1984; Isaksson et al. 1985; Kovacs et al. 1988; Posner et al. 1985; Roupas and Herington 1987; Wang and Teng 1987). The results of this study indicated that tissues may be capable of binding and responding to various fetal growth-related polypeptide hormones, e.g. GH, SM-C/

IGF-1, PRL and MIS. The fact that endogenous GH binding on Md can be recognized by polyclonal antibody against porcine GH indicates that: (*i*) the antibody is not species specific, and (*ii*) GH is involved in the differentiation of embryonic tissues. However, GH acts most likely through SM in its growth promoting function. Evidence is accumulating to show that SMs are involved in fetal growth. There is a positive correlation between SM levels and the birth size of human infants. Laron's dwarfs, who have low SM levels despite high GH levels, show a reduction in birth weight (Laron and Pertzelan 1969). Receptors for SM/IGF (IGF-R) have been detected in a variety of fetal tissues (E'Ercole et al. 1976, 1980). Those humans born slightly shorter than normals may have GH deficiency, however, the actual cause may be due to a SM deficiency (Laron and Pertzelan 1969). These events indicate that GH has some indirect effect on intrauterine growth (Laron 1983).

SM concentration is maintained by chorionic somatomammotropin instead of pituitary GH after hypophysectomy. Gluckman et al. (1983) suggesting that GH does not play a major role in the prenatal growth of mammals because of a lack of somatogenic receptors in the fetal liver. Moreover, GH binding to tissues displays a complex heterogeneity (Posner et al. 1985; Simpson et al. 1983). GH, prolactin (PRL) and placental lactogen all share common amino acids in their molecules, and there are also overlaps among receptor interactions of GH, PRL and the placental lactogen. The cross-reactions between human PRL-Ab and porcine GH, the GH-Ab and chick PRL as tested by the ELISA are negative. It is well established that PRL functions as a growth hormone in larval amphibians (Nicoll 1974). PRL has also been suggested to be a fetal growth promoting factor in non-mammalian (Nicoll 1979; van Zoelen et al. 1989) and mammalian species (Sinha and Vanderlaan 1982). These authors suggest that the growth rate of mice embryos is remarkably reduced under conditions of low PRL concentration which can be produced by treatment with PRL-Ab or by administration of PRL inhibitors, e.g. bromocriptine (CB-154) (Nicoll 1979). In contrast to GH, PRL seems to be more important to the growth of the embryo. The observations of early bindings of GH, PRL in 1 d chick embryonic discs support the concept that both GH and PRL are essential at early infancy for the normal growth and development (Sinha and Vanderlaan 1982; van Zoelen et al. 1989).

Accumulative evidences indicated that insulin (Rosenblum et al. 1986), SM/IGFs and IGF-R (Bassas et al. 1985; Smith et al. 1987) were identified in early embryonic stages, and also in an embryonic carcinoma cell line (van Zoelen et al. 1989). One of the SMs, SM-C/IGF-1 binding has been proven to be positive in the 2 d but negative on 1 d chick embryonic disc by this immunocytochemistry. These observations were supported by similar results from biochemical methods on 2 d chicken embryo discs (Bassas et al. 1985). These findings suggest the potential role of SM-C/IGF-1 being growth or differentiation mediators in early embryonic stage. However, SM-C/IGF-1 binding was negative on Mds of $7\ ^1/_2$ d and 10 d chick embryos. Possibly, the early actions of SM-C/IGF-1 in chick embryos could be an independent event to GH actions. Parkes and Hill (1985) also suggested that SM-C/IGF-1 is GH independent in the development of fetal lambs. However, it is now evident that the administration of GH alone improves growth rate. The experiment of Heggestad and Wells (1965) indicated that decapitation of rat fetuses showed a significant growth defect, which can be prevented by GH replacement. The fetal pituitary gland also showed a promotive effect on the growth of monkeys (Novy et al. 1981) and sheep (Bassas et al. 1985; Liggins and Kennedy 1968). GH has been reported to increase the levels of SM-C/IGF-1 secreted by adult rat fibroblasts (Bassas et al. 1985). SM-C/IGF-1 could afterwards, inhibit the release of GH in the pituitary gland (Lamberts et al. 1989), and even alters the mRNA levels of GH in vitro (Namba et al. 1989). According to this study, before the appearance of pituitary gland (13 d of incubation), a high degree of GH binding is related to the growth of the female right Md at the early stage of $7\ ^1/_2$ d of incubation. The steady increase of GH binding on the left Mds during development parallels the differentiation and stabilization of the ducts. The disappearance of SM-C/IGF-1 in late developmental stages of growing Mds indicated that SM-C/IGF-1 could be induced by GH when needed. Recently, Ernst and Froesch (1988) also suggested that in vitro stimulation of osteoblast-like cells is partially mediated by SM-C/IGF-1, and it is GH dependent. The origin of these growth-related hormones was still a mistrey. Recently, van Zoelen et al. (1989) suggested that IGF-1 is secreted by the embryonic cell it self, and an autocrine and paracrine growth stimulation processes existed at early embryonic development.

The glycoprotein hormone, MIS (Donahoe et al. 1976) or anti-Mullerian hormone (AMH, Josso 1986), is a Md regressor (Donahoe et al. 1976; Josso 1986; Teng et al. 1987; Wang and Teng 1987). However, the mechanism of Md regression induced by MIS and the basis for high secretory activity after hatching remains to be established. In chicks, purified MIS was found to be present as a monomer of 74000 Da by Teng and colleagues (Teng et al. 1987). MIS is secreted by Sertoli cells or granulosa cells in the embryonic gonads, testis or ovary, respectively (Hutson et al. 1981, 1985; Teng and Teng 1979; Teng et al. 1987; Tran and Josso 1982; Tran et al. 1987). The presence of MIS was also verified in embryonic or post-hatching testes by coculturing with Mds in vitro (Teng et al. 1987). MIS secretion reached its greatest level in 10–11 d embryonic gonads and decreasing during the following development (Wang and Teng 1987). At post-hatching stages, MIS reaches its highest level at about 12 weeks and disappears in adult testes (Teng 1987). Recent evidences which indicated that MIS is located in the gonads of embryos (Hutson et al. 1981; Teng 1987; Ueno et al. 1989), supported our EM immunocytochemical results.

According to our observations, MIS binds to the cell surface of its target tissue, the Mds, to a possible molecule of MIS-specific receptor, in order to initiate its physiological role. The highly purified and biologically active avian MIS has been obtained previously according to the method of Teng et al. (1987). Polyclonal antibodies were used mainly for the EM immunocytochemical labeling. The results of this study confirmed the specific binding of MIS to the liver as well as epithelial cells of Mds during development. The positive binding of MIS on the liver cell might indicate a possible way of degradation of the excess MIS in the embryo. The MIS binding was not evident on the epithelial cells of the Wd (Teng 1987). However, it is found on the embryonic disc as early as 2 d of incubation (Fig. 3c). The reason for early MIS bindings on the embryo

is unknown. The role of MIS-specific receptor in mediating MIS-Ab binding is also uncertain. Further work is undergoing to characterize the avian- or mammalian-specific MIS receptors.

There is no doubt that Md regression is under the control of MIS, whereas, the mechanism of hormone-triggered cell death (male and female right Md) (Price et al. 1977) and cell growth (female left Md) is still a mistery. It seems that the growing Md were protected by the binding of GH. It has been confirmed by the quantitative analysis of densitometer reading of immuno-peroxidase labeling that the regressing of male Mds and female right Md corresponds to the high intensity of MIS binding. The density changes of MIS binding patterns on the target cells also parallel the MIS content in embryonic chick gonads by an ELISA method (Teng 1987). The consistency of the two independent experiments and EM immunocytochemical evidences of MIS-PO bindings in the female gonad further supports the concept that the amount of MIS secreted from the gonads has a direct action on Mds by the binding of MIS to its target cells. These data also indicate that the direct detection of the bound hormones on target tissues is a dependable method (Wang et al. 1989).

The monoclonal antibody (MAb) for MIS has been produced in mammals (Vigier et al. 1984), and has been used to identify the intracellular sites of the hormone in Sertoli cells (Tran and Josso 1982; Tran et al. 1987). The MIS-MAbs raised in this laboratory has been shown to bind to the Mds, but is not suitable for quantitative analysis. MIS-MAb has been used to detect and characterize the epitopes of the hormone, especially to identify the variations presented by the avian and mammalian MIS molecules (unpublished data).

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