Expression of insulin, insulin-like growth factor I and glucocorticoid receptor in rat uterus and embryo during decidualization, implantation and organogenesis

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The significance of insulin, insulin-like growth factor I (IGF-I) and glucocorticoids to the early mammalian embryo is clear in that they are key regulators of both mitogenic and metabolic effects during development. In the present study, the temporal sequence of expression of the respective receptor proteins was investigated for the first time in the developing rat utero-embryonic unit between conception and day 12 of gestation using immunocytochemistry. Insulin, IGF-I and glucocorticoid receptor were expressed in embryonic tissues after the start of implantation, and were co-localized in the primary ectoderm, extraembryonic ectoderm as well as in the ectoplacental cone. The parietal endoderm was devoid of glucocorticoid receptor staining, whereas IGF-I receptor was absent in visceral endoderm. After completion of basic organogenesis, the neural tube, notochord, otic placode, Wolffian duct, mesonephros and intestinal tube

expressed insulin, IGF-I and glucocorticoid receptor. The glucocorticoid receptor was not expressed in heart tube and dorsal aortae. Considerable amounts of insulin receptor were detected in trophoblast-derived giant cells. In the uterus, luminal epithelium, endometrial stromal and myometrial smooth muscle cells immunoreacted with antisera against insulin, IGF-I and glucocorticoid receptor. Endometrial glands remained negative for the glucocorticoid receptor throughout the gestational period investigated. Uterine hormone receptor expression reached a peak at days 4 and 5 of gestation in endometrial stromal cells and decidua, respectively. In conclusion, the demonstrated ontogenetic pattern of insulin, IGF-I and glucocorticoid receptor expression indicates the potential sites of biological action of the respective ligands, providing supportive evidence for their critical importance during the course of embryogenesis in rats.

Introduction

From the earliest stages of embryogenesis, signalling occurs between the conceptus and the uterus, as well as between the cells of the endometrium itself (Salamonsen, 1992). Both the embryo and somatic cells in the maternal reproductive tract release growth factors, transcription regulators and classical hormones that may act in an autocrine or paracrine fashion. The optimal combination of these agents is important for establishing a receptive environment conducive to embryo implantation, subsequent maintenance of pregnancy and induction of specific developmental responses in the embryo (Schultz and Heyner, 1993). The precise roles of many of the factors in co-ordinating these processes are yet to be defined, but the central

role of insulin, insulin-like growth factor I (IGF-I) and glucocorticoids is now firmly accepted.

Insulin exerts its well-known anabolic effects (stimulation of glucose incorporation into macromolecules and protein synthesis, inhibition of lipolysis) on the embryo, and this is substantiated by the finding that the delay in development observed with diabetes is reversible by insulin administration (Diamond *et al.*, 1989; Moley *et al.*, 1991). In addition, this hormone promotes RNA and DNA synthetic activities and proliferation of embryonic cells (Kaye and Harvey, 1995). Insulin also appears to play very important roles in the regulation of synthesis of steroid and peptide hormones, such as oestradiol, progesterone, chorionic gonadotrophin and placental lactogen, during gestation (Desoye and Shafrir, 1994).

IGF-I exhibits a high degree of amino acid similarity with insulin and activates a signalling pathway that converges with that of insulin. However, its individual significance for normal development has been illustrated

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by the severe growth-retarded phenotype exhibited by IGF-I or IGF-I receptor knockout mice. IGF-I has been shown to participate in the regulation of all the processes necessary for organogenesis, that is cell proliferation, cell metabolism and cell differentiation (Cohran *et al.*, 1996). In uterine cells, IGF-I is considered to mediate the mitogenic effects of oestrogen throughout the oestrous cycle and during gestation (Girvigian *et al.*, 1994). Not least, IGF-I increases the glucose uptake response to insulin, thus having an 'insulin-sparing' effect in carbohydrate metabolism (Zierler, 1999).

Glucocorticoids are also potent morphogenetic factors providing key signals in cellular differentiation and are involved in the development and maturation of various fetal organ systems (Ballard, 1979). The hormones present interfere with the expression of insulin and IGF-I receptor and many cell cycle progression factors (Price *et al.*, 1992), generally opposing the mitogenic and metabolic actions of insulin and IGF-I (Reinisch *et al.*, 1978; Hahn *et al.*, 1999). In the uterus, glucocorticoids regulate the synthesis of prostaglandins that have been implicated to play critical roles during implantation by increasing stromal vascular permeability (Kennedy, 1983) and in the initiation of parturition (Lopez-Bernal *et al.*, 1995). Glucocorticoids contribute to preventing immunological rejection of the fetal semiallograft in the pregnant uterus by inhibiting eosinophil infiltration (Tchernitchin *et al.*, 1975). Moreover, glucocorticoids profoundly and specifically suppress expression of fibronectin and laminin, two extracellular matrix proteins that are important mediators of uterine-placental adherence (Guller *et al.*, 1994).

As a common adverse feature, insulin (Shafir, 1996), IGF-I (Langley-Evans *et al.*, 1996) as well as glucocorticoids (Barker, 1995; Hahn *et al.*, 1994) might imprint response patterns during specific windows of fetal development that persist throughout life and have been invoked to explain the links between early life events and later development of abnormal structure, function or serious disease, such as hypertension, non-insulin-dependent diabetes and ischaemic heart disease.

The rat is an important experimental model for the study of the molecular and cellular events leading to the co-ordinated proliferation and differentiation of embryonic and uterine cells during early mammalian development. As insulin, IGF-I and glucocorticoids can act along endo-, para- and autocrine pathways, it is essential to have a clear picture of the cellular distribution pattern of the respective receptors, because only their expression indicates the potential sites of biological action. There is surprisingly little information available on insulin receptor (IR), IGF-I receptor (IGF-IR) and glucocorticoid receptor (GR) expression of the rat pregnant uterus and early embryo. The scarce data from other species are of limited value, because of profound differences in embryonic development even between mice and rats (Zhang *et al.*, 1994; Kaye and

Harvey, 1995). The objective of the present study was therefore to document the expression of IR, IGF-IR and GR during the course of rat embryogenesis until day 12 of gestation.

Materials and Methods

Animal experiments were conducted in accordance with UK legal requirements. White Wistar rats (14–16 weeks old, mean body weights 205 ± 18 g) were kept under a 12 h light: 12 h dark cycle (light: 06:00–18:00 h) under standardized conditions (22°C; constant humidity $60\% \pm 5\%$) with free access to standard diet and tap water. They were mated between 08.00 and 10.00 h and impregnated females were identified by the presence of copulatory plugs 24 h after mating; this day was designated day 1 of gestation. Samples were collected each day from day 1 to day 9 of gestation and at day 12. Four rats were investigated at any one time at days 1–4, days 7–9 and day 12 of gestation. Eight rats were investigated at any one time at days 5 and 6 of gestation.

Immunocytochemistry was performed on paraffin wax embedded sections of the developing rat utero-embryonic unit. Briefly, 1 ml of 1% (v/v) Evans blue in 0.9% (w/v) NaCl was injected into the posterior right femoral vein of impregnated rats ($n=48$) under deep ether anaesthesia to identify the implantation areas within the uterus. Ten minutes after the injection, the anterior abdominal wall was opened and tissue samples were taken from parts of the uterus containing the dye. These samples were fixed in Bouin-Hollande's fixative (Romeis, 1989) for 4 h, embedded, and serial sections (6 μ m) were collected on SuperFrost Plus slides (Novoglas, Berne). After rehydrating, samples were transferred to 0.01 mol citrate buffer l^{-1} (pH = 6) and subsequently heated in a microwave oven for 2×5 min at 750 W for retrieval of antigen. After cooling for 20 min at room temperature, the sections were washed with PBS. Endogenous peroxidase activity was removed by keeping sections in 3% (v/v) H_2O_2 diluted in methanol for 20 min and afterwards washing with PBS. Sections were incubated for 60 min at room temperature in a moist chamber with 1:100 diluted rabbit antisera against amino acid sequences mapping at the β subunit cytoplasmic domains of IR (Transduction Laboratories, Lexington, KY), IGF-IR (Santa Cruz Biotechnology, Heidelberg) and the amino terminus of GR (Santa Cruz Biotechnology). Labelling was visualized using the DAKO EnVision + System (Dako, Carpinteria, CA) based on a dextran polymer to which about 20 secondary antibodies and 100 horseradish peroxidase molecules were bound.

Each section was examined at low power to identify regions of interest. Fields within the chosen region were randomly selected for scoring by putting the slide out of focus, moving the microscope stage and refocusing at $\times 400$ magnification. Staining intensities were scored

Table 1. Distribution of insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR) and glucocorticoid receptor (GR) in the rat utero-embryonic unit

Table 1a

Day of gestation	Uterus epithelium	Endometrial stroma	Endometrial glands	Myometrial smooth muscle	Decidual zones	Parietal endoderm	Visceral endoderm	Primary ectoderm	Extraembryonic ectoderm	Ectoplacental cone	Mesoderm	Ectoderm	Receptor
1	1.2 ± 0.4	0.8 ± 0.3	0.9 ± 0.5	1.9 ± 0.4									IR
	2.1 ± 0.6	1.0 ± 0.4	2.1 ± 0.4	0.7 ± 0.2									IGF-IR
	1.1 ± 0.4	1.0 ± 0.3	0.1 ± 0.2	1.4 ± 0.5									GR
2	0.6 ± 0.3	1.8 ± 0.4	1.3 ± 0.4	2.3 ± 0.5									IR
	2.0 ± 0.2	1.3 ± 0.3	2.2 ± 0.3	1.0 ± 0.2									IGF-IR
	0.8 ± 0.4	0.7 ± 0.4	0.3 ± 0.3	0.8 ± 0.5									GR
3	1.7 ± 0.6	1.9 ± 0.3	1.1 ± 0.3	2.0 ± 0.3									IR
	2.1 ± 0.3	2.1 ± 0.4	2.3 ± 0.4	0.7 ± 0.3									IGF-IR
	1.9 ± 0.3	2.0 ± 0.1	0.3 ± 0.2	1.2 ± 0.6									GR
4	2.0 ± 0.3	2.7 ± 0.6	1.0 ± 0.5	1.8 ± 0.3	2.8 ± 0.6								IR
	1.9 ± 0.4	2.8 ± 0.7	2.2 ± 0.5	0.7 ± 0.3	2.9 ± 0.7								IGF-IR
	2.7 ± 0.5	2.4 ± 0.6	0.4 ± 0.3	1.3 ± 0.4	2.3 ± 0.7								GR
5	2.2 ± 0.3	2.8 ± 0.6	1.1 ± 0.4	1.8 ± 0.4	2.8 ± 0.5								IR
	2.0 ± 0.2	2.7 ± 0.3	1.9 ± 0.3	0.8 ± 0.4	2.9 ± 0.3								IGF-IR
	2.6 ± 0.6	2.3 ± 0.6	2.8 ± 0.6	1.3 ± 0.5	2.8 ± 0.4								GR
6	0.1 ± 0.3	2.1 ± 0.5	0.6 ± 0.4	1.8 ± 0.4	1.9 ± 0.2	0.9 ± 0.3	2.1 ± 0.4	2.3 ± 0.5	1.9 ± 0.4	0.8 ± 0.4			IR
	1.2 ± 0.2	1.2 ± 0.5	2.2 ± 0.3	0.9 ± 0.3	1.0 ± 0.4	1.4 ± 0.4	0.4 ± 0.5	2.9 ± 0.4	2.1 ± 0.2	1.8 ± 0.7			IGF-IR
	2.1 ± 0.3	2.9 ± 0.6	0.4 ± 0.2	1.2 ± 0.3	2.7 ± 0.4	0.3 ± 0.3	1.1 ± 0.1	0.7 ± 0.3	2.4 ± 0.7	1.3 ± 0.4			GR
7	0.8 ± 0.4	2.0 ± 0.1	0.9 ± 0.5	2.1 ± 0.3	1.8 ± 0.4	1.2 ± 0.4	1.8 ± 0.3	2.1 ± 0.4	2.4 ± 0.5	1.9 ± 0.2			IR
	2.3 ± 0.4	2.1 ± 0.2	1.7 ± 0.6	0.9 ± 0.3	2.2 ± 0.5	0.8 ± 0.2	0.2 ± 0.5	2.7 ± 0.6	2.6 ± 0.8	1.8 ± 0.3			IGF-IR
	1.9 ± 0.5	2.7 ± 0.5	0.5 ± 0.6	1.1 ± 0.4	2.8 ± 0.3	0.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.3	0.8 ± 0.3	1.0 ± 0.2			GR
8	0.7 ± 0.4	1.1 ± 0.2	1.1 ± 0.2	2.0 ± 0.3	1.1 ± 0.3	0.7 ± 0.3	1.0 ± 0.2	0.7 ± 0.3	1.2 ± 0.3	1.4 ± 0.3			IR
	0.9 ± 0.2	1.2 ± 0.1	2.3 ± 0.5	1.2 ± 0.4	1.2 ± 0.2	0.8 ± 0.4	0.4 ± 0.7	2.9 ± 0.4	1.8 ± 0.4	2.1 ± 0.4			IGF-IR
	2.0 ± 0.3	2.8 ± 0.4	0.2 ± 0.3	0.9 ± 0.2	2.7 ± 0.4	0.3 ± 0.2	0.9 ± 0.3	1.2 ± 0.4	1.2 ± 0.4	1.0 ± 0.3			GR
9	0.8 ± 0.3	2.3 ± 0.2	0.7 ± 0.4	2.2 ± 0.4	2.3 ± 0.5	2.0 ± 0.3	1.8 ± 0.4			2.1 ± 0.2	2.8 ± 0.3	2.9 ± 0.6	IR
	1.1 ± 0.2	1.2 ± 0.3	2.3 ± 0.4	1.4 ± 0.6	2.1 ± 0.5	1.0 ± 0.2	0.3 ± 0.4			1.9 ± 0.3	2.0 ± 0.1	1.9 ± 0.6	IGF-IR
	2.2 ± 0.5	2.2 ± 0.7	0.4 ± 0.3	0.7 ± 0.4	1.8 ± 0.3	1.2 ± 0.3	0.9 ± 0.2			0.9 ± 0.2	1.2 ± 0.4	1.3 ± 0.4	GR

Hormone receptors in rat uterus and embryo

Table 1b

Day of gestation	Neural tube	Notochord	Otic placode	Dorsal aortae	Heart tube	Wolffian duct	Mesonephros	Intestinal tube	Giant cells	Parietal yolk sac epithelial cells	Visceral yolk sac epithelial cells	Endometrial stromal cells	Receptor
12	1.8 ± 0.4	1.1 ± 0.2	2.3 ± 0.5	1.3 ± 0.3	0.9 ± 0.2	0.8 ± 0.2	1.3 ± 0.4	1.2 ± 0.3	1.9 ± 0.5	2.1 ± 0.5	2.7 ± 0.5	2.7 ± 0.3	IR
	2.1 ± 0.5	0.7 ± 0.4	1.1 ± 0.2	1.8 ± 0.3	2.2 ± 0.4	1.2 ± 0.3	1.3 ± 0.5	1.2 ± 0.2	0.5 ± 0.4	0.9 ± 0.4	1.7 ± 0.5	2.1 ± 0.3	IGF-IR
	0.8 ± 0.4	0.9 ± 0.3	1.2 ± 0.5	0.3 ± 0.3	0.2 ± 0.3	0.8 ± 0.4	1.2 ± 0.2	0.7 ± 0.4	0.4 ± 0.3	0.5 ± 0.4	1.9 ± 0.2	2.8 ± 0.7	GR

Mean ± SD values for staining intensities of IR, IGF-IR and GR in the developing rat utero-embryonic unit between day 1 and day 12 of gestation, scored on a scale of 0 (no staining) to 3 (intense staining). Empty cells in the table indicate that the tissue concerned had either not yet differentiated or was not investigated at the respective day of gestation.

on a scale of 0 to 3: no visible or very low ambiguous staining (borderline) was scored as 0; weak staining as 1; and clearly visible, progressively intense, staining as 2 (moderate) or 3 (strong). Two observers unaware of which receptor the samples were stained for, analysed the sections independently. From each rat a minimum of four sections per receptor was examined. Inter-animal variation did not reach the level of statistical significance by one-way non-parametric ANOVA (Kruskal–Wallis procedure) followed by the Mann–Whitney U test.

For negative controls, sections from each developmental stage were incubated with IGF-IR and GR antisera pre-adsorbed with corresponding peptides based on the sequences used for the immunization of the antibody-generating rabbits. In addition, specimens were incubated with non-immune rabbit serum (Dako) instead of the primary antibodies. The IR antiserum used in this study was produced by immunizing rabbits with a 20.6 kDa protein fragment corresponding to positions 441–620 of the mature receptor protein. Because of the size of the antigen, it seemed reasonable to assume that the antiserum contained antibodies against several different epitopes. Therefore, it was not useful to apply the pre-adsorption method and negative control experiments for IR immunoreactivity were performed only by replacing the primary antiserum with normal rabbit serum (Dako).

Blood cells present in the sections were used as internal positive controls. The sections were counterstained with hemalaun (Merck, Darmstadt) and mounted with Kaiser's glycerol gelatin (Merck). Photographs were taken with an Axiophot microscope (Zeiss, Oberkochen).

Results

The distribution of IR, IGF-IR and GR in the rat utero–embryonic unit (Table 1) showed the following major findings.

Days 1–3 of gestation

During the first 2 days of gestation the IR (Fig. 1a) and IGF-IR antisera immunoreacted with the uterine epithelium, the endometrial stromal cells, the epithelium of the endometrial glands and also the myometrial smooth muscle cells. GR distribution differed in that the antibodies did not label the endometrial glands, which

remained negative throughout the whole gestational period investigated. IR and GR staining became more apparent at day 3 of gestation in the uterine epithelium and endometrial stroma, whereas IGF-IR labelling intensity increased only in the endometrial stroma.

Days 4 and 5 of gestation

Implantation starts at day 5 of gestation and the rat blastocyst that became visible in the sections (Fig. 1b) was immunopositive for the three receptors examined. At this stage, the first two cell lineages are established and the first intraembryonic fluid formed. The inner cell mass gives rise to the embryo proper and the trophoctoderm forms the placenta. With the exception of the parietal and visceral endoderm, respectively, the reaction pattern of subsequently emerging embryonic cell populations remained roughly constant until day 8 of gestation, apart from further increases in IGF-IR staining intensity (see Fig. 1e,g). Uterine IR (Fig. 1b) and IGF-IR immunoreactivity peaked in endometrial stromal cells with progressing decidualization. In contrast to the homogeneously distributed IR signal within the luminal uterus epithelial layer of the newly impregnated rats (Fig. 1a), IR staining in the uterus epithelium at days 4 and 5 of gestation was more pronounced in the antimesometrial region (Fig. 1b) where implantation usually takes place. In addition, the uterus epithelium showed high GR content. Adjacent primary endometrial decidual cells expressed abundant IR (Fig. 1b), IGF-IR and GR.

Day 6 of gestation

IR (Fig. 1d) and IGF-IR (Fig. 1e) expression was markedly reduced in the uterus at day 6 of gestation. In contrast, GR staining was increased even in endometrial stromal cells. Primary as well as the newly emerging secondary decidual cells also showed significant amounts of GR (Fig. 1f). In general, maternal tissue GR was detectable predominantly in the nuclei, whereas embryonic GR was located in the cytoplasm. Parietal endodermal, visceral endodermal, primary ectodermal, extraembryonic ectodermal cells as well as the ectoplacental cone immunoreacted with the IR antiserum. IGF-IR was strongly expressed in the primary ectoderm, but was absent in visceral endodermal cells

Fig. 1. Transverse sections of the developing rat utero–embryonic unit from days (a) 1, (b,c) 5, (d–f) 6 and (g,h) 7 of gestation, immunolabelled for insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR) or glucocorticoid receptor (GR). Red arrows, uterine luminal epithelium; green arrows, endometrial glands; white arrows, ectoplacental cone; yellow arrows, parietal endoderm; black arrows, visceral endoderm; black arrowhead, embryo; green arrowheads, primary ectoderm; pink arrowheads, extraembryonic ectoderm. DEC, decidua; ESC, endometrial stromal cells; UL, uterus lumen. Sections incubated with peptide pre-adsorbed IGF-IR and GR antisera or non-immune rabbit serum (as negative control for IR staining) are shown as insets. Scale bars represent (a) 100 μ m, (b) 60 μ m, (c) 50 μ m, (d–f) 20 μ m and (g,h) 40 μ m.

Fig. 1. For legend see opposite page.

Fig. 2. For legend see opposite page.

(Fig. 1e). The parietal endoderm represented the site of lowest hormone receptor expression, as it was completely devoid of GR (Fig. 1f).

Days 7–9 of gestation

The IR, IGF-IR and GR expression of the uterus at days 7–9 of gestation roughly resembled that at day 6. Significant GR labelling was found in primary and secondary decidual cells (Fig. 1h), whereas IR and IGF-IR (Fig. 1g) of the decidual zones were detected at more moderate amounts. In visceral endodermal, primary ectodermal and extraembryonic ectodermal cells, IR staining was slightly reduced at day 8 of gestation when compared with days 6 and 7. Conspicuous IR expression was visualized with the beginning of organogenesis at day 9 of gestation in ectodermal and mesodermal cells of the embryo, accompanied by immunoreactivity with IGF-IR and GR (Fig. 2a).

Day 12 of gestation

Among the organ anlagen investigated, notochord, Wolffian duct, mesonephros and intestinal tube of the rat embryo were weakly labelled for IR (Fig. 2b), IGF-IR (Fig. 2c) and GR at this mid-organogenesis stage (day 12 of gestation). Neural tube and the otic placode immunoreacted more intensively for IR (Fig. 2b), as did neural tube, heart tube and dorsal aortae for IGF-IR (Fig. 2c). GR could not be detected in the heart tube and dorsal aortae. The trophoblast-derived giant cells were exclusively labelled with IR antiserum and remained negative for IGF-IR and GR (Fig. 2d–f). Parietal as well as visceral yolk sac epithelial cells were endowed with IR and IGF-IR (Fig. 2d,e). Both growth factor receptors stained more intensively in the latter cells. GR was visualized in visceral, but not in parietal yolk sac epithelial cells (Fig. 2f). The tendency to declining IR and IGF-IR labelling demonstrated between days 6 and 9 of gestation in the endometrial stromal cells was found to be reversed at day 12 (Fig. 2d,e). The expression of GR was continuously high in this tissue (Fig. 2f).

Controls

Replacement of the antisera with non-immune rabbit serum or pre-incubation of the IGF-IR and GR antisera with the immunizing peptides, respectively, resulted in

the complete absence of immunoreactivity or at least a drastically reduced staining (insets in Figs 1 and 2). Labelling of fetal blood cells present in the heart tube (Fig. 2b,c) and of maternal leukocytes that infiltrated the endometrial stroma (Fig. 2d–f) provided internal positive controls for receptor immunoreactivity.

Discussion

Insulin, IGF-I and glucocorticoids are present in the maternal reproductive tract (for review, see Kaye and Harvey, 1995; Heyner, 1997), and are thus able to act as key regulators of mitogenic and metabolic effects not only to cells of the uterus, but also to the growing embryo even before the chorioallantoic circulation is established. The responsiveness of a tissue to these hormones is primarily determined by the number of receptors in target cells. Although the rat has proven to be a highly informative model for investigating early mammalian development in numerous studies, the present work is the first to delineate the cellular sites of expression of IR, IGF-IR and GR from conception until completion of basic organogenesis in this species.

IR is a heterotetrameric protein comprising two α subunits that bind the ligand and two β subunits that confer the signalling properties to the receptor by its intrinsic tyrosine-specific kinase. There is evidence that at least an alternatively spliced form of IR can interact with IGFs in addition to insulin, implicating IR in their growth-promoting effects (Frasca *et al.*, 1999). In view of the 'housekeeping' role of the IR gene (Seino *et al.*, 1989), the localization of IR protein on all rat uterine and embryonic tissue and types of cell investigated here was not unexpected, yet it has not been described to date. As all attempts to detect insulin in preimplantation embryos of commonly used laboratory animals have failed so far (Kaye and Harvey, 1995), it is currently believed that maternal insulin exerts growth-promoting effects during preimplantation development and there is no reason to assume that this effect stops after implantation and placentation (Desoye and Shafrir, 1996). This would allow the mother to control fetal and placental growth until the fetus has developed its own endocrine system. With the establishment of a primitive placental circulation and an active fetal pancreas, the fetus can take over growth control (Desoye *et al.*, 1994; Jones *et al.*, 1997).

Fig. 2. Transverse sections of the developing rat utero-embryonic unit from (a) day 9 of gestation and (b–f) day 12 of gestation, immunolabelled for insulin receptor (IR), insulin-like growth factor-I receptor (IGF-IR) or glucocorticoid receptor (GR). Yellow arrow, parietal endoderm; black arrow, visceral endoderm; yellow arrowhead, ectoderm; blue arrowhead, mesoderm. AC, amniotic cavity; DEC, decidua; DA, dorsal aortae; EC, ectoplacental cavity; EEC, extraembryonic cavity; ESC, endometrial stromal cells; GC, giant cell; HT, heart tube; IT, intestinal tube; M, mesonephros; MBC, maternal blood cells; NT, neural tube; N, notochord; OP, otic placode; PYS, parietal yolk sac; VYS, visceral yolk sac; WD, Wolffian duct. Sections incubated with peptide pre-adsorbed IGF-IR and GR antisera or non-immune rabbit serum (as negative control for IR staining) are shown as insets. Scale bars represent (a–c) 70 μ m and (d–f) 100 μ m.

IR immunoreactivity detected already in the rat embryo at day 5 of gestation is in good agreement with preceding expression of the respective transcript in rat one-cell and two-cell embryos and in blastocysts (Zhang *et al.*, 1994). Peri-implantation rat embryos have already switched their preferred substrate of fuel metabolism from the more oxidized pyruvate to glucose, which might be taken up by an insulin-sensitive glucose transporter isoform from the extracellular space (Hahn and Desoye, 1996). It is therefore intriguing that embryonic tissues staining for IR in the present study are also richly endowed with the GLUT4 and GLUT8 glucose transporters (Carayannopoulos *et al.*, 2000; Korgun *et al.*, 2001), which are known to translocate from intracellular storage vesicles to the cell membrane in response to insulin.

The uterus undergoes extensive remodelling in order to prepare for the implantation of the embryo. The experimental data showing most abundant IR expression in endometrial cells around the implantation period and pronounced IR labelling of uterine epithelium in the antimesometrial region where implantation takes place, implies that insulin triggers cellular proliferation and differentiation during decidualization. These events impose acute metabolic demands on the cells, that again can largely be met by the utilization of glucose. Thus, considering the expression of insulin-regulatable glucose transporters in the rat uterus (Korgun *et al.*, 2001), it is conceivable that insulin not only promotes mitogenic effects in this organ, but also stimulates uterine glucose uptake.

The trophoblast-derived giant cells pave the way for implantation in that they invade the decidua, erode maternal capillaries and expand the embryonic cavity by histolysis and phagocytosis. Interestingly, these cells, which are endocrinologically active, were found in the present study to express IR, but were generally devoid of IGF-IR and GR.

IGF-IR shows striking similarities to IR with respect to subunit structure, subunit size and disulphide crosslinks, although both receptors are distinct molecules with subtle differences in their polypeptide compositions (Ullrich *et al.*, 1986). The present study revealed abundant IGF-IR expression in the embryo from blastocyst stage onward, which coincides with the detection of the transcript in the preimplantation period reported by Zhang *et al.* (1994). In contrast, the growth factor itself has not been found in preimplantation embryos (Zhang *et al.*, 1994). Data showing that the rat uterus contains IGF-I in high concentrations, surpassed in this respect only by the liver (Girvigian *et al.*, 1994), indicate that a paracrine regulatory pathway brings about IGF-I effects in the early embryo. During organogenesis, peak IGF-IR amounts were observed in the neural tube, consistent with mRNA signals demonstrated in embryonic brain at day 13 of gestation (Baron-van Evercooren *et al.*, 1991). In addition, in other organ

anlagen, expression of IGF-IR was found to be quite high, supporting the hypothesis that the biological role of IGF-I is related to morphogenesis. The placenta may represent the exception proving this rule, as trophoblast (the direct progenitor tissue of the placenta) was only weakly labelled for IGF-IR until day 12 of gestation.

Uterine IGF-IR expression was most abundant in endometrial stromal cells, which is contradictory with data showing the receptor predominantly in smooth muscle cells of the myometrial layer of the rat uterus (Ghahary and Murphy, 1989). IGF-IR staining in the mouse uterus differs completely from that of the rat uterus in which immunoreactivity is restricted to glandular epithelium (Henemyre and Markoff, 1999).

The GR is a 95 kDa cytoplasmic transcription factor forming a functional heterocomplex with heat shock protein 90, which may organize or control the subcellular GR distribution. As the maturation of the rat fetal adrenal does not start before day 6 of gestation (Dupont *et al.*, 1991), the fetus is not capable of synthesizing its own glucocorticoids until relatively late in gestation. Therefore, most of the corticosterone influencing early rat embryogenesis is of maternal origin. In accordance with the proposed role of glucocorticoids in the maturation of fetal organs and measurable GR mRNA in rat embryo at day 10 of gestation that underwent rapid ontogenetic increase in the ensuing days (Ghosh *et al.*, 2000), the GR antiserum used in our experiments labelled neural tube, notochord, otic placode, Wolffian duct, mesonephros and intestinal tube of the rat embryo as well as its progenitor germ layers. In view of these results, complete absence of GR mRNA in all these tissues as revealed at day 12 of gestation by *in situ* hybridization (Kitraki *et al.*, 1997) might have been caused by inadequate sensitivity of the detection method.

Data presented here for the yolk sac are not consistent to a large extent with a study that used a binding assay to analyse GR (Carbone *et al.*, 1986). The parietal endoderm and giant cells as constituents of the parietal yolk sac remained negative for GR in the present study, whereas Carbone *et al.* (1986) found evidence for specific receptor binding in the parietal yolk sac as a whole at day 11 of gestation and quantifiable GR amounts in parietal endodermal and giant cells at day 14 of gestation. The lack of signal in trophoblast observed in the present study is perplexing, as GR transcript (Waddell *et al.*, 1998) and protein (Heller *et al.*, 1988) have been unambiguously identified in the rat placenta.

In line with the presence of GR mRNA in rat uterus (Ho *et al.*, 1999), in the present study the receptor protein was localized in all uterine cell populations, except the endometrial glands. Major sites of expression were the luminal epithelium and, in particular, decidual cells in the stromal compartment of the endometrium. These findings closely resemble GR distribution in the human uterus (Bamberger *et al.*, 2001) and may indicate an

autocrine function of glucocorticoids in the process of decidualization, as human decidual cells are net producers of cortisol (Giannopoulos *et al.*, 1982). The subcellular localization of GR differed markedly between embryonic and maternal tissues; it was cytoplasmic in embryonic tissues and nuclear in maternal tissues. The intracellular distribution of GR has not yet been firmly established (for review, see Akner *et al.*, 1995), but the demonstrated results are consistent with the hypothetical model of two independent GR populations in the nucleus and cytoplasm, respectively, with the extranuclear population exerting effects in the cytoplasm without involving nuclear genomic transcription (Akner *et al.*, 1994).

In conclusion, the expression of receptors for insulin, IGF-I and glucocorticoids is regulated in a tissue-specific manner as well as spatio-temporally in the developing rat utero-embryonic unit. The presented data support the concept that optimal embryonic development is obtained by interaction of specific receptors in the embryo or uterine cells with factors released from the physiological environment acting in a paracrine or autocrine mode.

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