0021-972X/98/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 83, No. 4
Printed in U.S.A.

RETINOID RECEPTORS EXPRESSION IN HUMAN TERM PLACENTA: INVOLVEMENT OF RXRα IN RETINOID INDUCED-hCG SECRETION

J. GUIBOURDENCHE [1], E. ALSAT [1], F. SONCIN [1], C. ROCHETTE-EGLY [2], D. EVAIN-BRION [1] *

[1]*Unité INSERM 427, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes Paris V, 4 Avenue de l'Observatoire, 75006 Paris;* [2]*CNRS / INSERM / Université Louis Pasteur, 67404 Illkirch, France.*

ABSTRACT: To investigate the role of retinoids on human placental development and functions, we characterized the spatial distribution of retinoid receptors in human term chorionic villi. *In situ* hybridization with ^{35}S labeled sense and antisense probes for the RARs, α, β, γ and RXRs, α, β, γ, specifically detected only RARα and RXRα. Both RARα and RXRα mRNA were preferentially expressed in the trophoblast cell layer. This syncytiotrophoblast expression was confirmed by immunohistochemical analyses using anti-RARα and RXRα antibodies. Using trophoblast cells in culture, we then studied the effect on hCG secretion of 0.1 μM RA physiological forms and of selective RARα and RXRα synthetic agonists. Only RXRα specific ligands such as physiological 9-*cis* RA and synthetic Ro 25-7386 stimulated hCG secretion (doubled). These results suggest an important role for RXRα in human placental development and function.

During gestation, vitamin A and its metabolite, retinoic acid (RA), play a key role in embryonic development as shown by the spectrum of fetal abnormalities observed in vitamin A deficiency in rodents (1) and the well established teratogenic effect of retinoids in humans (2, 3). However, the possible role of retinoids on human placental development and function is poorly understood. *In vitro*, RA has been shown to stimulate human Chorionic Gonadotropin (hCG) secretion in choriocarcinoma cells (4) and placental lactogen hormone in normal human trophoblast cells (5).

In recent years, two families of nuclear receptors for retinoids have been characterized, the RARs (RARα, β and γ, activated by all natural forms of RA) and the RXRs (RXRα, β and γ, activated only by 9-cis RA), demonstrating the complexity of the molecular machinery transducing the retinoid signal. These receptors function as ligand-activated transcription factors and regulate gene expression by binding as dimers to DNA response elements associated with their target genes (6-8). RXRs not only form homodimers, but can also heterodimerize with RARs and a variety of other nuclear receptors.

To investigate the effect of retinoids on human placenta, we characterized the spatial expression of retinoid receptors in human chorionic villi. Using the *in vitro* culture model of human trophoblast cells, we then studied the effect of physiological forms of RA and selective synthetic RARα and RXRα agonists, on hCG secretion.

MATERIALS AND METHODS

Placentas

Term placentas were obtained after elective section from healthy mothers near term with uncomplicated pregnancies. Placental fragments were placed in isopentane cooled in liquid nitrogen for *in situ* hybridization and immunohistochemical studies, as described elsewhere (9). Villous tissue was dissected free of membranes and vessels, rinsed and minced in Ca2+, Mg2+ free Phosphate Buffered solution (PBS) for cytotrophoblast cell isolation and culture.

In situ hybridization

The plasmid used to generate ^{35}S-labeled riboprobes from cDNAs has been described elsewhere (10, 11). Briefly, the RARs probes used were synthesized from cDNAs covering the entire reading frame for RARs (10) and RXRs (11). To establish the spatial distribution of the corresponding transcripts, 8 μm thick cryosections were hybridized to these probes. The procedures for probe synthesis and *in situ* hybridization were as described previously (9).

Immunohistochemical procedure

Affinity-purified rabbit polyclonal antibodies (12) against RARα and RXRα were used as the primary antibody (dilution 1/5000). A biotinylated donkey anti-rabbit IgG F(ab')2 fragment was used as the secondary antibody (1/100) and detection was performed using the streptavidin-fluorescein complex (1/200, Amersham, Les Ulis, France). A drop of fluorescent Dapimounting medium (Vector Laboratories, Burlingame, CA) was added to each section on a slide, and a coverslip was placed on top of the section. The slides were then examined in an epifluorescence microscope. To check the specificity of the immunological reactions, adjacent control sections were subjected to the same method except that the primary antibodies were replaced by human serum or peptide preabsorbed antibody. No positive staining of control samples was observed.

Cell culture

Cytotrophoblast cells were prepared as previously described (13-14). The cells were plated in triplicate, on 60 mm culture dishes ($3x10^6$ cells/dish) in 3 ml of D-MEM. They were incubated at 37°C in a humid atmosphere

* correspondence and reprint requests

containing 5% CO2/ 95% air, and were allowed to aggregate, fuse and form syncytia. The cells were treated with the indicated dose of RA, 9-*cis* RA, Ro 257386 (Ro 25, agonist of RXRα) or Ro 406055 (Ro 40, agonist of RARα) or were left untreated. 9-*cis* RA, Ro 25 and Ro 40 were a gift from Dr M. Klaus (F. Hoffmann-La Roche SA, Basel, Switzerland). Stock solution of retinoids were made up in ethanol. Control cultures were treated with the same volume of ethanol (1 per 1000). At the used dose, retinoids did not affect cell viability (using blue trypan exclusion) or cell morphology. The culture media were collected and stored at -20°C.

Hormone assays

Human chorionic gonadotropin (hCG): hCG concentration was determined by an enzyme-linked fluorescence assay,Vidas System, BioMerieux, Marcy l'Etoile, France)

Data analyses

The data are expressed as means ± SEM of triplicate determinations. Significant differences were identified using Student's *t* test.

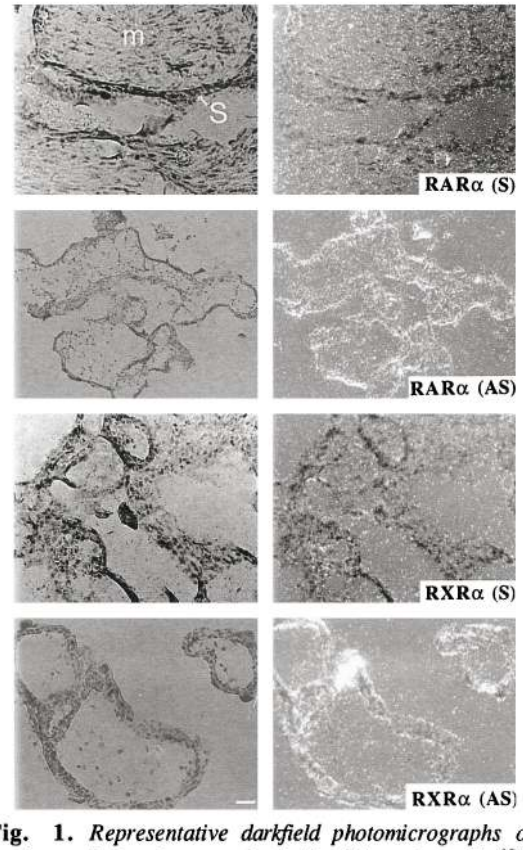

Fig. 1. *Representative darkfield photomicrographs of sections of term human chorionic villi probe with ^{35}S-labeled RARα and RXRα sense (S) or antisense (AS) oligonucleotides. The corresponding histological sections are shown in the lef-hand panel.*

RESULTS

Expression of RARα and RXRα mRNA and protein in the chorionic villi.

In situ hybridization of cryosections of human chorionic villi from term placenta showed that RARα and RXRα genes were specifically expressed in term human placenta (Fig.1). RARβ and γ, RXRβ and γ, were not labeled or gave only a diffuse, non specific labeling pattern in placental tissues (data not shown). Both RARα and RXRα transcripts were preferentially expressed in the trophoblast cell layer of the villi. This is the exchange and endocrine zone of the placenta, the syncytiotrophoblast. RARα was also weakly but specifically labeled in the mesenchymal core of the villi. Northern blot analysis of human placental mRNA showed there was one (5.6 kb) transcript for RARα and two (2.8, 3.8 kb) transcripts for RXRα (data not shown).

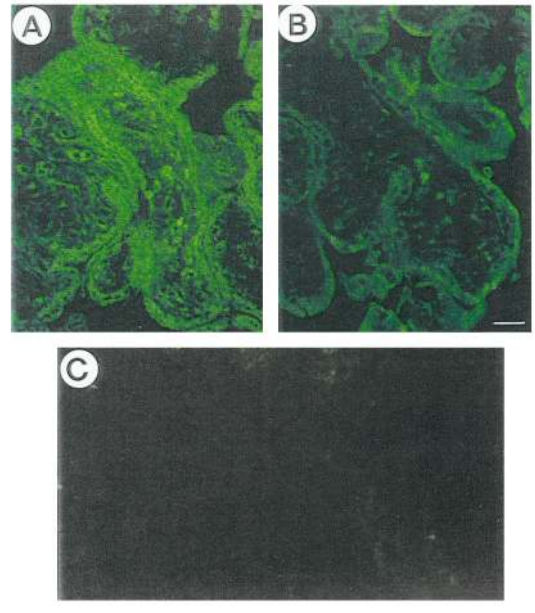

Fig. 2. *Immunostaining of term chorionic villi with anti-RARα (A) or anti-RXRα (B) antiserum. C: Control with peptide preabsorbed antibodies. Scale bar: 25 μm*

Figure 2 shows immunohistochemical analyses of term human placenta using anti-RARα (Fig. 2A) and anti-RXRα (Fig. 2B) antibodies. Specific immunostaining was observed in the syncytiotrophoblast layer. Faint immunostaining was observed in the mesenchymal core with the anti-RARα antibody.

Effect of RA physiological forms and of selective synthetic RARs and RXRs agonists, on hCG secretion.

The effect of RA, 9-*cis* RA and selective synthetic RARα and RXRα agonists on hCG secretion by cultured human trophoblast cells was studied (Fig. 3A-B). Isolated cytotrophoblasts from term placenta differentiate *in vitro* into syncytiotrophoblast; this morphological differentiation is associated with a progressive increase in hCG secretion from 22 ± 0.8 mIU/mL at 24h to 496 ± 13 mIU/mL at 96h of culture. 0.1 μM RA did not affect hCG secretion during the 4 days of culture (Fig. 3-A), 9-*cis* RA doubled hCG secretion. These results suggest that RXRα rather than RARα is involved in the stimulation of hCG secretion by retinoids. Such a role for RXRα was confirmed using synthetic retinoids that act as specific agonists of RARα or RXRα. The RXRα specific ligand, Ro 25, doubled hCG secretion over the four days of culture (Fig 3-B). The synthetic RARα agonist (Ro 40) did not stimulate hCG secretion.

DISCUSSION

Recent studies in mice hemotrichorial placenta have pointed the major expression of RARα and RXRα retinoid receptors (15). This study showed, for the first time, the specific *in situ* expression of RARα and RXRα in human hemochorial term placenta. The retinoid receptor transcripts and proteins were mostly present in the syncytiotrophoblast layer. These results are in agreement with our previous work in which we used immunoblotting to demonstrate the presence of these two types of retinoid receptor in cultured trophoblasts cells (16). The syncytiotrophoblast, which is bathed in maternal blood, is the endocrine unit of the human placenta. It secretes steroids and polypeptide hormones (see 17 for review) of which hCG plays a major role in the maintenance of pregnancy (18). More recent studies have also demonstrated its involvement in syncytiotrophoblast differentiation (19). HCG secretion is stimulated *in vitro* by cAMP (18), growth factors and hormones such as EGF, PTH (20, 21) and is inhibited by hypoxia (22,23). In this study, we showed that physiological 9-*cis* RA or synthetic ligand to RXRα stimulated hCG secretion, whereas RA and specific ligands to RARα had no effect.

RA acts on specific target genes via RARs and RXRs which bind as heterodimers to a variety of response elements (RAREs or RXREs). RAREs consist of a direct repeat of two core motifs (5'-A/GGTTCA-3' or a closely related sequence) separated by few base pairs.The analysis of the promoter region of hCG-β genes 5 and 6 identified direct repeats of RARE or RXRE like motifs. This supports possible interaction of RXRα with such a response element in the hCG promoter region. The characterization of such elements is in progress.

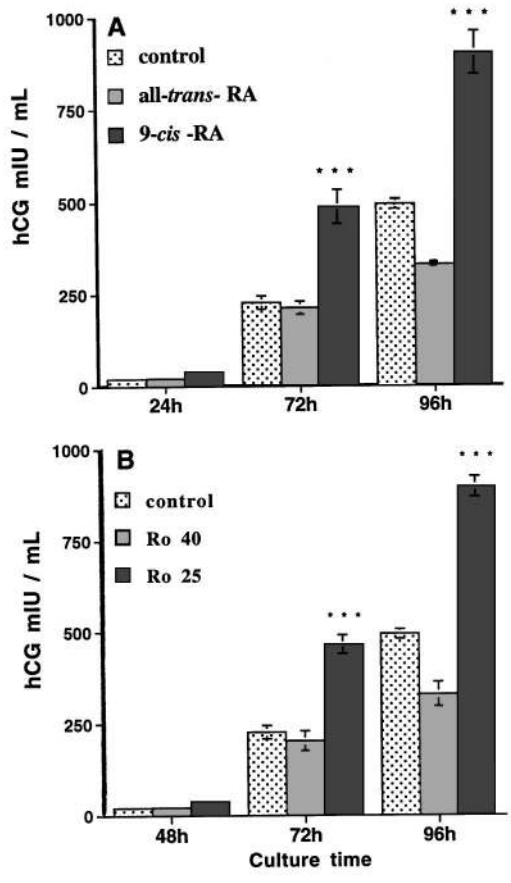

Fig. 3. *Effect of physiological RA and 9-cis RA (A) and synthetic RARα and RXRα agonists (B) on hCG secretion; Human trophoblast cells were cultured in the absence control) or presence of 0.1 μM RA, 9-cis RA, Ro 25, or Ro 40 for the times indicated. The data are the mean values for 3 separate cultures ± SEM. ***p<0.001, significantly different from controls.*

In addition, the possible regulation of human cytotrophoblast differentiation by RXR-specific ligands stimulating hCG secretion cannot be ruled out.

In conclusion, human chorionic villi at term specifically express two retinoid receptors, RARα and RXRα. A major role for RXRα in placental function is suggested by the effect of its specific ligands on hCG secretion.

Acknowledgements

This study was approved by the local ethical committee. We would like to thank P. Chambon, IGBMC, for the gift of RAR and RXR probes and antibodies and the staff of Saint Vincent de Paul Obstetrics Department for providing us with placentas. This work was supported by a grant from La Fondation de France and by the CRC 95266.

REFERENCES

1. **Wilson JG, Roth CB, Warkany J.** 1993 An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. Am J Anat. 92:89-217.
2. **Lammer EJ, Chen DT, Hoar RM, et al.** 1985 Retinoic acid embryopathy. N Engl J Med. 313:837-841.
3. **Rothman KJ, Moore LL, Singer MR, Nguyen U-SDT, Mannino S, Milunsky A.** 1995 Teratogenicity of high vitamin A intake. N Engl J Med 333:1369-1373.
4. **Kato Y, Braunstein GD.** 1991 Retinoic acid stimulates placental hormone secretion by choriocarcinoma cell lines *in vitro*. Endocrinology. 128: 401-407.
5. **Stephanou A, Handwerger S.** 1995 Retinoic acid and thyroid hormone regulate placental lactogen expression in human trophoblast cells. Endocrinology. 136:933-938.
6. **Petkovitch M.** 1992 Regulation of gene expression by vitamin A: the role of nuclear retinoic acid receptors. Annu Rev Nutr. 12:443-471.
7. **Chambon P.** 1993 The molecular and genetic dissection of the retinoid signalling pathway. Gene. 135:223-228.
8. **Giguère V.** 1994 Retinoic acid receptors and cellular retinoid proteins: complex interplay in retinoid signaling. Endocr Rev. 15:61-79.
9. **Décimo D, Georges-Labouesse E, Dollé P.** 1995 *In situ* hybridization of nucleic acid probes to cellular RNA. In: Hames BD, Higgins S, eds. Genes Probes 2, a pratical Approach. Oxford: Oxford University Press; 183-210.
10. **Dollé P, Ruberte E, Kastner P et al.** 1989 Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature. 342:702-705.
11. **Dollé P, Fraulob V, Kastner P, Chambon P.** 1994 Developmental expression of murine retinoid X receptor (RXR) genes. Mech Dev. 45:91-104.
12. **Ghyselinck NB, Dupé V, Dierich A, et al.** 1997 Role of the retinoic acid receptor beta (RARβ) during mouse development. Int J Dev Biol. 42:425-447.
13. **Alsat E, Haziza J, Evain-Brion D.** 1993 Increase in epidermal growth factor and its messenger ribonucleic acid levels with differentiation of human trophoblast cells in culture. J Cell Physiol. 154:122-128.
14. **Patel N, Alsat E, Igout A et al.** 1995 Glucose inhibits human placental GH secretion, *in vitro*. J Clin Endocrinol Metab. 80:1743-1746.
15. **Sapin V, Ward SJ, Bronner S, Chambon P, Dollé P.** 1997 Differential expression of transcripts encoding retinoid binding proteins and retinoic acid receptors during placentation of the mouse. Dev Dynamics. 208:199-210.
16. **Roulier S, Rochette-Egly C, Rebut-Bonneton C, Porquet D, Evain-Brion D.** 1994 Nuclear retinoic acid receptor characterization in cultured human trophoblast cells: effect of retinoic acid on epidermal growth factor receptor expression. Mol Cell Endocrinol. 105:165-173.
17. **Simpson ER, MacDonald PC.** 1981 Endocrine physiology of the placenta. Ann Rev Physiol. 43:163-188.
18. **Jameson JL, Hollenberg AN.** 1993 Regulation of chorionic gonadotropin gene expression. Endocr Rev. 14:203-221.
19. **Cronier L, Bastide B, Hervé JC, Delèze J, Malassiné A.** 1994 Gap junctional communication during trophoblast differentiation: influence of human chorionic gonadotropin. Endocrinology. 135:402-408.
20. **Morrish DW, Bhardwaj D, Dabbagh LK, Marusyk H, Siy O.** 1987 Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. J Clin Endocrinol Metab. 65:1282-1290.
21. **Alsat E, Mirlesse V, Fondacci C, Dodeur M, Evain-Brion D.** 1991 Parathyroid hormone increases epidermal growth factor receptors in cultured human trophoblastic cells from early and term placenta. J Clin Endocrinol Metab. 73:288-295.
22. **Esterman AL, Lee JD, Dancis J.** 1994. Response of trophoblast in culture to hypoxia Placenta, 15:A.
23. **Alsat E, Wyplosz P, Malassiné A, Guibourdenche J, Porquet D, Nessmann C, Evain-Brion D.** 1996 Hypoxia impairs cell fusion and differentiation process in human cytotrophoblast, *in vitro*. J Cell Physiol. 168:346-353.