Accumulation of chromotrope 2R positive cells in equine endometrium during early pregnancy and expression of transforming growth factor- β 2 (TGF- β 2)

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Endometrial tissue from the gravid uterine horn of pregnant mares was examined by northern analysis and *in situ* hybridization for mRNA that hybridized to cDNA and RNA probes generated from a mouse TGF- β 2 1.2 kb cDNA clone. The mouse cDNA probe hybridized to characteristic TGF- β 2 mRNA transcripts on a northern blot of total RNA isolated from horse endometrium collected at day 45 of gestation. Two major 4.0 and 3.5 kb transcripts and possibly a minor 1.6 kb transcript were observed, consistent with specific hybridization to equine TGF- β 2 mRNA. By *in situ* hybridization, riboprobes transcribed from the same fragment used in northern analysis hybridized to clusters of cells scattered between endometrial glands at days 38, 40, 42, 43, 78 and 81 of gestation. Positive cells were absent before day 38. From day 38 to day 43 there was marked hybridization over maternal leucocytes in the region of the developing endometrial cups, and at later stages (day 78 and day 81) clusters of cells positive for mRNA encoding TGF- β 2 were localized within the dense band of leucocytes at the periphery of the degenerating endometrial cups. There was no hybridization to invasive or non-invasive trophoblast or to fully differentiated endometrial cup cells. Approximately 90% of the TGF- β 2 positive cells detected in the sections taken at day 78 also stained with chromotrope 2R used to detect eosinophils and the morphology of approximately 50% of these cells was characteristic of eosinophils. These cells are known to accumulate around endometrial cups and are therefore a possible source of mRNA encoding TGF- β 2 in the endometrium after day 38 of gestation.

Introduction

Human and mouse trophoblast display highly invasive properties during the establishment of their haemochorial placentae, whereas the trophoblast in animals like sows and mares with epitheliochorial placentae shows no penetration of the endometrium over most of the uterine surface (Samuel *et al.*, 1975). However, in mares, a discrete, annulate portion of the trophoblast, the chorionic girdle, does invade the endometrium during the formation of the hormone-secreting endometrial cups. This reaction is unique to equids and occurs at day 36–38 when the chorionic girdle peels off the fetal membrane and becomes attached to the overlying endometrial epithelium. The girdle cells actively penetrate the luminal epithelium and then pass down the endometrial glands before crossing the basement membrane to enter the endometrial stroma (Allen *et al.*,

1973). Here, they round up, enlarge greatly, acquire a second nucleus and aggregate together to form the chorionic gonadotrophin (eCG)-secreting endometrial cups (Hamilton *et al.*, 1973). These cups have a lifespan of 80–100 days during which they are surrounded by increasing numbers of lymphocytes, eosinophils, plasma cells and other leucocytes. From around day 70 of gestation, the accumulated leucocytes begin to invade the cup tissue and destroy the large eCG-secreting fetal cup cells. Eventually, at day 100–120, the necrotic cup and its admixed eCG-rich exocrine secretion is sloughed from the surface of the endometrium (Allen, 1982).

Before, and for a few days after, their invasion of the endometrium, expression of surface paternal major histocompatibility complex (MHC) antigens is high on chorionic girdle cells (Donaldson *et al.*, 1990; Antczak *et al.*, 1994); these antigens stimulate a strong humoral alloantibody response in almost all pregnant mares (Allen, 1979; Antczak *et al.*, 1984). Nevertheless, the endometrial cups survive for 40–80 days in most mares and it is suggested that they may be partially protected by an immunoprotective-type response possibly

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manifest by a population of suppressor cells (Antczak and Allen, 1989). In support of this theory, Kydd and Allen (1986) reported a subpopulation of endometrial cup leucocytes that exhibit suppressor, rather than cytotoxic, activity *in vitro*.

A local intrauterine immunoprotective response has been reported in mice in which resorption (abortion) is associated with a deficiency of local decidual suppressor activity predominantly mediated by a molecule closely related to transforming growth factor subtype $\beta 2$ (TGF- $\beta 2$) (Clark *et al.*, 1986, 1991a). During normal mouse pregnancy, bioactive decidual TGF- $\beta 2$ is released at the beginning of the post-implantation period when the fetus and surrounding trophoblast express MHC and non-MHC antigens of paternal origin (Lea *et al.*, 1992). Despite the presence of these antigens on mouse trophoblast, it is resistant to killing by effector cells such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, but can be killed by lymphokine-activated killer (LAK) cells, that is, NK cells that have become activated by interleukin-2 and tumour necrosis factor α (TNF- α) (Head, 1989). Furthermore, abortion in mice has been associated with an infiltration of NK-lineage cells (Gendron and Baines, 1988). As decidual TGF- $\beta 2$ is highly suppressive, and as it is released just before the stage of pregnancy when most resorptions occur, Clark *et al.* (1991a) proposed that successful pregnancy may depend, in part, on the presence of local uterine suppressor activity mediated by TGF- $\beta 2$ when maternal non-specific effectors may become activated (reviewed in Clark *et al.*, 1991a; Lea *et al.*, in press, a).

Human first trimester endometrial tissue also contains mRNA encoding TGF- $\beta 2$, and some women who suffer recurrent early abortions have fewer TGF- $\beta 2$ mRNA positive cells in their decidualized endometrium, as determined by *in situ* hybridization (Lea *et al.*, in press, b). In addition to these immunosuppressive effects, TGF- β may play a role in inhibiting the growth and invasiveness of trophoblast, and may also promote the differentiation of this tissue (Hunt *et al.*, 1989; Clark *et al.*, 1990; Graham *et al.*, 1992; Lea *et al.* in press, a).

Since TGF- $\beta 2$ has been shown to be significantly suppressive in the gravid uteri of mice and women, the present study was designed to determine whether this cytokine is also present in the equine uterus during early pregnancy and, if so, whether its expression is associated with the expression of MHC class I antigens by invading chorionic girdle cells before their transformation into mature, hormone-secreting endometrial cup cells.

Materials and Methods

Tissues

Samples of endometrium with and without allantochorion attached were collected during surgical hysterectomy from the gravid uterine horn of ten pony mares on days 29 (two mares), 34, 35, 38, 42, 43, 45, 78 and 81 of gestation. Biopsies of endometrial cups with and without attached allantochorion were also taken on days 38, 40, 42, 43, 78 and 81. These tissues were immediately fixed in 10% (v/v) phosphate buffered formal saline and stored in 70% (v/v) ethanol. They were then trimmed and mounted in paraffin wax for *in situ* hybridization studies. In addition, approximately 1 g of endometrium was

removed from the gravid horn of the uterus of a mare at day 45 of gestation and frozen immediately in liquid nitrogen for northern blot analysis. A sample of liver was obtained *post mortem* from a nonpregnant pony mare for use as negative control tissue.

Hybridization probes

The bluescript KSII + vector containing a 1.2 kbp insert corresponding to the complete coding region of mouse TGF- $\beta 2$ was used. The plasmid was linearized with Sal I and used as a template for the T7 polymerase-directed synthesis of ^{35}S -labelled cRNA antisense probes. Sense probes, serving as negative controls, were transcribed from Xba I linearized plasmid with T3 polymerase. All riboprobes were transcribed using the riboprobe gemini II core system (Promega, Southampton). Each transcription used 2 μg of DNA and the probes had specific activities of 1×10^7 – 10^9 c.p.m. μg^{-1} .

Northern blot analysis

Total RNA was extracted from the frozen tissue samples by the method of Chomczynski and Sacchi (1987) and 20 μg per track of each sample was loaded for standard formaldehyde/agarose gel electrophoresis of RNA. The fractionated RNA was visualized with ethidium bromide, photographed to assess evenness of loading and then blotted onto a nylon membrane (Hybond-N, Amersham International, Amersham). The mouse cDNA encoding TGF- $\beta 2$ insert was isolated and labelled with ^{32}P using the random oligonucleotide priming method and hybridization to the blots was carried out at 58°C for 4–6 h in Quick Hyb[®] (Stratagene Ltd, Cambridge). The blots were washed in $2 \times \text{SSC}$ ($0.3 \text{ mol NaCl l}^{-1}$ and $0.03 \text{ mol sodium citrate l}^{-1}$; pH 7.0) at 58°C with several changes for a total of 1–2 h and placed in contact with X-ray film for 5–16 h at -80°C (Sambrook *et al.*, 1989). RNA molecular size markers (Gibco-BRL, Paisley) were used to estimate the sizes of the RNA transcripts.

In situ hybridization

In situ hybridizations were performed as described by Tron *et al.* (1988) with some modifications. Sections (3–5 μm) were cut and floated onto 3-aminopropyl triethoxysilane-coated slides. They were dewaxed through xylene, rehydrated through an ethanol series and soaked in $2 \times \text{SSC}$ for 10 min. The slides were then immersed in freshly prepared 0.25% (v/v) acetic anhydride in $0.1 \text{ mol triethanolamine buffer l}^{-1}$ (pH 8.0) at room temperature for 10 min (acetylation), followed by 10 min in $0.1 \text{ mol glycine l}^{-1}$ in $0.1 \text{ mol Tris-HCl l}^{-1}$ (pH 7.4). The sections were then dehydrated through an ethanol series and air-dried for 1 h before hybridization. Some sections were incubated in $20 \mu\text{g RNase-A ml}^{-1}$ for 30 min at 37°C just before acetylation to provide 'negative' controls.

The hybridization solution contained the riboprobe labelled with ^{35}S UTP (Amersham International) at a concentration of 1×10^6 c.p.m. ($30 \mu\text{l l}^{-1}$), $2 \times \text{SSC}$, 50% (v/v) formamide, Denharts ($\times 0.5$), $10 \text{ mmol dithiothreitol l}^{-1}$ (DTT), 1 mg salmon sperm DNA ml^{-1} , and 0.1 mg *Escherichia coli* tRNA

ml⁻¹ (Boehringer-Mannheim, Dorval, Quebec). An aliquot (30 μ l) of this solution was applied to each slide and the hybridizations were allowed to proceed at 48°C for 14–16 h. The slides were then washed in 0.1 $\times$ SSC/50% (v/v) formamide at 42°C for 30 min, treated with RNase-A (0.1 μ g ml⁻¹) in 2 $\times$ SSC at room temperature, and then further washed in 0.1 $\times$ SSC/50% (v/v) formamide at 42°C for 30 min and in 0.1 $\times$ SSC for 10 min at room temperature. Before autoradiography, some slides were immersed in carbol chromotrope for 45 min to stain eosinophils (Ohno *et al.*, 1992). Finally, the slides were dipped in nuclear track emulsion (Kodak NTB-2; Eastman Kodak, Rochester, NY or K5 emulsion; Ilford Limited, Mobberley, Cheshire) diluted with an equal volume of distilled water, dried in an upright position overnight and transferred to a light-sealed box for 10–14 days at 4°C. After development using Kodak D-19 developer, the slides were counterstained with haematoxylin and eosin and examined by water and oil immersion light microscopy as appropriate. Those slides immersed in chromotrope 2R were counterstained with haematoxylin.

Statistical analyses

The numbers of cells overlain by clusters of silver grains in the sense and antisense sections and at different gestational stages were compared using the Normal approximation to the Poisson distribution. Assuming that there are equal numbers of cells in each field and that the distribution of cells overlain with silver grains is the same across the fields, the total number of such cells in each tissue will have a Poisson distribution. The small number of cells overlain with silver grains and the large number of fields validates this assumption. The Normal approximation to the Poisson distribution can then be used to test for a difference between the mean number of cells overlain with silver grains in different tissues (Mood *et al.*, 1974). This analysis also assumes that the differences observed are not due to the fact that the tissues are situated on different slides.

Results

Detection of TGF- β 2 mRNA transcripts in endometrium of pregnant mares

Although the nucleic acid sequence of equine mRNA encoding TGF- β 2 is unknown, TGF- β subtypes have a high degree of cross-species similarity (Roberts and Sporn, 1990). We therefore carried out a northern blot to determine if the mouse TGF- β 2 cDNA clone would hybridize to equine endometrial RNA. As adult mouse liver is reported to be negative for mRNA encoding TGF- β 2 by northern analysis using a mouse TGF- β 2 riboprobe, a liver sample from a nonpregnant pony mare was selected as a source of negative control RNA (Miller *et al.* 1989, 1990).

Two major transcripts at 4.0 and 3.5 kb, and possibly a minor transcript at 1.6 kb, were observed in the endometrial RNA sample taken at day 45 and there was no specific hybridization to the liver sample (Fig. 1). The presence of more than one transcript is characteristic of TGF- β 2. Indeed, many others have detected similar sized transcripts in mouse

Fig. 1. Northern blot analysis using total RNA (20 μ g per track) prepared from day 45 pregnant horse endometrium (track 1) and horse liver (track 2). The upper panel shows two major transcripts, at 4.0 and 3.5 kb, and a minor transcript, at 1.6 kb (arrows), in the endometrial samples but no specific signal in the liver sample. The lower panel shows the ethidium bromide-stained RNA gel before transfer; the 28S and 18S ribosomal RNA bands are indicated on the gel and their position on the autoradiogram is shown in the upper panel.

endometrial RNA and we have previously detected a 4.0–4.2 kb and what appears to be a faint 1.0–1.6 kb transcript in RNA isolated from mouse decidua (Miller *et al.*, 1989; Gatherer *et al.*, 1990; Millan *et al.*, 1991; Schmid *et al.*, 1991; Lea *et al.*, 1992; Das *et al.*, 1993). The northern analysis illustrated in Fig. 1 shows that the mouse TGF- β 2 cDNA probe detected a limited number of known TGF- β 2 transcripts in equine endometrium. These findings validated our use of the mouse riboprobe in the cross-species detection of equine TGF- β 2 mRNA encoding TGF- β 2.

Detection of endometrial mRNA encoding TGF- β 2 by *in situ* hybridization

The localization of TGF- β 2 mRNA positive cells in pregnant horse endometrium and the kinetics of expression were investigated by performing *in situ* hybridization using the mouse TGF- β 2 riboprobe transcribed from the same fragment used for northern analysis. Sections of preimplantation gravid horn endometrium taken from two mares at day 29 and one at day 35 of gestation showed no hybridization after being incubated with the mouse TGF- β 2 riboprobe (Figs 2a, 3). By contrast, marked hybridization was seen when the same riboprobe was used on sections of endometrial tissue recovered on days 38,

Fig. 3. Kinetics and localization of cells positive in the endometrium of pregnant mares. The number of positive cells per 1000 high power water emersion fields are shown for endometrial sections through the band of leucocytes surrounding the endometrial cup trophoblast (■) and for sections of gravid horn endometrium distant from the cups (□). ^aThe specialized trophoblast cells of the chorionic region of the placenta invade the maternal endometrium at days 36–38 to form the gonadotrophin-secreting endometrial cups. Endometrial tissues taken earlier in gestation were negative for mRNA encoding TGF- β 2, for example, ^btwo mares at day 29 and ^cone mare at day 35. ^dA day-78 biopsy adjoining the endometrial cups trophoblast contained significantly more positive cells than a biopsy distant from the cups taken from the same pregnant uterus. * $P < 0.001$. ^eOnly one day-81 endometrial biopsy with adjoining trophoblast was available for examination.

40, 42, 78 and 81 of gestation (Figs 2, 3, 4). Within each of these sections, dense accumulations of silver grains were clearly localized over individual cells when the antisense probe was used. Some low level nonspecific binding of the sense probe to cells with a more random distribution in the tissue was also observed. This phenomenon is characteristic of the sense riboprobe strand for cytokine mRNA; however, the number of cells exhibiting an overlay of silver grains with higher density than background was significantly lower than the number of distinct and more dense silver grain clusters seen with the antisense probe ($P < 0.001$). It is recognized that it may be possible to reduce the degree of nonspecific binding of the sense probe by adjusting the experimental conditions. How-

ever, it is not clear whether sensitivity and specificity would be optimal if the negative was completely negative, that is, some positive signals could be lost. Positive cells were scattered between the endometrial glands throughout the stroma in the later stage samples but in the day 38 sample showing early endometrial cup development they appeared to be clustered more densely in the *stratum compactum* (Fig. 2b–d).

Some endometrial samples were taken adjacent to endometrial cups so that sections contained both endometrial cup and adjoining normal endometrium. Other endometrial samples were taken from different regions of the gravid horn and were associated with the noninvasive trophoblast of the allanto-chorion. These endometrial tissues were divided accordingly and compared in terms of their expression of mRNA encoding TGF- β 2 (Fig. 3). Positive cells were observed within the clusters of mononuclear cells at the periphery of healthy, recently developed endometrial cups at days 40 and 43 of gestation (Fig. 2e–g) and also in the dense band of leucocytes surrounding the more advanced day 78 and 81 endometrial cups, which were in the early stages of degeneration (Figs 4, 5). In contrast, none of the large fetal cup cells of trophoblast origin showed any evidence of hybridization (Fig. 2g,h; day 43).

Figure 2e shows a low power photomicrograph of the junction between the transformed invasive trophoblast cells of the endometrial cup and the interface between the noninvasive trophoblast of the allanto-chorion and the endometrial epithelium. Hybridization can be seen over some cells within the endometrial stroma and over two cells situated within the bulk of the adjacent endometrial cup tissue. Other cells that were positive for mRNA encoding TGF- β 2 were observed within the endometrial cup tissue situated close to the endometrial glands (Fig. 2f–h).

In the day 78 specimen, clusters of cells positive for mRNA encoding TGF- β 2 were distributed between the dilated endometrial glands and among the dense band of leucocytes surrounding the degenerating cup (Fig. 4). In contrast, a sample of endometrium and attached allanto-chorion recovered some distance from the endometrial cups in the same day 78 uterus, had very few positive cells (Fig. 3). These findings indicate that maternal cells positive for mRNA encoding TGF- β 2 are part of the characteristic leucocyte response to the fetal endometrial cups.

Fig. 2. *In situ* hybridization showing localization of cells positive for mRNA encoding TGF- β 2 in horse endometrium between days 29 and 43 after ovulation. Antisense probes were labelled with [³⁵S]UTP and tissue sections were exposed for 12–14 days. (A) Section of endometrium from the gravid uterine horn of a pony mare on day 29 of gestation; e: luminal epithelium; g: endometrial glands in the *stratum spongiosum*; s: stroma ($\times 100$). Although the background signal is slightly higher than normal in this figure, no cell-associated signal was observed. (B) Low power section of the endometrium from the gravid uterine horn of a pony mare on day 38 of gestation; just after invasion by the chorionic girdle cells to form the endometrial cups. Arrows show a concentration of labelled cells in the *stratum compactum* beneath the luminal epithelium, and a more scattered distribution between the endometrial glands in the deeper *stratum spongiosum* ($\times 40$). (C) High power view of (B) showing positive cells ($\times 100$). (D) Higher power view of (C) showing the close proximity of the positive cells to endometrial glands in the *stratum spongiosum* ($\times 400$). (E) Low power section of the interface between an endometrial cup and the endometrium in the gravid uterine horn of a mare at day 43 of gestation. The arrowheads point to positive cells, both within the cup tissue and beneath the luminal epithelium of the adjacent endometrium; t: fetal endometrial cup cells ($\times 40$). (F) Cross section of the same endometrial cup in (E), with arrows pointing to positive cells, both within and beneath the cup tissue; t: fetal endometrial cup cells, g: distended endometrial glands ($\times 40$). (G) High power view at the base of (F) showing labelled cells accumulated in the stroma beneath the cup cells ($\times 100$). (H) Very high power view of labelled cell within the endometrial cup tissue at day 43 of gestation; t: large eCG-secreting endometrial cup cells; g: endometrial gland ($\times 400$). Sections incubated with negative control sense probes (not shown) exhibited random low level nonspecific binding and had significantly fewer silver grain clusters than sections incubated with antisense probes ($P < 0.001$). Preimplantation endometrium (A) incubated with antisense probe also served as negative control.

Fig. 4. (A) Section at the periphery of an endometrial cup at day 78 of gestation after *in situ* hybridization using the mouse TGF- β 2 riboprobe, followed by counterstaining with chromotrope 2R. Note the cluster of cells positive for mRNA encoding TGF- β 2 (large arrow) within the dense aggregation of maternal leucocytes surrounding the dilated endometrial glands, g. The small arrows point to individual cells positive for mRNA encoding TGF- β 2 at the periphery of the band of leucocytes; s: endometrial stroma. Scale bar represents 100 μ m. (B) Higher magnification of (A) showing very precise hybridization (arrows) to only the larger cells with chromotrope 2R-positive cytoplasm. Approximately 50% of these cells also had bilobed nuclei characteristic of eosinophils (see Fig. 5). Ninety-three percent of cells positive for mRNA encoding TGF- β 2 were chromotrope 2R-positive. The phenotype of the remaining 7% was uncertain. Scale bar represents 100 μ m.

The distribution of cells positive for mRNA encoding TGF- β 2 in the day 78 sections resembled that of eosinophils (Amoroso, 1955; Allen, 1975). Thus, after *in situ* hybridization and before autoradiography, some day 78 sections were counterstained with chromotrope 2R, a stain regarded generally as being specific for eosinophils (Lendrum, 1944). Approximately 90% of the chromotrope 2R-stained cells were also positive for mRNA encoding TGF- β 2 and a representative cluster of these is shown in Fig. 4. Close examination of these sections by high power oil emersion microscopy revealed that approximately 50% of chromotrope 2R positive cells also had the bilobed nuclei characteristic of eosinophils (Fig. 5). In addition, approximately 7% of cells positive for mRNA encod-

ing TGF- β 2 did not stain with chromotrope 2R. However, this last observation may reflect the plane in which the tissue was cut. In total, these results suggest that there may be more than one type of endometrial cell that is positive for mRNA encoding TGF- β 2.

Kinetics of expression of mRNA encoding TGF- β 2 in endometrium of pregnant mares

The kinetics of expression of mRNA encoding TGF- β 2 in the endometrium was investigated further by calculating the number of positive cells per 1000 high power water emersion fields both in the band of leucocytes surrounding the endometrial cup and in gravid horn endometrium distant from the cups (Fig. 3). The results indicated that more cells positive for mRNA encoding TGF- β 2 were present around the periphery of recently formed endometrial cups (day 40) than around ageing cups at later stages of gestation (days 78, 81).

For the purpose of this study, it was only feasible to collect tissues from one mare on most of the days studied. The marked difference between the three mares before, and the five mares after, chorionic girdle invasion was so convincing that larger numbers at each time point were not necessary. Where possible, all comparisons were justified statistically. To minimize trauma to the mares, no biopsies were taken from the nonpregnant horn.

Discussion

The results of this study suggest that endometrial tissue from the gravid horn of the uterus of the mare harbours a population of chromotrope 2R positive cells which contain TGF- β 2 mRNA. There appears to be more than one population of these cells, of which approximately 50% have bilobed nuclei and are probably eosinophils. These cells are predominantly localized around the periphery of healthy endometrial cups.

The northern analysis of total RNA from day 45 pregnant endometrium revealed two major transcripts at 4.2 kb and 3.5 kb and a possible minor transcript at 1.6 kb. The major transcripts are characteristic of TGF- β 2; however, this is the only study to report a 1.6 kb band. Although the detection of this band is reproducible, it is uncertain whether it represents a unique TGF- β 2 transcript or a degradation product. Variability in the number of TGF- β 2 mRNA transcripts from similar sources of RNA is commonly reported in the literature (Lea *et al.*, 1992). The lack of a 3.5 kb transcript in our earlier studies in mice (Lea *et al.*, 1992) may reflect a species difference in uterine expression of mRNA encoding TGF- β 2 or our use of a simian TGF- β 2 riboprobe in the mouse experiments compared with a mouse TGF- β 2 riboprobe in the horse. A 3.5 kb TGF- β 2 mRNA transcript has, however, been reported by other investigators using mouse cDNA probes and riboprobes against mouse uterine and placental total RNA and whole mouse polyadenylated RNA (Miller *et al.*, 1989; Schmid *et al.*, 1991; Das *et al.*, 1992, 1993).

By *in situ* hybridization, clusters of positive cells were found scattered between the endometrial glands in the stroma in samples from mares at days 38, 40, 42, 78 and 81 after ovulation. In contrast, both the invasive and noninvasive

Fig. 5. High power oil emersion view of cells positive for mRNA encoding TGF- β 2 stained positive for chromotrope 2R (shown in Fig. 4) in day 78 endometrium. (A,C) Clusters of silver grains depict hybridization to the underlying cells in the endometrium. (B,D) View of the same cells as (A) and (C), respectively, only focused on the nucleus rather than the silver grains. Note the bilobed nucleus in (B; eosinophil) and the non-bilobed nucleus in (D). Scale bar represents 10 μ m.

components of trophoblast exhibited no evidence of hybridization at any of the stages of pregnancy examined. However, the TGF- β 2 riboprobe hybridized to a few of the maternal leucocytes accumulated around newly formed endometrial cups at days 40 and 43 after ovulation. Later in gestation, at days 78 and 81, definitive clusters of cells positive for mRNA encoding TGF- β 2 were present within the dense band of leucocytes surrounding dilated endometrial glands at the periphery of the endometrial cups. This clustering mimicked the known accumulation of eosinophils around the degenerating cups and this was confirmed with respect to at least 50% of the cells positive for mRNA encoding TGF- β 2. About 4% of the chromotrope 2R positive cells were negative for mRNA encoding TGF- β 2 and about 7% of the positive cells did not stain with chromotrope 2R. The phenomenon that not all eosinophils in a tissue express a detectable gene product has been observed previously for TGF- β 1 in nasal polyps (Ohno *et al.*, 1992).

In pregnant mice, TGF- β 2 has been immunolocalized to uterine stroma and epithelium (Das *et al.*, 1992). In contrast, the mouse riboprobe used in the present study did not show this localization. By northern analysis the long 1.2 kb mouse riboprobe has specificity for a unique assortment of two (possibly three) TGF- β 2 transcripts. It is recognized that there are multiple molecular weight species of TGF- β 2 and various groups using probes of different length and origin have published different results (reviewed in Lea *et al.*, 1992). The specificity in the northern analysis therefore probably accounts for the specificity in the *in situ* hybridization studies in which no hybridization to uterine epithelium was observed. The *in situ* hybridization studies suggest that the probe of longer length hybridizes with greater specificity to the cells of interest. This supports the general observations that probes of longer length hybridize with greater specificity (Singer *et al.*, 1986).

Most leucocytes surrounding endometrial cups at day 54 are lymphocytes that express either the CD4 or the CD8 antigens

in an overall CD4:CD8 ratio of 4:1 (Antczak *et al.*, 1994). Macrophages in human pregnancy decidua may also express CD4, unlike macrophages in the endometrium, and it is therefore possible that some of the CD4 positive cells associated with endometrial cups in mares are actually macrophages (Clark and Daya, 1988; Clark, 1993). A possible role for eosinophil-derived TGF- β 2 could be to suppress the potential cytotoxic activities of LAK cells attacking endometrial cup tissue; it is also possible that the small population of TGF- β 2 mRNA positive cells in equine endometrium that did not stain with chromotrope 2R could be a subset of macrophages.

In mice, the detection of mRNA encoding TGF- β 2 *in situ* correlates closely with the release of TGF- β 2-related suppressor factor from decidual tissue cultured *in vitro* (Lea *et al.*, 1992). Our mouse studies have suggested that TGF- β 2 released by asialo-GM1⁻, MAC1⁻, 1E5/B5.1⁺ bone marrow-derived natural suppressor cells present in postimplantation decidua plays an important role in suppressing the activation of nonspecific maternal effector cells that would be capable of attacking the fetus (Clark *et al.*, 1990, 1991a, b, 1993). It has been further suggested that class I antigens expressed on murine trophoblast may act as a target for anti-MHC antibodies and that these, in turn, stimulate suppression and prevent spontaneous abortion (Raghupathy *et al.*, 1981; Clark *et al.*, 1987; Chaouat *et al.*, 1988). In this regard, it is interesting that low concentrations of TGF- β can promote IgG as well as IgA responses (Zettel *et al.*, 1991).

The data presented in this study suggest that TGF- β 2 may play a similar role in horses. MHC class I antigens are expressed in increasing concentration between 25 and 35 days after ovulation on the trophoblast cells of the preinvasive chorionic girdle (Donaldson *et al.*, 1990) but our *in situ* hybridization studies did not reveal any cells positive for mRNA encoding TGF- β 2 in endometrial tissue at this stage of gestation. However, immediately after the invasion of the class I

positive girdle cells into the endometrium on day 36, cells positive for mRNA encoding TGF- β 2 were clearly visible in the surrounding endometrial tissue on days 38 and 40. After their brief phase of invasion, the transformed, mature endometrial cups downregulate their expression of MHC class I antigens such that expression is switched off completely by as early as day 45 (Donaldson *et al.*, 1990; Antczak *et al.*, 1994). This phenomenon may account for the greater number of cells positive for mRNA encoding TGF- β 2 in day 40 endometrium adjoining endometrial cup tissue compared with later gestational stages. Preliminary *in situ* hybridization studies of other TGF- β subtypes in equine endometrium suggest a similar distribution of TGF- β 3 positive cells with fewer cells positive for TGF- β 1 (R. G. Lea, W. R. Allen, I. Ohno, M. Jordana and D. A. Clark, unpublished). In general, however, our data suggest that only TGF- β 2 is of particular interest with respect to immunoregulation within the mammalian uterus (Lea *et al.*, 1992, in press, b).

In conclusion, our data suggest that clusters of cells positive for mRNA encoding TGF- β 2, including a population of eosinophils, are present in pregnant endometrial stroma in close proximity to developing and healthy chorionic gonadotrophin-secreting endometrial cups. The immunohistochemical localization of TGF- β 2 in equine endometrium is clearly an important component of future studies and the concurrence of chromotrope 2R positivity with *in situ* hybridization requires further investigation.

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References

- Allen WR (1975) The influence of fetal genotype upon endometrial cup development and PMSG and progestagen production in equids *Journal of Reproduction and Fertility Supplement* **3** 405–413
- Allen WR (1979) Maternal recognition of pregnancy and immunological implications of trophoblast–endometrium interactions in equids. In *Maternal Recognition of Pregnancy* (Ciba Foundation Series No. 64), pp 323–352, Excerpta Medica, Amsterdam.
- Allen WR (1982) Immunological aspects of the endometrial cup reaction and the effect of xenogeneic pregnancy in horses and donkey *Journal of Reproduction and Fertility Supplement* **31** 57–64
- Allen WR, Hamilton DW and Moor RM (1973) The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast *Anatomical Record* **177** 485–502
- Amoroso EC (1955) The endocrinology of pregnancy *British Medical Bulletin* **17** 117–125
- Antczak DF and Allen WR (1989) Maternal immunological recognition of pregnancy in equids *Journal of Reproduction and Fertility Supplement* **37** 69–78
- Antczak DR, Miller J and Remick LH (1984) Lymphocyte alloantigens of the horse. II. Antibodies to ELA antigens produced during equine pregnancy *Journal of Reproductive Immunology* **6** 283–287
- Antczak DF, Maher JK, Grunig G, Donaldson WL, Kydd J and Allen WR (1994) MHC gene expression in placentas of domestic animals. In *Immunobiology of Reproduction* pp 153–169 Ed. J Hunt. Springer-Verlag, New York
- Chaouat G, Clark DA and Wegmann TG (1988) Genetic aspects of the CBA $\times$ DBA/2 and B10 $\times$ B10.A models of murine pregnancy failure and its prevention by lymphocyte immunisation. In *Early Pregnancy Loss: Mechanisms and Treatment* pp 89–102 Eds RW Beard and F Sharp. Peacock Press, Ashton-under-Lyne
- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction *Analytical Biochemistry* **162** 156–159
- Clark DA (1993) Cytokines and uterine bleeding. In *Steroid Hormones and Uterine Bleeding* pp 263–275 Eds N Alexander and C d'Arcangues. AAAS Press, Washington
- Clark DA and Daya S (1988) Macrophages and other migratory cells in endometrium: relevance to uterine bleeding. In *Contraception and Mechanism of Uterine Bleeding* pp 363–382 Eds C d'Arcangues, I Fraser, J Newton and V Olind. Cambridge University Press, Cambridge
- Clark DA, Chaput A and Tutton D (1986) Active suppression of host-versus-graft reaction in pregnant mice. VII. Spontaneous abortion of allogeneic DBA/2 $\times$ CBA/J fetuses in the uterus of CBA/J mice correlates with deficient non-T suppressor cell activity *Journal of Immunology* **136** 1668–1675
- Clark DA, Chaput A, Slapsys R, Brierley J, Daya S and Allerdyce R (1987) Suppressor cells in the uterus. In *Immunoregulation and Fetal Survival* pp 63–47 Eds TG Wegmann and TJ Gill. University Press, New York
- Clark DA, Flanders K, Banwatt D, Millar-Book W, Manuel J, Stedronska-Clark J and Rowley B (1990) Murine pregnancy decidua produces a unique immunosuppressive molecule related to transforming growth factor β 2 *Journal of Immunology* **144** 3008–3014
- Clark DA, Lea RG, Podor T, Daya S, Banwatt D and Harley CB (1991a) Cytokines determining the success or failure of pregnancy. In *Frontiers in Human Reproduction* pp 524–544 Eds M Seppala and L Hamberger. NY Academy of Sciences, New York
- Clark DA, Lea RG, Rowley B, Denburg J, Banwatt D, Manuel J, Damji N, Underwood J, Michel M, Mowbray J, Daya S and Chaouat G (1991b) Transforming growth factor- β 2-related suppressor factor in mammalian pregnancy decidua: homologies between the mouse and human in successful pregnancy and in recurrent unexplained abortion. In *Maternofetal Relationship: Molecular and Cellular Biology* Vol. 212 pp 171–179 Ed. G Chaouat. John Libbey Eurotext, Paris
- Clark DA, Flanders KC, Hirte H and Dosch JR (1993) Murine and human decidua-associated natural suppressor cells release a novel type of transforming growth factor beta type-2 *Journal of Immunology* **150** 4A (Abstract)
- Das SK, Flanders KC, Andrews GK and Dey SK (1992) Expression of transforming growth factor- β isoforms (β 2 and β 3) in the mouse uterus: analysis of the periimplantation period and effects of ovarian steroids *Endocrinology* **130** 3459–3466
- Das SK, Paria BC, Andrews GK and Dey SK (1993) Effects of 9-ene-tetrahydrocannabinol on expression of β -type transforming growth factors, insulin-like growth factor-1 and c-myc genes in the mouse uterus. *Journal of Steroid Biochemistry and Molecular Biology* **45** 459–465
- Donaldson WL, Zhang CH, Oriol JG and Antczak DF (1990) Invasive equine trophoblast expresses conventional class I Major Histocompatibility Complex antigens *Development* **110** 63–71
- Gatherer D, Dijke PT, Baird DT and Akhurst DJ (1990) Expression of TGF- β 2 isoforms during first trimester human embryogenesis *Development* **110** 445–460
- Gendron RL and Baines MG (1988) Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice *Cellular Immunology* **113** 261–267
- Graham CH, Lysiak JJ, McRae KR and Lala PK (1992) Localisation of transforming growth factor- β at the human fetal–maternal interface: role in trophoblast growth and differentiation *Biology of Reproduction* **46** 561–572
- Hamilton DW, Allen WR and Moor RM (1973) The origin of equine endometrial cups. III. Light and electron microscopic study of fully developed equine endometrial cups *Anatomical Record* **177** 503–517
- Head JR (1989) Can trophoblast be killed by cytotoxic cells?: *in vitro* evidence and *in vivo* possibilities *American Journal of Reproductive Immunology* **20** 100–105
- Hunt J, Soares MI, Lei M, Smith RN, Wheaton D, Atherton R and Morrison DC (1989) Products of lipopolysaccharide modify DNA synthesis by rat trophoblast cells exhibiting the 80 kDa lipopolysaccharide-binding protein *Journal of Immunology* **143** 1606–1613
- Kydd JH and Allen WR (1986) Suppressor activity in the equine endometrial cup reaction *Journal of Reproductive Immunology Supplement* **119** 125 (Abstract)
- Lea RG, Flanders KC, Harley CB, Manuel J, Banwatt D and Clark DA (1992) The release of a TGF- β 2-related suppressor factor from post-implantation murine decidua can be correlated with the detection of a subpopulation of cells containing RNA for TGF- β 2 *Journal of Immunology* **148** 778–787

- Lea RG, Vince G, Flanders KC, McIntyre S, Michel M, Mowbray J, Underwood J, Baird J and Clark DA Uterine TGF- β 2 in human normal and pathological pregnancies. In *Early Embryo Development: Uterus Preparation and Role of Cytokines in Implantation and Labour* Eds G Chaouat, A Hazout, R Frydman. INSERM Colloque Foundation Marcel Merieux (in press, a)
- Lea RG, Underwood J, Flanders K, Hirte H, Banwatt D, Finotto S, Ohno I, Daya S, Harley C, Michel M, Mowbray JF and Clark DA A subset of patients with recurrent spontaneous abortion is deficient in transforming growth factor β 2-producing "suppressor cells" in uterine tissue near the placental attachment site *American Journal of Reproductive Immunology* (in press, b)
- Lendrum AC (1944) The staining of eosinophil polymorphs and enterochromaffin cells in histological sections *Journal of Pathology and Bacteriology* **56** 441
- Millan FA, Denhez F, Kondaiah P and Akhurst RJ (1991) Embryonic gene expression patterns of TGF- β 1, β 2 and β 3 suggests different developmental functions *in vivo Development* **111** 131–144
- Miller DA, Lee A, Pelton RW, Chen EY, Moses HL and Derynck R (1989) Murine transforming growth factor β 2 cDNA sequence and expression in adult tissues and embryos *Molecular Endocrinology* **3** 1108–1114
- Miller DA, Pelton RW, Chen EY and Moses HL (1990) Transforming growth factor- β : a family of growth regulatory peptides. In *Annals of the New York Academy of Sciences, Transforming Growth Factor- β s: Chemistry, Biology and Therapeutics* Vol. 593 pp 208–217 Eds KA Piez and MB Sporn. New York Academy of Sciences, New York
- Mood AM, Grayhill FA and Boes DC (1974). Special parametric families of invariate distributions In *Introduction to the Theory of Statistics* pp 85–120 Eds AM Mood, FA Grayhill and DC Boes. McGraw Hill International Student Edition, Tokyo
- Ohno I, Lea RG, Flanders KC, Clark DA, Banwatt D, Dolovich J, Denburg J, Harley CB, Gauldie J and Jordana M (1992) Eosinophils in chronically inflamed human upper airway tissues express transforming growth factor β 1 gene (TGF- β 1) *Journal of Clinical Investigation* **89** 1662–1668
- Raghupathy R, Singh B, Leigh JB and Wegmann TG (1981) The ontogeny and turnover kinetics of paternal H-2k antigenic determinants on the allogeneic murine placenta *Journal of Immunology* **127** 2074–2079
- Roberts AB and Sporn MB (1990) The transforming growth factor-betas. In *Peptide Growth Factors and their Receptors* Vol. 95 Eds MB Sporn and AB Roberts. Springer-Verlag, Heidelberg
- Sambrook J, Fritsch EF and Maniatis T (1989) Hybridization and radiolabeled probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes. In *Molecular Cloning*, pp 9.52–9.55. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Samuel CA, Allen WR and Steven DH (1975) Ultrastructural development of the equine placenta *Journal of Reproduction and Fertility Supplement* **23** 575–578
- Schmid P, Cox D, Bilbe G, Maier R and McMaster GK (1991) Differential expression of TGF- β 1, β 2 and β 3 genes during mouse embryogenesis. *Development* **111** 117–130
- Singer RH, Laurence JB and Villnave C (1986) Optimisation of *in situ* hybridisation using isotopic and non-isotopic detection methods *Biotechniques* **4** 230–242
- Tron VAC, Harley CB, Caussy D and Sauder DN (1988) *In situ* detection of interleukin-1 mRNA in human monocytes *Molecular Immunology* **25** 439–445
- Zettl LA, Clark DA and Ernst PB (1991) TGF- β enhances antibody response by a contrasuppression-like mechanism *International Immunology* **9** 975–984