Accumulation of chromotrope 2R positive cells in equine endometrium during early pregnancy and expression of transforming growth factor-β2 (TGF-β2)

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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-β2 1.2 kb cDNA clone. The mouse cDNA probe hybridized to characteristic TGF-β2 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-β2 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-β2 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-β2 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-β2 in the endometrium after day 38 of gestation.

Introduction

Human and mouse trophoblast display highly invasive properties during the establishment of their haemochorial placenta, 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
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 β2 (TGF-β2) (Clark et al., 1986, 1991a). During normal mouse pregnancy, bioactive decidual TGF-β2 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-β2 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-β2 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-β2, and some women who suffer recurrent early abortions have fewer TGF-β2 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-β2 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 blueprints K11 + vector containing a 1.2 kbp insert corresponding to the complete coding region of mouse TGF-β2 was used. The plasmid was linearized with Sal 1 and used as a template for the T7 polymerase-directed synthesis of 35S-labelled cRNA antisense probes. Sense probes, serving as negative controls, were transcribed from Xba 1 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 × 107–109 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-β2 insert was isolated and labelled with 32P 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 × SSC (0.3 mol NaCl l−1 and 0.03 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 5–10 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 dehydrated through xylene, rehydrated through an ethanol series and soaked in 2 × SSC for 10 min. The slides were then immersed in freshly prepared 0.25% (v/v) acetic anhydride in 0.1 mol triethanolamine buffer l−1 (pH 8.0) at room temperature for 10 min (acetylation), followed by 10 min in 0.1 mol glycine l−1 in 0.1 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 µg 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 [35S] UTP (Amersham International) at a concentration of 1 × 106 c.p.m. (30 µl−1), 2 × SSC, 50% (v/v) formamide. Denharts (~0.5), 10 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 µl) 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 x SSC/50% (v/v) formamide at 42°C for 30 min, treated with RNase-A (0.1 µg ml⁻¹) in 2 x SSC at room temperature, and then further washed in 0.1 x SSC/50% (v/v) formamide at 42°C for 30 min and in 0.1 x 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-β2 mRNA transcripts in endometrium of pregnant mares

Although the nucleic acid sequence of equine mRNA encoding TGF-β2 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-β2 cDNA clone would hybridize to equine endometrial RNA. As adult mouse liver is reported to be negative for mRNA encoding TGF-β2 by northern analysis using a mouse TGF-β2 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-β2. Indeed, many others have detected similar sized transcripts in mouse 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-β2 cDNA probe detected a limited number of known TGF-β2 transcripts in equine endometrium. These findings validated our use of the mouse riboprobe in the cross-species detection of equine TGF-β2 mRNA encoding TGF-β2.

Detection of endometrial mRNA encoding TGF-β2 by in situ hybridization

The localization of TGF-β2 mRNA positive cells in pregnant horse endometrium and the kinetics of expression were investigated by performing in situ hybridization using the mouse TGF-β2 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-β2 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 leukocytes surrounding the endometrial cup trophoblast (■) and for sections of gravid horn endometrium distant from the cups (○). "The specialized trophoblast cells of the chorionic region of the placenta invade the maternal endometrium at days 36–38 to form the gonadotropin-secreting endometrial cups. Endometrial tissues taken earlier in gestation were negative for mRNA encoding TGF-β2, for example, 2 mares at day 29 and 1 mare at day 35. A 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. "Only 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. However, 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 allantochorion. These endometrial tissues were divided accordingly and compared in terms of their expression of mRNA encoding TGF-β2 (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 leukocytes 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 allantochorion 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-β2 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-β2 were distributed between the dilated endometrial glands and among the dense band of leukocytes surrounding the degenerating cup (Fig. 4). In contrast, a sample of endometrium and attached allantochorion 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-β2 are part of the characteristic leukocyte response to the fetal endometrial cups.

Fig. 2. In situ hybridization showing localization of cells positive for mRNA encoding TGF-β2 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: a: luminal epithelium; g: endometrial glands in the stratum spongiosum; s: stroma (×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 (×40). (C) High power view of (B) showing positive cells (×100). (D) Higher power view of (C) showing the close proximity of the positive cells to endometrial glands in the stratum spongiosum (×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 (×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 (×40). (G) High power view at the base of (F) showing labelled cells accumulated in the stroma beneath the cup cells (×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 (×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.
The distribution of cells positive for mRNA encoding TGF-ß2 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-ß2 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 encoding TGF-ß2 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-ß2.

Kinetics of expression of mRNA encoding TGF-ß2 in endometrium of pregnant mares

The kinetics of expression of mRNA encoding TGF-ß2 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-ß2 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 mare harbours a population of chromotrope 2R positive cells which contain TGF-ß2 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-ß2, 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-ß2 transcript or a degradation product. Variability in the number of TGF-ß2 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-ß2 or our use of a simian TGF-ß2 riboprobe in the mouse experiments compared with a mouse TGF-ß2 riboprobe in the horse. A 3.5 kb TGF-ß2 mRNA transcript has, however, been reported by other investigators using mouse cDNA probes and riboprobe 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
components of trophoblast exhibited no evidence of hybridization at any of the stages of pregnancy examined. However, the TGF-β2 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-β2 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-β2. About 4% of the chromotrope 2R positive cells were negative for mRNA encoding TGF-β2 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-β1 in nasal polyps (Ohno et al., 1992).

In pregnant mice, TGF-β2 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-β2 transcripts. It is recognized that there are multiple molecular weight species of TGF-β2 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-β2 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-β2 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-β2 in situ correlates closely with the release of TGF-β2-related suppressor factor from decidual tissue cultured in vitro (Lea et al., 1992). Our mouse studies have suggested that TGF-β2 released by asialo-GM1−, MAC1−, IEl/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; Chauvat 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-β2 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-β2 in endometrial tissue at this stage of gestation. However, immediately after the invasion of the class I

Fig. 5. High power oil emersion view of cells positive for mRNA encoding TGF-β2 stained positive for chromatrop 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 μm.
positive girdle cells into the endometrium on day 36, cells positive for mRNA encoding TGF-ß2 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-ß2 in day 40 endometrium adjoining endometrial cup tissue compared with later gestational stages. Preliminary in situ hybridization studies of other TGF-ß subtypes in equine endometrium suggest a similar distribution of TGF-ß3 positive cells with fewer cells positive for TGF-ß1 (R. G. Lea, W. R. Allen, I. Ohno, M. Jordana and D. A. Clark, unpublished). In general, however, our data suggest that only TGF-ß2 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-ß2, 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-ß2 in equine endometrium is clearly an important component of future studies and the concurrence of chromo¬tropic 2R positivity with in situ hybridization requires further investigation.

The authors thank K. C. Flanders, Laboratory of Chemoprevention, NIH, Bethesda, MD, USA for supplying the bluescript KSII + vector containing the mouse TGF-ß2 insert. They also thank L. Brett, Department of Pathology, Western General Hospital for technical help with the photography and C. Lea for secretarial assistance.

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