The effect of maternal undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe

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Abstract

The placenta is a highly efficient multifunctional organ, mediating the exchange of nutrients, gases and waste products between the dam and fetus. This study investigated the effects of chronic maternal undernutrition (70% of estimated requirement) on the placental growth trajectory in the ewe on days 45, 90 and 135 of gestation. The insulin-like growth factor (IGF) system was investigated using in situ hybridisation analysis to determine if nutritionally mediated alterations in placental growth were regulated through modifications in placental IGF expression. Placental weight increased between days 45 and 90 (P<0.01), accompanied by a reduction in maternal placentome IGF binding protein (IGFBP)-3, -5 and -6 expression (P<0.05), although IGF-II mRNA levels in maternal villi remained unchanged. Placentome number was unaffected by diet or gestational age. Placental weight remained constant between days 90 and 135 in ewes on 100% maintenance rations but decreased over this period (P<0.05) in ewes on the 70% rations. Gross morphology also altered, so the underfed ewes had more type C and type D placentomes and fewer type B placentomes than their well-fed counterparts on day 135 (P<0.05). These changes were accompanied by higher IGFBP-6 mRNA expression in the maternal placental villi in undernourished ewes (P<0.05). The change in shape from a type A to a type C placentome was accompanied by flattening of the placentome and a reduction in the ratio of the area of unattached fetal allantochorion to interdigitated maternal and fetal villi. Within the intercotyledonary endometrium, expression of IGFBPs-3 and -5 mRNA in the glandular epithelium increased between days 45 and 90, showing an opposite trend with time to that found in the adjacent placentomes. This indicates tissue-specific control of IGFBP expression. In conclusion, this study has shown clear time-related changes in the uterine IGFBP system during pregnancy, which accompany changes in placental growth. Altered IGFBP expression may play a role in determining placental size in relation to nutritional status, but is unlikely to be the only mediator.


Introduction

The placenta is an important determinant of fetal growth, with placental size and birthweight being positively correlated in many species, including sheep (Mellor 1983, Kelly 1992). The ewe has a cotyledonary placenta where fetomaternal exchange takes place at discrete sites known as placentomes. The placentomes reach a maximum weight by approximately day 75 to 80 of gestation (term ~150 days) whilst 80% of fetal growth occurs in the final third of gestation (Ehrhardt & Bell 1995, Wallace et al. 1999). In early to mid-pregnancy, insults such as inappropriate maternal nutrition and thermal stress can significantly disrupt placental development and subsequent fetal growth (McCrab et al. 1991, 1992, Vatnick et al. 1991a, Galan et al. 1999). The factors responsible for modifying placental growth are poorly understood.

Evidence suggests that the insulin-like growth factor (IGF) system, a nutritionally sensitive group of proteins, has a fundamental role in placental and fetal development (Reynolds et al. 1997, Wathes et al. 1998, Han & Carter 2000). These findings are supported by gene deletion studies in mice (Baker et al. 1993, Liu et al. 1993). The IGF axis consists of two single chain polypeptides, IGF-I and IGF-II, which mediate cellular proliferation, differentiation and metabolism (Jones & Clemmons 1995, Hossner et al. 1997). The type 1 IGF-receptor (IGF-1R) primarily mediates the biological activities of IGF-I and IGF-II, whilst the type 2 IGF receptor (IGF-2R) is considered to act as a degradative pathway, removing excess IGF-II from the circulation (Jones & Clemmons 1995, Stewart & Rotwein 1996, Braulke 1999). A family of high affinity insulin-like growth factor binding proteins (IGFBPs), designated IGFBP-1 to -6, regulate the biological activities of the IGFs (Rechler & Clemmons 1998, ...
Ferry et al. (1999). It is not known whether nutritionally mediated alterations in placental growth occur as a result of the IGF axis becoming modified. The IGFs were thus studied in the placentomes and intercotyledonary region (the endometrial area between the placentomes) using in situ hybridisation at three critical time points during pregnancy: day 45 (during the establishment of the placentomes), day 90 (following the development of the placenta) and day 135 (during the period of rapid fetal growth). The associated changes in circulating metabolic hormone concentrations and in fetal growth from animals in this study have been reported previously (Osgerby et al. 2002).

Materials and Methods

Experimental design and nutritional treatments

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and took place within the normal seasonal breeding cycle. Forty-eight multiparous Welsh Mountain ewes were individually housed under conditions of natural light and ambient temperature on wheat straw bedding with free access to water. Their body condition scores were standardised to 2.5 before the start of the experiment. Ewes were fed a complete diet of pelleted sheep nuts calculated on an individual ewe basis to provide them with 100% of their daily maintenance requirements (based on the criteria of the Meat and Livestock Commission 1988). The complete diet was fed in two equal portions at 0800 h and 1600 h and supplied 10.8 MJ metabolizable energy and 150 g crude protein per kg dry matter.

Following 30 days of acclimatisation, oestrus was synchronised by withdrawing progesteragen impregnated sponges (60 mg medoxyprogesterone acetate, Vermix; Upjohn, Crawley, Sussex, UK) 12 days after their insertion. At sponge withdrawal, ewes received an injection of a prostaglandin (PG) F₂α analogue, Estrumate (0.5 ml i.m. Schering-Plough Animal Health, Uxbridge, Middlesex, UK) and were presented to the ram 48 h later. Synchronisation was staggered into 3 groups of 16 so that the same ram could be used to fertilise all the ewes and thus minimise genotypic variation. The ram was raddled and the ewes checked four times daily. Day 0 of gestation was taken as the first date at which ewes were observed to have an obvious raddle mark. Pregnancy was confirmed by measuring plasma progesterone concentrations on day 16 of gestation using an enzyme immunoassay kit (Ridgeway Science Ltd, Alvington, Glos, UK). The fetal number was determined as the first date at which ewes were observed to have a pregnancy: day 45 (during the establishment of the placentomes), day 90 (following the development of the placenta) and day 135 (during the period of rapid fetal growth). All the placentomes were removed post-mortem through a midline incision at a consistent point along the length of the cervix at three time points during gestation - day 45 \( (n=16) \), day 90 \( (n=16) \) and day 135 \( (n=16) \). From the body of the uterus four placentomes and six pieces of intercotyledonary endometrium (with fetal membranes attached) were dissected. All tissue samples were wrapped in aluminium foil, rapidly frozen in liquid nitrogen-tempered isopentane and stored at \(-80^\circ\text{C}\) until required for sectioning. The remaining placentomes were dissected from the uterine wall, counted and individually weighed. Following a procedure developed by Vatnick et al. (1991b), placentomes from the day 135 pregnant ewes were typed according to their gross morphology. According to (i) the overall shape of the placentome i.e. rounded or flat and (ii) the position of the fetal tissue (cotyledon) in relation to the maternal portion of the placentome (caruncle), the placentome was classified as type A, B, C or D (see Fig. 1). Type A placentomes are rounded or concave in shape, with the fetal cotyledon level with the top of the caruncle. In type B placentomes the fetal cotyledon is raised, protruding from the centre of the rounded cotyledon. Type C placentomes are more flat when viewed from the top, with the fetal cotyledon covering the top of the caruncle. The lower part of the caruncle is still visible when viewed from the side. Type D placentomes are flat, with the fetal cotyledon completely covering the caruncle such that it is no longer visible when viewed from the side. These changes in shape are only apparent in late gestation, so only placentomes from day 135 were classified in this way.

In a follow-up experiment to examine placentome type in more detail, one further ewe taken from the university flock and carrying a singleton pregnancy was killed on day 135 of gestation. All the placentomes were removed and sorted according to type. Twelve placentomes of each of types A, B and C were then selected at random, weighed and fixed in 10% formalin. As only three type D placentomes were present, these were excluded from the analysis. All the fixed placentomes were subsequently cut through the midline, and a section was stained with haematoxylin and eosin for histological analysis. Using an image analysis system (SeescanPLC, Cambridge, Cambs, UK), measurements were made on each section of the
following parameters: total width of the placentome, distance between the tips of the placentome, width of the area of interdigitation, width of the capsule, area of interdigitated maternal and fetal villi and area of central core of allantochorion (Fig. 1).

Oligonucleotide probes
All probes used for in situ hybridisation were single stranded oligonucleotides (Babraham Institute, Cambridge, Cambs, UK). Sense probes were always included as negative controls as any signal produced on applying this probe could be regarded as non-specific. In the placentomes IGF-II, IGFBP-2, IGFBP-3, IGFBP-5 and IGFBP-6 mRNA were investigated. In the intercotyledonary tissue (the areas between the placentomes) IGFBP-3, IGFBP-5 and IGFBP-6 mRNA were studied. These probes were chosen based on our previous studies on the localisation of the placental IGF system during ovine pregnancy (Reynolds et al. 1997, Gadd et al. 2000a,b). All probes used in the study are described in Table 1.

Localisation of mRNA by in situ hybridisation
This procedure was performed as described previously by Reynolds et al. (1997). All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) unless otherwise stated. In summary, frozen placentome and intercotyledonary tissue sections were cut (10 µm thick) and thaw mounted onto 1 mg/ml

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**Figure 1** Photomicrographs of four placentomes stained with haemotoxylin and eosin to illustrate differences in dimensions according to placentome type. Details of the classification system are given in the text. Measurements taken for analysis included: (a) width of placentome, (b) distance between tips and (c) width of interdigitation of fetal and maternal villi. AC, fetal allantochorion; V, area of interdigitating maternal and fetal villi; PC, placentome capsule. Scale bar represents 1 cm.
poly-l-lysine (M, >300 000)-coated glass slides. Sections were fixed in 4% (wt/vol) paraformaldehyde in 0.01 M PBS at pH 7.0 for 5 min at room temperature, prior to three washes in 0.01 M PBS and sequential dehydration in 70% and 95% ethanol.

The oligonucleotide (5 ng) was labelled with 35S dATP (Amersham International, Aylesbury, Bucks, UK) at the 3′-end using deoxynucleotidyl transferase (Pharmacia Biotech, St Albans, Herts, UK) at 34 °C for 60 min. The labelled probe was subsequently diluted to a final concentration of 1 100 000 c.p.m/ml in hybridisation buffer and 100 µl were added to each section. The sections were incubated in a humidified box overnight at 42.5 °C. Following incubation, slides were washed in 1 × saline sodium citrate (1 × SSC) 0.2% (wt/vol) sodium thiosulphate-phantahydrate solution at room temperature for 30 min, then at a higher stringency in 1 × SSC 0.2% (wt/vol) sodium thiosulphate-phantahydrate solution for 60 min at 57.5 °C. Sections were finally rinsed in 1 × SSC, 0.1 × SSC, 70% ethanol, and 95% ethanol, for 1 min each. Slides were then air dried for at least 2 h and exposed to β-max hyperfilm (Amersham International) for the time indicated in Table 1. Uterine samples shown to be positive for each of the probes (Reynolds et al. 1997, Wathes et al. 1998) were included in each experiment as a positive control.

Photographic emulsions

Slides previously exposed to X-ray film were coated with a photographic emulsion (LM1, Amersham International) according to the manufacturer’s instructions and stored at 4 °C for the time indicated in Table 1. Slides were then developed in 20% phenosol, fixed in 1:9 M sodium thiosulphate-pentahydrate and counterstained with haematoxylin and eosin to confirm microscopically the cellular localisation of the radioactive signal.

**Table 1** The sequence of the antisense probes and exposure time for *in situ* hybridisation, X-ray films and emulsions

<table>
<thead>
<tr>
<th>Probe</th>
<th>Antisense sequence</th>
<th>X-ray film</th>
<th>Emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II</td>
<td>554–598 of ovine IGF-II gene (O’Mahoney &amp; Adams 1989)</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>5′-AAG-TGG-AGG-GTG-TCC-ACC-AGC-TCC-CCG-CCG-AGA-GTC-TGG-CTG-3′</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>5′-CAG-AGT-GCT-CTG-CCG-CAT-TGT-CTG-CAA-CCT-GCT-CTG-CCG-GGC-CCG-3′</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>5′-AGC-CTG-ATT-CTG-CTG-CAT-GCT-GTC-GTC-3′</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>5′-TCC-GAG-ATG-CCG-GTG-TCC-CCG-CCG-AAG-ATG-GTC-GGC-CCG-3′</td>
<td>30</td>
<td>37</td>
</tr>
</tbody>
</table>

Optical density (OD) quantification

An image analysis system (Seescan PLC) was used to quantify the level of radioactive signal as OD units using a linear grey scale of 0–2.1 as described previously (Reynolds et al. 1997). To determine a background reading of the autoradiograph under analysis, a blank section of the film was placed under the image analyser lens and measured. Measurements were made of the antisense (AS) and sense (S) images obtained from four sections per sample. For each region where localisation had been confirmed, at least six readings per section were taken, giving a minimum of 24 readings per region per sample. The S values were subtracted from the AS values to produce a mean value of specific hybridisation for a particular region for that sample. The detection limit was taken as an OD greater than 0.01. The coefficients of variation for duplicate measurements of the pairs of slides were as follows: IGF-II, 6.5%; IGFBP-2, 11.3%; IGFBP-3, 8.9%; IGFBP-5, 11.4%; IGFBP-6, 6.3%. All samples for each probe were processed in the same batch to avoid any possible inter-batch variation. While this system provides accurate comparisons between animals using the same probe, note that it is not valid to make comparisons of OD units between different probes.

Statistical analysis

All values are given as means ± S.E.M. The statistical analysis was performed using Statistical Package for the Social Sciences version 7.0 (SPSS, Chicago, IL, USA). All data were tested for homogeneity of variance and log transformed if necessary. The IGF expression data in a given uterine region were analysed by univariate analysis of variance with ration (100% or 70%) and day of gestation (45, 90 or 135) as factors. *Post hoc* tests were by Tukey honestly significantly different (HSD). Data on the histological analysis of placentome shape (A, B or C) were
analysed by one-way ANOVA with post hoc least significant difference tests. Data were considered statistically significant when P values were equal to or less than 0·05.

Results

Maternal data

Of the 48 ewes mated, 27 had singleton pregnancies and 21 had twins. To avoid any nutritional effects of fetus number, the data presented in this study will concern only those ewes that had singleton pregnancies on day 45 (100%, n=5; 70%, n=4), day 90 (100%, n=4; 70%, n=5) and day 135 (100%, n=4; 70%, n=5). The well-fed ewes maintained their body condition score of 2·5 at mating until the end of the study whereas the undernourished ewes (on the 70% ration) dropped approximately half a body condition score by day 135 of gestation.

Uterine and placental data

The total number of placentomes and the mean placen-
tome weight were unaltered by dietary treatment at each time point investigated (Table 2). However, on day 135 the total uterine weight and the total placental weight tended to be lower in undernourished ewes (Table 2; a>b, P<0·08).

Over time the placental growth trajectory differed between the two dietary treatments. Initially both groups of ewes experienced a significant increase in their total and mean placental weights between days 45 and 90 (Table 2; g h, i j, P<0·01). The well-fed ewes maintained these weights to day 135 whilst the placentae from the undernourished ewes significantly decreased from their day 90 weights (Table 2; k P<0·05). The placental number remained unchanged by gestational age. The placentae from the undernourished mothers had significantly more type D and type C placentomes and fewer type B placentomes than their well-fed counterparts (Table 2; c f, P<0·05).

Expression of components of the IGF axis in placentomes

In the placentomes, IGF-II mRNA was localised in the mesoderm of the fetal villi (OD 0·18±0·007, n=26) and at lower levels in the maternal placentome capsule (OD 0·06±0·006) (Fig. 2A). In both regions, expression was unaltered by ration or stage of gestation.

IGFBP-2, -3, -5 and -6 were all localised in the maternal (but not the fetal) component of the placentomes, in both the villi and the surrounding capsule. IGFBP-2 mRNA expression was higher in the capsule (OD 0·17±0·015, n=25) compared with the maternal villi (OD 0·06±0·006) (Fig. 2C, D, Fig. 3A-C) and expression was unaltered by time or ration in either location. IGFBP-3 mRNA was expressed in the placente
capsule (OD 0.10 ± 0.008, n=27) and in the maternal villi (Fig. 2E, F, Fig. 3D, E). IGFBP-3 expression in the maternal villi, IGFBP-5 expression in the maternal villi (Fig. 4A–C, Fig. 5A–C) and IGFBP-6 mRNA expression in the placentome capsule (Fig. 4E, F) all showed time-related changes, with a major reduction in expression between 45 and 90 days of gestation (Fig. 6). IGFBP-5 expression in the capsule also decreased with time but not until after day 90. None of these measurements was affected by ration. IGFBP-6 mRNA expression in the maternal villi (Fig. 4E) also decreased over time, but in this case there was also an effect of ration, with expression lower on days 90 and 135 in sheep on the 100% compared with the 70% ration (Fig. 7).

Expression of components of the IGF axis in intercotyledonary endometrium

IGF-II mRNA expression was detected in both the luminal epithelium (OD 0.07 ± 0.007, n=25) and the...
Figure 3 Sections were hybridised with antisense (AS) (A, C–G) and sense (S, control) (B, H) oligonucleotide probes, coated with photographic emulsion and counterstained with haematoxylin and eosin. The silver grains confirmed that IGFBP-2 (A–C) and IGFBP-3 (D, E) mRNA is expressed in the placental capsule (PC) and caruncular stroma (CS) in the ovine placentome of well-fed ewes on days 45 and 90 of gestation respectively. In the intercotyledonary region, IGFBP-3 (F–H) mRNA was localised in the luminal epithelium (LE) and endometrial glands (EG) in well-fed ewes on day 90 of gestation. SC, stratum compactum. Scale bars represent 200 μm.
underlying stratum compactum (OD 0.12 ± 0.023). As in the placentomes, expression was not altered by gestation stage or ration. IGFBPs-3 and -5 mRNA were detected in the luminal epithelium (IGFBP-3, OD 0.08 ± 0.008, n = 26; IGFBP-5 0.37 ± 0.027, n = 23) and glandular epithelium (Fig. 3G, Fig. 4D, Fig. 5D). In the glands, expression of IGFBP-3 and IGFBP-5 mRNA both showed changes related to stage of gestation but in the opposite direction to those observed in the maternal villi, as concentrations increased between days 45 and 90 of gestation (Fig. 6). IGFBP-6 mRNA was also expressed in the glandular epithelium (OD 0.06 ± 0.004, n = 26) and at a higher level in the stratum compactum (OD 0.21 ± 0.011) (Fig. 5F). Within the intercotyledonary

Figure 4 Autoradiographs showing the localisation of IGFBP-5 and IGFBP-6 mRNA in the pregnant ovine uterus. Sections were hybridised with antisense (AS) (A, C, D, E) or sense (S, control) (B, F) oligonucleotide probes. Sections A to C show placentome sections taken from well-fed ewes on day 45 (A and B) and day 135 of gestation (C). IGFBP-5 mRNA is expressed in the placentome capsule (PC) and caruncular stroma (CS) with levels decreasing between days 45 and 135 of gestation (A-C). Section D demonstrates the expression of IGFBP-5 mRNA in the luminal epithelium (LE) and endometrial glands (EG) in the intercotyledonary region of a well-fed ewe on day 90 of gestation. Sections E and F show the expression of IGFBP-6 mRNA in the placentome of a well-fed ewe on day 45 of gestation where levels are confined to the placentome capsule (PC) and caruncular stroma (CS). Scale bars represent 1.25 mm.
endometrium, no significant changes in expression of the IGF system were detected due to ration.

Changes in placentome shape

Results of the main experiment indicated that there was a shift to more C and D type placentomes on the restricted diet by day 135 gestation. In order to examine the implication of such a change on placental histology, 12 placentomes of each of types A, B and C were collected from one ewe on day 135. There were insufficient type D placentomes present for statistical analysis. Examination of cross sections through the centre of each placentome suggested that the main change with type was an opening out and flattening of the placentome from type A to type D (Fig. 1). This was associated with an increase in weight. In this process, the overall width of the placentome and the distance between the tips both increased significantly (Table 3). There were no significant changes in the width of the area of interdigitation between maternal and fetal villi or in the width of the capsule. The total area of interdigitation and of the central core of fetal allanto chorion both increased. However, the ratio of interdigitated to fetal only tissue also increased between types A and C.

Figure 5 Sections were hybridised with antisense (AS) (A, C, D, F) or sense (S, control) (B, E) oligonucleotide probes, coated with photographic emulsion and counterstained with haematoxylin and eosin. IGFBP-5 mRNA was localised in the placentome capsule (PC) and caruncular stroma (CS) in the placentome (A–C), and in the luminal epithelium (LE) and endometrial glands (EG) in the intercotyledonary region (D, E) of a well-fed ewe on day 45 of gestation. IGFBP-6 mRNA was detected in the stratum compactum (SC) underlying the luminal epithelium (LE) in the intercotyledonary region of a well-fed ewe on day 90 of gestation (F). Scale bars represent 200 μm (A–C, F) and 800 μm (D, E).
Figure 6 Histograms showing changes in IGFBP expression with stage of gestation in different regions of the ovine placenta. (a) IGFBP-6 and (b) IGFBP-5 mRNA expression in the placentome capsule (CAP). (c) IGFBP-3 and (d) IGFBP-5 mRNA expression in the maternal villi (MV). (e) IGFBP-3 and (f) IGFBP-5 mRNA in the glandular epithelium (GE). Values are measured as OD units from autoradiographs on days 45, 90 and 135 (n=9 ewes at each stage of gestation) and are shown as means ± S.E.M. The effect of stage of gestation on mRNA expression is indicated: b > a P < 0.001; d > c, P < 0.06. None of the IGFBPs illustrated was affected by ration. Note that with the in situ hybridisation system used, it is not valid to make comparisons of OD units between different probes.
This implied that there was proportionately less uninterdigitated fetal allantochorion present in the type C placentomes.

Discussion

During pregnancy, inappropriate maternal nutrition can significantly disrupt the placental growth trajectory (Kelly 1992, Wallace et al. 1999) but the factors that instigate these changes remain unknown. The IGF axis, localised in the reproductive tract and placentae of many species, may play a fundamental role in placental and fetal growth (sheep: Reynolds et al. 1997, cow: Robinson et al. 2000, rodent, primate, human: Han & Carter 2000). Recent studies have demonstrated the nutritional sensitivity of the IGFs, both systemically (Thissen et al. 1994, Bauer et al. 1995, Sohlström et al. 1998) and locally within the uterine tract (Gadd et al. 2000a). In this study we have investigated the utero-placental IGF system using in situ hybridisation to enable tissue-specific localization of mRNA. We did not attempt to co-localise protein expression in the different uterine regions as locally synthesised protein cannot be distinguished from that which has arrived at the region via the circulation. However, by focusing upon gene expression we realise that the post-transcriptional regulation of the protein cannot be addressed.

The total and average placentome weights increased between days 45 and 90 of gestation. Earlier studies have demonstrated that placentome proliferation reaches a maximum rate between days 50 and 60 of gestation, with placentomes attaining a maximum weight by days 75 to 80 (Ehrhardt & Bell 1995). Nutritional insults during this period have thus been associated with changes in placental growth (McCrabb et al. 1992), with maternal factors including weight, age and body condition influencing this interaction (DeBarro et al. 1992, Wallace et al. 1997, Osgerby et al. 2003a,b). In this study, placentome weight was not, at this early stage, influenced by nutrition although we have previously reported that the 70% reduction in ration did result in altered fetal growth by day 90, with more obvious signs of fetal growth retardation apparent by day 135 of gestation (Osgerby et al. 2002). Ration did, however, significantly affect the placental growth trajectory between days 90 and 135 of gestation, with the total placentome weight decreasing in underfed ewes whilst remaining unchanged in their well-fed counterparts.

The factors responsible for regulating placentome size remain to be elucidated. IGF-I mRNA was not

![Figure 7 Histograms showing changes in IGFBP-6 mRNA expression with stage of gestation and ration in maternal villi. Values are measured as OD units from autoradiographs on days 45, 90 and 135. Ewes were fed either 100% (open bars) or 70% (solid bars) of their estimated nutrient requirements for the stage of gestation, with 4 or 5 ewes per group. Values are means ± S.E.M. Differences due to stage of gestation are indicated by the superscript letters a > b (P<0.01). Differences due to ration at a particular stage are indicated by *P<0.05.](image)

This implied that there was proportionately less uninterdigitated fetal allantochorion present in the type C placentomes.

Table 3 Comparison of measurements of placentomes of different types from the same ewe

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Type A (n=12)</th>
<th>Type B (n=12)</th>
<th>Type C (n=12)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Width of placentome (mm)</td>
<td>18.7 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.5 ± 1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.3 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>Distance between tips (mm)</td>
<td>4.9 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.3 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>4.9 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>Width of interdigation (mm)</td>
<td>5.0 ± 0.19</td>
<td>4.6 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.016</td>
</tr>
<tr>
<td>Width of capsule (mm)</td>
<td>0.67 ± 0.32</td>
<td>0.60 ± 0.018</td>
<td>0.63 ± 0.038</td>
<td>NS</td>
</tr>
<tr>
<td>Area of interdigitated villi (mm²)</td>
<td>118 ± 9.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>123 ± 13.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>178 ± 12.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>Area of central allantochorion (mm²)</td>
<td>33.5 ± 3.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.2 ± 5.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.9 ± 6.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003#</td>
</tr>
<tr>
<td>Ratio V:AC</td>
<td>3.7 ± 0.30</td>
<td>2.9 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.045#</td>
</tr>
</tbody>
</table>

Within rows, values with different superscript letters were significantly different. For rows marked with #, analysis was on log transformed data.

NS, not significant.
investigated as previous studies had demonstrated that levels were below the limit of detection after implantation (Reynolds et al. 1997). However, IGF-II mRNA was localised in the placentome capsule and mesoderm of the fetal villi as previously described (Reynolds et al. 1997, Gadd et al. 2000a). Mouse embryos carrying null mutations for IGF-II exhibit placental growth retardation, emphasising the significant role which this growth factor has in placental development (Baker et al. 1993). However, in this study maternal ration had no significant effect on placental IGF-II mRNA and this lack of effect due to nutrition is consistent with two previous studies (Gadd et al. 2003a, Osgerby et al. 2003b). IGF-II mRNA levels were also unaltered by gestational age.

The IGFBPs localised in the placentome capsule and stroma of the maternal villi as previously described (Reynolds et al. 1997, Gadd et al. 2000a,b) may, however, regulate the mitogenic activity of IGF-II in the placentome. IGFBP-3 and IGFBP-5 expression in the caruncular stroma of the maternal villi and IGFBP-6 mRNA in the placentome capsule all decreased significantly between days 45 and 90 of gestation. These reductions in IGFBP expression may result in lower levels of protein being translated. Consequently, the availability and thus the mitogenic capacity of the IGFs may rise, contributing to the increase in placental mass between days 45 and 90 of gestation.

The reduced total placentome weight in underfed ewes between days 90 and 135 was associated with higher IGFBP-6 mRNA expression in maternal villi. However, IGFBP-2, IGFBP-3 and IGFBP-5 expression in the caruncular stroma of the maternal villi and IGFBP-6, -5 and -6 mRNA in the placentome capsule remained unaltered by ration. IGFBP-3 has previously been shown to decrease in the plasma of undernourished animals whilst circulating IGFBP-2 is inversely regulated by insulin, increasing in response to dietary restriction (Lee et al. 1997, Maxwell et al. 1998, Oldham et al. 1999). In our study, the insulin concentration in late gestation was reduced on the restricted ration (Osgerby et al. 2002). Although circulating concentrations of IGFBPs were not measured, it is thus possible that raised IGFBP-2 levels in the plasma of undernourished ewes coupled with higher IGFBP-6 expression in the maternal villi may have been responsible for reducing placental weight by inhibiting the IGF-II mediated proliferation. This is supported by findings in the guinea pig, where placental weight and maternal plasma IGFBP-2 concentrations were negatively correlated (Roberts et al. 2001).

A reduction in placental weight between mid and late pregnancy has previously been described (Vatnick & Bell 1992, Ehrhardt & Bell 1995, Schneider 1996, Jenkinson et al. 1999). This may have significant implications on subsequent milk production as placental lactogen and progesterone, important mediators of mammary gland development, are produced from the placenta at levels that are proportional to the placental mass (Mellor 1987).

The shapes of the placentomes on day 135 of gestation were also modified by diet. Undernourished ewes had significantly fewer type B placentomes and more types C and D placentomes than well-fed ewes. Similar structural adaptations have previously been reported in ewes that have been undernourished (Crowe et al. 1996), undergone a unilateral fetectomy (Vatnick et al. 1991b) or been exposed to hypoxic conditions (Penninga & Longo 1998). Women living at high altitude also have an increased incidence of irregular shaped placentas (reviewed by Penninga & Longo 1998). It has been suggested that this change in shape is due to a compensatory increase in fetal vascularisation in response to nutrient restriction (Hoet & Hanson 1999). Histological analysis in this study showed that the structural change was due to an opening of the placentome which was not accompanied by a proportionate increase in the area of fetal allantochorion. The trend was rather in the reverse direction. This result corresponds to a study by Heasman et al. (1999) who manually separated fetal and maternal components of the placentomes and found that dietary restriction to 50–60% of maintenance energy requirements between 30 and 80 days of gestation caused a significant decrease in the weight of the fetal component, whereas the weight of the maternal component remained the same. The shift from the type A to the type C placentome in the single ewe examined in the follow-up study described here was accompanied by an increase in placentome weight. We have, however, also observed small type D placentomes (J C Osgerby, T S Gadd and D C Wathes, unpublished observations) and in the main study the shift from type B to type D placentomes was accompanied by an overall decrease in placental weight. It is therefore unlikely that the flattening process is caused just by an increase in size and as we did not assess the expression of the IGF axis in relation to placentome type it remains uncertain what does cause the flattening.

The number of placentomes that formed was unaltered by diet and gestational age. Holst et al. (1992) similarly found that a moderate nutritional restriction implemented for four weeks from days 79, 87 and 95 of gestation exerted a negligible effect on placentome number, which is thought to be fixed by day 56 of gestation (Wallace 1948). There are, however, exceptions with infusions of bovine somatotrophin from days 97 to 124 of gestation increasing the placentome number (Stelwagen et al. 1994) while Heasman et al. (1999) found that feeding ewes 50–60% maintenance energy on days 30 to 80 of gestation caused a significant increase in placentome number in the nutrient-restricted group. The factors that prevent further placentomes from developing in late gestation are unclear. Neither body condition score at mating nor ration (100 or 70% of maintenance requirements) affected placentome number at day 65 of gestation (Osgerby et al. 2003a,b). Placentome number at this time was, however, positively related to the circulating maternal IGF-I concentration.
and negatively related to IGFBP-3 expression in the luminal epithelium (Osgerby et al. 2003b). It is thus possible that a high local concentration of IGFBP-3 can inhibit placental formation.

In the intercotyledonary region, the expression of IGFBPs-3 and -5 mRNA increased between days 90 and 135 of pregnancy. In contrast, expression of both these binding proteins in the placentomes decreased as pregnancy progressed. Therefore, there were opposite trends in closely adjacent maternal uterine tissues, which were presumably exposed to similar circulating hormone concentrations. In the Dorset ewe in mid gestation we have previously reported reduced expression of both IGFBPs-3 and -5 mRNA in the glandular epithelium of ewes of fat compared with thin body condition (Osgerby et al. 2003a). Surprisingly little is known about local factors regulating IGFBP expression in different tissues. In liver, growth hormone (GH), IGF-I and insulin concentrations all contribute to the control of IGFBP-3 synthesis (Rajaram et al. 1997, Phillips et al. 1998). Type 1 IGF receptors are localised to the epithelium but not the placentome in the pregnant ovine uterus (Reynolds et al. 1997), and GH receptor expression is also highest in this location (Klemp et al. 1993) providing a possible differential control mechanism. Uterine IGFBP-5 mRNA is up-regulated by an embryonic factor in early pregnancy (Gadd et al. 2000b) with evidence from other systems suggesting that this could be IGF-II (e.g. Matsumoto et al. 1996). However, local expression of IGF-II in this study did not change with time. In the quail, Fu et al. (2001) reported that retinoic acid could cause a rapid up-regulation in IGFBP-5 mRNA expression in a tissue-specific manner. Further work is therefore required to determine both the control mechanisms involved and the functional significance of changing IGFBP expression in the uterus during pregnancy.

In summary, the 70% ration used in this study only had a relatively small effect on placental size although we have shown previously that it did result in fetal growth retardation (Osgerby et al. 2002). The increase in placental weight between days 45 and 90 of gestation was accompanied by a reduction in placental IGFBPs-3, -5 and -6 expression whilst IGF-II mRNA levels remained unchanged. The placental weight increased in underfed ewes between days 90 and 135 of gestation and the gross placental morphology on day 135 was altered with fewer type B and more type C and D placentomes expressed than in well-fed animals. This was accompanied by a higher expression of IGFBP-6 mRNA in the maternal villi over this period but other aspects of the placental IGF system were not altered by the reduced ration. These data suggest that changing placental expression of IGF binding proteins may affect placental growth but additional factors probably contribute to the fine-tuning in size associated with diet.

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