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Variation in Macrophage Migration Inhibitory Factor [MIF] immunoreactivity during bovine gestation

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ABSTRACT

Macrophage Migration Inhibitory Factor (MIF) is a proinflammatory cytokine involved in several aspects of the immune response. MIF appears to play important roles in materno-fetal immuno-tolerance during placental establishment, modulation and growth as studied in epitheliochorial porcine and hemochorial human and mouse placentae. Here we studied the bovine placenta being multiplex, villous and synepitheliochorial with a low degree of invasion, to see if MIF could be involved. Placental tissues sampled from 12 cows at 9 stages of gestation (days 18–250), and endometrial tissues from two non-pregnant animals were processed for immunohistochemistry. Bovine MIF was detected by Western blot using anti-human MIF monoclonal antibodies. An immunoreactive band of approximately 12 kDa confirmed similarities between bovine and human MIFs. Compared to the non-pregnant stage with very faint staining, the caruncular epithelium during pregnancy showed stronger staining for MIF. The intercaruncular epithelium in non-pregnant endometrium showed some reaction apically with increasing intensity at uterine gland openings; in contrast, at day 18 of gestation this staining was markedly increased. During gestation both caruncular and trophoblast epithelium of the placentomes were positive with different intensity in relation to the gestational stage. In the uterine glands, some strongly stained cells were present. The mature binucleated trophoblast giant cells were negative throughout pregnancy. During reestablishment of vascularisation, the vasculature in the caruncular area showed MIF reactivity. While supporting involvement of MIF in different placental types, the spatio-temporal variation in the bovine placenta suggests a regulatory role for MIF mainly in the interhemal barrier and during vascular development.

1. Introduction

Macrophage Migration Inhibitory Factor (MIF), first demonstrated as a factor capable of inhibiting the random migration of macrophages *in vitro*, is now recognised as a proinflammatory cytokine involved in several aspects of the immune response [1]. MIF is produced by a number of immune and non-immune cells including macrophages, lymphocytes and fibroblasts as well as those of the endocrine, nervous and reproductive systems [1]. Recent literature has shown that MIF is involved in different reproductive processes mainly in humans and mice [2–4]. MIF mRNA and protein are highly expressed in the human endometrium throughout the menstrual cycle and in early pregnancy in predecidualized stromal cells [5]. MIF expression in human endometrium was found to increase during the mid-late proliferative phase reaching a maximum around ovulation and decrease in the mid-secretory phase before increasing again in the late secretory phase [6]. In the human placenta, MIF mRNA and protein are highly expressed mainly by the villous and extravillous cytotrophoblast [7]. Placental MIF levels were higher in the very early stages of gestation and declined at late first trimester [8]. In addition, other studies in humans have shown that decreasing levels of maternal serum MIF were associated with first trimester miscarriages [9].

In mice, MIF mRNA is expressed in uteri during the pre-implantation period and throughout the estrous cycle as well as in early embryos [10]. MIF mRNA and protein are also expressed by the fetal placental components with an increase at gestational day 10.5, the stage at which the placenta assumes the three-layered organization and the fetal blood circulation begins [11]. The importance of MIF in early murine gestation is supported by the

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findings of Bondza et al. [12] who showed that an intraperitoneal injection of recombinant MIF on the day after mating resulted in an enhancement of the pregnancy rate.

All these data suggest that MIF is a key cytokine involved in uterine receptivity and pregnancy in species with a hemochorial type of placenta. Studies in other species with different placental type are limited. In the diffuse folded epitheliochorial placenta of the pig, it was shown that MIF is mainly expressed by trophoblast and maternal epithelium throughout gestation with a dramatic decrease in the maternal epithelium in late gestation, whereas immunostaining in trophoblast remained relatively high [13]. In a recent extensive study in the sheep [14], MIF was characterized and localised in all compartments of the female genital tract as well as in the placenta from early and late stages, but without a more detailed description of placental MIF throughout gestation. In ruminants, the placenta is cotyledonary, villous and epitheliochorial but it is a subtype, namely synepitheliochorial, as the binucleated trophoblast giant cells (TGC) migrate to fuse with one maternal epithelial cell forming a multinucleated hybrid cell [15,16]. The TGC have high secretory activity and release hormonal products including progesterone, prostacyclins, prostaglandins, placental lactogen and pregnancy specific glycoproteins to the maternal compartment [17–19]. The invasion does not go beyond the basal lamina of the maternal epithelium and the hormonal products are released close to the maternal circulation. At present there is no evidence in the literature of MIF in the bovine placenta, however MIF has been localised on days 1–3 of the estrus cycle in the luminal epithelium of the bovine endometrium and in uterine glands [20]. The aim of this study is therefore to investigate the expression of MIF in the bovine placenta at various gestational stages from initial placentation to late pregnancy.

2. Materials and methods

2.1. Animals

We used material from 16 cows, where the early stages (days 18–42) were defined from the time of insemination i.e. post-insemination (pi); in the later stages crown rump length (CR) was used to assess the day pi. Placental tissues were obtained from 12 cows at 9 stages of gestation from day 18 pi up to close to term (days 18, 26, 36, 40, 42, 110, 160, 177, 250 pi). Gestation time in cows is 280 ∓2 days. Endometrial tissues were also taken from 2 non-pregnant animals around estrus. The early stages up to days 40–42 pi were immersion fixed in situ via the uterine lumen with 4% buffered formalin, and one uterus from day 26 pi was perfusion fixed from the maternal uterine artery. From these early stages the caruncular/placentomal as well as the intercaruncular/interplacentomal tissues were collected, however no fetal membranes were conserved in contact with the endometrium at the very early stages (days 18–26 pi). At the later stages the placentomes were perfusion fixed from the fetal side with the same fixative.

Endometrium, alone or with adherent fetal membranes and placentomal tissues, was cut out (6–9 blocks of tissues per fetus) and post-fixed in the same fixative for 24 h, stored in phosphate buffered saline (PBS), and subsequently embedded in paraffin.

Additionally, placentomes were taken from two animals at approximately day 110 of gestation (23 and 26 cm crown-rump length, CRL) and manually separated into fetal cotyledon and maternal caruncle parts. These tissues were cut into smaller pieces (1 × 1 cm) and immediately frozen in liquid nitrogen for antibody validation by Western blot.

2.2. Immunohistochemistry

Immunohistochemical staining was performed on 5 μm thick sections of formalin-fixed, paraffin embedded tissues at each of the gestational stages as well as in non-pregnant uteri as described above. After deparaffination in Bioclear (Bio-Optica – Milan, Italy) and rehydration in serial dilutions of ethanol, the histological sections were washed in Tris buffered saline (TBS) pH 7.6 and pre-incubated with normal rabbit serum to prevent non-specific binding. The slides were first incubated overnight 4 °C with mouse anti-human MIF monoclonal (1:100) antibody (R&D System Abingdon, U.K.). Slides were then incubated with a secondary *rabbit anti-mouse* antibody, labeled with biotin (Dako, Copenhagen, Denmark) (1:500 in TBS) and finally with Streptavidin–AP complex (Dako) diluted 1:300. Each incubation was performed for 30 min at room temperature and was followed by three washes in TBS. The alkaline phosphatase reaction was revealed using SIGMAFAST™ (Sigma Aldrich, St. Louis, MO, USA) as substrate. The sections were then washed for 5 min in running tap water and mounted with aqueous mountant. No counterstaining was undertaken. Controls were performed for each tissue by substituting the primary antibody with TBS. First trimester human placental tissues were used as positive controls for MIF staining. Evaluation and micrographs were performed with a Leica DMR light microscope.

2.3. Western blot

Specificity of the anti-human MIF monoclonal antibodies on bovine placentomal tissues was assessed by Western blot.

For protein isolation, manually separated and snap-frozen placentomal tissues from two animals (CRL 23 and 26 cm) were homogenized in ice-cold BLP (50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1% (v/v) Nonidet P40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride) supplemented with complete proteinase inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). After centrifugation at 15000 × *g* for 5 min at 4 °C, the supernatant was assayed for total protein concentration [21] and MIF detection by Western blot analysis. The total protein (25 μg or 75 μg) for each tissue was separated on 4–12% NuPAGE Gel with MES buffer (Invitrogen Paisley, Scotland UK) in the presence of SDS according to the procedure of Laemmli [22] and the gel was run at 200 V for 40 min. Proteins were then blotted onto nitrocellulose membranes at 30 V for 1.5 h. Uniform blotting was confirmed by staining the membrane with Ponceau S (0.2% Ponceau S in 3% acetic acid) (Sigma Aldrich) for 3 minutes. Afterwards, the membrane was blocked with 5% nonfat dry milk in TBST (TBS, pH 7.4, 0.1% Tween 20) for 1.5 h. Primary monoclonal antibodies were used at 1:500 for mouse anti-human MIF (R&D System) and 1:2000 for beta-actin (Sigma Aldrich) in TBS, or omitted (negative control). The membrane was then incubated with an alkaline phosphatase conjugated secondary antibody diluted 1:1000 (Invitrogen Paisley, Scotland UK). The bands were finally detected with a BCIP/NBT Substrate-Kit (Invitrogen Paisley, Scotland UK) according to the manufacturer's instructions.

3. Results

3.1. Immunohistochemistry

The bovine placenta consists of up to 80–120 placentomes composed of maternal caruncles forming crypts complementary to fetal villi assembled in cotyledons. The uterine glands open only between the caruncles (Figs. 1A and B).

In the non-pregnant uterus the uterine epithelium of the caruncles (Figs. 2A) exhibited no or in some cells a very faint MIF reactivity, whereas the intercaruncular epithelium (Fig. 2B) showed no reaction or reaction apically in a few cells being more intense in some cells at the uterine gland openings. The uterine glands themselves showed only a weak immunostaining in a few cells. In the stratum compactum and stratum reticularis only very few cells showed some immunostaining.

At days 18 and 26 pi only endometrium could be studied as the fetal membranes were lost.

At day 18 pi, MIF showed a stronger staining in the caruncular epithelium (Fig. 2C) compared to that in the intercaruncular region (Fig. 2D) and in the cyclic stage. In the uterine glandular epithelium some strongly stained cells were present. In the stratum compactum of the caruncular and intercaruncular regions the cellular reactivity differed, being high in the caruncles (Fig. 2C). In the intercaruncular region, a thin layer of the stratum compactum adjacent to the epithelium was unstained (Fig. 2D). In the remaining stratum compactum and in the stratum reticularis only very few strongly stained cells were seen.

At day 26 pi vascularisation of the caruncular part had increased remarkedly, being clearly visible especially in the perfusion fixed tissue (Fig. 2E). The caruncular epithelium appeared very fragile due to the close apposition to the trophoblast and migration of TGC. The caruncular epithelium showed a strong to moderate immune staining and the cells in the stratum compactum were seen in a less broad band than seen at day 18 pi and showed a weaker reactivity. In the intercaruncular region (Fig. 2F), the uterine epithelium