Introduction

Abnormal trophoblast physiology in late canine gestation can lead to postpartum complications including subinvolution of placental sites and placental retention. Understanding normal late gestation canine trophoblast physiology provides a basis for future studies aimed at characterizing postpartum placenta-related disorders. The objective of the current study was to characterize term canine trophoblast migration, invasion, and proliferation in vitro. Further, the effects of interleukin-8 (IL-8) and tissue inhibitor of metalloproteinase-2 (TIMP-2) on trophoblast physiology were examined. Both IL-8 and TIMP-2 are known to play key roles in tissue remodeling in late gestation in several species. IL-8 stimulated term canine trophoblast migration by 35% compared to the control (P<0.01), while TIMP-2 had no significant effect on migration (P=0.38). TIMP-2 decreased term canine trophoblast invasion by 57% (P<0.05). However, IL-8 had no significant effect on term canine trophoblast invasion (P=0.42) or proliferation (P=0.18). This was the first investigation of in vitro canine trophoblast physiology. This study revealed that both IL-8 and TIMP-2 play a role in regulating trophoblast function at the end of gestation in the dog.

Keywords: Chorioallantois Dog; Matrix-Metalloproteinases; Placenta; Wound Healing Assay
TIMP-2 expression during late gestation is a necessary step in the sequence of successful placental release in cattle [9]. Canine peripartum trophoblasts also express TIMP-2, however, its expression does not significantly decrease prior to successful placental release [8]. Therefore, the influence of TIMP-2 on canine trophoblasts warrants further investigation to determine its role in placental physiology at the end of gestation.

Interleukins are dependent on MMP activity [10]. Interleukin-8 (IL-8/CXCL8), an 8 kDa inflammatory chemokine, mediates its effect through the IL-8 receptors, CXCR1 and CXCR2 [11]. Human trophoblasts constitutively secrete IL-8 throughout the entire duration of pregnancy [12] and express CXCR1 [13,14]. Studies using first trimester human trophoblasts show that IL-8 stimulates in vitro proliferation, migration, and invasion through the up-regulation of MMP-2 [14]. The canine pre-implantation embryo also expresses IL-8 [7], but its role in late gestation has not been examined.

Factors

Recombinant human IL-8 was obtained from Peprotech (Rocky Hill, NJ, USA) and was used at a concentration of 10 ng/ml for each assay. Recombinant human TIMP-2 (Peprotech) was used at a concentration of 0.5 µg/ml for each assay. Concentrations selected for IL-8 and TIMP-2 were based on dosages in previous studies found to elicit maximum effects with human trophoblasts [14,15].

Tissue collection

Term canine placentas were collected following elective C-sections (n=5 dogs) at prepartum luteolysis, the period preceding the onset of the first stage of labor at which there is a marked decrease in progesterone below 2.5 ng/ml. Following tissue collection, the villous chorioallantois was dissected from placental tissues. Villous chorioallantois was minced and washed in a cell isolation medium as previously described [16] until further processed for primary trophoblast isolation.

Isolation of TCT and cell culture

Term canine trophoblasts (TCT) were isolated from the villous chorioallantois as previously described [16]. Briefly, following serial collagenase and trypsin tissue digestions, cells were separated by Percoll density gradient centrifugation. The trophoblast-containing gradient layer was washed twice and re-suspended in cell culture medium. The cells were identified using immunostaining cytokerin-7 to observe cellular morphology as previously described [16]. The cell culture conditions were the same for all assays at 37°C in 5% CO₂.

Migration assay

The migration (wound healing) assay was performed as previously described [17]. Briefly, primary TCT (1000X10³ cells/well) were suspended in 2 ml of a Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and cultured in 12-well tissue culture plates. Cells were grown to a confluent monolayer. A wound was added with a sterile 200µl pipette tip. The monolayer was rinsed with the same media to remove loosened cells. Media with no factor (control), with IL-8 or with TIMP-2 was then added. Photomicrographs of the wound were taken with phase-contrast microscopy (100X magnification) after 8 h in culture (Fig. 1). The area of the wound was measured using ImageJ v.1.34 software [18]. The experiment was performed in triplicate.

Invasion assay

An invasion (Matrigel) assay was performed as previously described [15] using Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA). Briefly, primary TCT (250X10³ cells/well) were suspended in 0.5 ml of a modified DMEM media containing 10% FBS with no factor (control), with IL-8 or with TIMP-2 and then incubated for 22 h. Non-invading cells were removed from the Matrigel-coated filter membrane and filters were fixed and stained with DiffQuik (Harleco, EMD Chemicals, Inc. Gibbstown, NJ, USA). Cells from ten randomly selected non-overlapping fields were counted under light microscopy (400X magnification). The experiment was performed in triplicate.

Proliferation assay

The proliferation (((3-)4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyl tetrazolium bromide) (MTT) assay (Cell growth determination kit, MTT based, Sigma), was performed as previously described [19]. Primary TCT (100X10⁴ cells/well) were suspended in 100 µl of a modified DMEM media with 10% FBS with no factor (control), with IL-8 or with TIMP-2 and cultured in 96-well tissue culture plates for 28 h.) MTT 50 µg was added then to each well for 4 h. MTT and media was replaced with 100 µL 1-propanol and the plates were vigorously shaken for 5 min. Using a microplate reader (SpectraMax 190, Molecular Devices, LLC, Sunnyvale, CA, USA), absorbance was measured at 570 nm and 690 nm. Measurements were analyzed with SoftMax Pro 5.2 program SoftMax® Pro Data Acquisition & Analysis Software (Molecular Devices, LLC, Sunnyvale, CA). The experiment was performed in quadruplicate.

Statistical analysis

The data were analyzed statistically by Repeated Measures or Two-Way ANOVA in PROC MIXED using SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) for all assays. For the migration assay, the mean of the control wound area at 0
h was set to 0% wound closure and the data were expressed as the percent closure of the control wound area for each dog. For the invasion assay and proliferation assay, the mean of the controls was set to 100% and the data were expressed as the percentage of the control for each dog. For the proliferation assay, measurements at 690 nm were subtracted from measurements at 570 nm to account for background. In all experiments, data were expressed as mean ± SEM and significance was defined as P<0.05.

Results

Results from the experiment are summarized in table 1. Representative wells for the migration assay and invasion assay are illustrated in Fig. 1 and 2, respectively. IL-8 increased term canine trophoblast (TCT) migration by 35% compared to the control (P=0.003). TIMP-2 had no significant effect on TCT migration (P=0.38). There was no significant effect of IL-8 on invasion compared to the control (P=0.42). However, TIMP-2 decreased TCT invasion by 57% compared to the control (P=0.03). For the proliferation assay, IL-8 at the concentration studied had no significant effect on TCT proliferation (P=0.18).

Table 1. Percent Migration, Invasion, and Proliferation of Control.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IL-8</th>
<th>TIMP-2</th>
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<tbody>
<tr>
<td>Migration</td>
<td>23.39 ± 3.1 %</td>
<td>31.96 ± 4.6 %*</td>
<td>28.66 ± 2.2 %</td>
</tr>
<tr>
<td>Invasion</td>
<td>100 ± 1.2 %</td>
<td>63 ± 1.1 %</td>
<td>139 ± 1.5 %*</td>
</tr>
<tr>
<td>Proliferation</td>
<td>0.96 ± 0.2 %</td>
<td>0.14 ± 0.09 %</td>
<td>^</td>
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^TIMP-2 was not used for proliferation assay because it is not involved in cell proliferation

* indicates statistical significance

Figure 1. Representative wells illustrating the wound at time 0 and 8 h for the control, IL-8-treated, and TIMP-2-treated term canine trophoblast cells in primary culture (100X).

Figure 2. Representative fields of cell invasion for control, IL-8-treated, and TIMP-2-treated cells (400X). Arrows indicate the trophoblast nuclei and arrowheads indicate the filter membrane pores.
Discussion

In vitro canine trophoblast physiology has not been previously described. Therefore, the objective of this study was to characterize normal in vitro term canine trophoblast (TCT) physiology in response to IL-8 and TIMP-2 using migration, invasion, and proliferation assays. Similar to observations in first trimester human trophoblasts (HTR-8/SVneo) where IL-8 increased migration [14], IL-8 increased TCT migration in the current study. Previous studies have also shown that IL-8 increases invasion and proliferation in first trimester human trophoblasts [13,14]. However in the current study, IL-8 did not increase invasion and proliferation in TCT primary cultures. It is possible that canine trophoblasts do not retain the IL-8 receptors (CXCR1 and CXCR2) at term. A decrease in expression of the IL-8 CXCR1 receptor has been established as a normal condition of human myometrial tissue during parturition [20]. It is not known if the IL-8 receptors are similarly decreased in expression in the dog at term, but this warrants further investigation.

Whereas the effects of IL-8 are dependent on MMP activity [14], MMP activity is regulated by TIMPs in human trophoblast invasion [15,21]. Down-regulation of TIMP-2 mRNA increases migration and invasion of first trimester human trophoblasts (HTR-8/SVneo) [21]. In the current study, TIMP-2 significantly decreases invasion, but not migration of normal TCT. This study has laid the groundwork for future investigations to examine responses to TIMP-2 in abnormal TCT conditions (e.g. subinvolution of placental sites, retained placenta). Studies in cattle have reported that a reduction of TIMP-2 at the end of gestation is essential for the separation of fetal membranes from the endometrium in normal parturition [9]. Also in cattle, the expression of MMP-2 is higher in those with retained placenta compared to those with a normal placenta, further supporting the regulatory role of TIMP-2 [22].

Conclusion

In conclusion, this is the first study to examine canine trophoblast physiology in vitro. The current study provides evidence that IL-8 promotes migration, whereas TIMP-2 decreases invasion in normal primary TCT. This information provides a basis for in vitro investigations to understand trophoblast pathologies that occur during the canine postpartum period.

Acknowledgements

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Footnotes

86.5% DMEM with 1% Hepes (Life Technologies), 1% Glutamine Plus, 1% Penicillin/Streptomycin, 0.5% Gentamycin, and 10% FBS.
86.5% DMEM with 1% Hepes, 1% Glutamine Plus, 1% Penicillin/Streptomycin, and 0.5% Gentamycin

References


18. Rasband WS, Image J. U. S. National Institutes of Health, Bethesda, Maryland, USA.


