Molecular mechanisms facilitating bovine conceptus attachment to the uterine epithelium : a reexamination with insights from lymphocyte homing

論 文 の 内 容 の 要 旨

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論文題目 Molecular mechanisms facilitating bovine conceptus attachment to the uterine epithelium: a reexamination with insights from lymphocyte homing
（ウシ胚の子宮内膜上皮細胞への接着の分子機構：リンパ球ホーミングからの解析）

In the bovine, a significant fraction of embryonic losses occurs during peri-implantation period, resulting from insufficient biochemical communication between the elongating conceptus and the uterus (Diskin & Morris 2008). The implantation process is generally considered as a highly coordinated process including hatching, apposition, attachment (pre-implantation period) and adhesion (implantation period), before placentation. During the peri-implantation period, trophoblasts become oval shape on day 13 (day 0 = day of estrus) and start to elongate on day 14-15. Conceptus attachment is initiated when the blastocyst elongation slows down on day 18-19 during which the maternal endometrium becomes “receptive” to conceptus attachment. It is therefore expected that uterine environments differ during this time period. Using two-dimensional gel electrophoresis, uterine fluids were investigated and found that uterine components differ between the stage of estrous cycle and/or pregnancy in the bovine species (MacLaughlin et al. 1986). The similar differences are demonstrated in human uterine fluids when the uterine fluids from mid proliferative phase are compared to those of the mid secretory phase (Chen et al. 2009).

All these results indicated that biochemical communication between the developing conceptus and uterus must be characterized if one can find a way to reduce embryonic losses before placental formation.

Data accumulated so far are not sufficient to reduce embryonic losses or to develop methods in reducing embryonic losses in cattle. For these reasons, the mechanisms of implantation processes in the bovine species need to be characterized from the factors not identified or over-looked. Instead of taking common approaches in studying the peri-implantation events, I decided to look at the phenomenon from a different angle, the way implantation processes has not been studied. In my dissertation, therefore, I examined the implantation processes through the events of lymphocyte homing; the phenomenon often seen in the blood stream when lymphocyte flowing slows down and eventually trapped by the endothelial cells of blood vessels. My dissertation consists of three parts: studies of L-selectin (SELL) in chapter 1, vascular cell adhesion molecule (VCAM-1) in chapter 2, and epithelium mesenchymal transition (EMT) in chapter 3 because SELL is required for slowing down of lymphocytes, VCAM-1 is required for lymphocyte adhesion to the endothelial cells and finally EMT, allowing epithelium and epithelium interactions.

Objectives of chapter 1 were to determine if selectins and their ligands were expressed in the bovine conceptus and/or uterus during the peri-attachment period, and to study whether selectins were associated with conceptus attachment to the uterine epithelium. Through the RNA-seq analysis of bovine conceptuses on days 17, 20 and 22 (day 0=day of estrus) only SELL ligand, podocalyxin (PODXL), was found in these conceptuses. Quantitative PCR analysis revealed that SELL mRNA detected in the uterine epithelium, but not in conceptuses, increased during the peri-attachment period. SELL protein was localized to the uterine epithelium of day 25 pregnant animals. In the co-culture system with EECs and bovine trophoblast CT-1 cells, SELL expression in EECs was effectively reduced by its siRNA, however, IFNT, a marker for CT-1 cell attachment to EECs, was not reduced. In the cultured endometrial epithelial cells (EECs), SELL transcript was up-regulated when uterine flushings from day 20 pregnant animals were placed onto these cells. SELL was also up-regulated when cultured EECs were treated with progesterone, EGF or bFGF, but not with estrogen or IFNT. The SELL protein was increased in EECs treated with EGF but not bFGF. These observations suggest that the conceptus could attach to the uterine epithelium through the interaction between endometrial SELL and conceptus PODXL, possibly initiating conceptus attachment process in the bovine species.

In chapter 2, to clarify the mechanism of conceptus attachment, we focused on VCAM-1

to determine if it was expressed in bovine conceptuses or endometrium during the peri-attachment period. Uterine *VCAM-1* expression was minimal in day 17 (day 0 = day of estrus) cyclic and pregnant animals, but increased between days 20 and 22 of pregnancy. In cultured endometrial epithelial cells (EECs), *VCAM-1* expression was up-regulated when treated with growth factor or uterine flushings, while its protein was increased in the cell surface of EECs that were cocultured with bovine trophoblast CT-1 cells. *VCAM-1* expression in CT-1 cells was also up-regulated with the use of uterine flushings, and further increased when these cells were cocultured with EECs. Expression of VCAM-1 receptor, integrin alpha 4 (*ITGA4)*, increased significantly in day 22 conceptuses. Immunohistochemical detection revealed that in day 22 pregnant uteri, VCAM-1 protein was found in both EECs and conceptuses, but ITGA4 was localized only in trophoblasts. These observations indicate that in the bovine species, cell-cell interactions between conceptuses and uterine epithelial cells are required for sufficient VCAM-1 and ITGA4 expressions, and suggest that uterine VCAM-1 and conceptus ITGA4 play a role in the establishment of conceptus adhesion to the uterine endometrium.

In chapter 3, during the course of experiments to identify and characterize factors that function in bovine conceptuses during peri-attachment periods, various transcripts related to the epithelial-mesenchymal transition (EMT) were found. In this study, RNA was extracted from different sets of days 17, 20 and 22 (day 0 = day of estrus) bovine conceptuses and subjected to real-time PCR analysis as well as western blotting, from which abundances of N-cadherin, vimentin, *MMP2* and *MMP9* mRNAs were determined on Day 22, concurrent with E-cadherin, mRNA and protein, down-regulation. Transcription factors in EMT processes were then analyzed and changes in *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* transcripts were found in day 22 conceptuses, while confirming SNAI2 expression by western blotting. Immunohistochemical analysis revealed that the day 22 trophectoderm expressed mesenchymal markers N-cadherin and vimentin as well as an epithelial marker cytokeratin. In attempts to identify molecular mechanisms by which the trophectoderm expressed EMT-related genes, growth factor receptors associated with EMT were analyzed. Up-regulation of growth factor receptor transcripts, *FGFR1*, *PDGFRA*, *PDGFRB* and *TGFBR2* mRNAs, was found on day 22. The analysis was extended to determine integrin (ITG) transcripts and found high levels of *ITGA4*, *ITGA8*, *ITGB3* and *ITGB5* mRNAs on day 22. These observations indicate that after the conceptus-endometrium attachment, EMT-related transcripts as well as an epithelial marker cytokeratin were present in the bovine trophectoderm, and suggest that the implantation process for non-invasive

trophoblasts requires not only extracellular matrix expression but also partial EMT.

Above all, molecules present in uterine flushings during peri-implantation period, such as EGF, induced SELL and VCAM in endometrium through the endometrial receptors. The up-regulated SELL together with the ligands expressed on conceptus may be involved in the initial step in conceptus attachment to the uterine endometrium. In addition to the cell-cell attachment stimulation between trophectoderm cells and uterine epithelial cells, efficiency of VCAM expression at the cell surface is significantly increased, which interacting with the receptor, ITGA4, is likely to be required for conceptus stable adhesion to the endometrial epithelium in the bovine species. Moreover, during the implantation period significantly up-regulated PODXL, VCAM, ITGA4, EGF, EGFR together with marker of EMT in trophectoderm cells were found, suggesting that in addition to ECM bindings, partial EMT is also required for proper adhesion of trophoblasts in non-invasive placentation.

The studies described in my dissertation here provide new insights for the elucidation of mechanisms associated with conceptus implantation to the maternal endometrium in the bovine species, and with the application of these factors, the chance of implantation could be promoted, resulting in improved pregnancy rate in cattle and beyond.