

Immunophenotypical and functional properties of equine uterine neutrophils of mares with or without degenerative endometrial changes

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Summary

A model system for equine endometritis was developed. The system is based on the controlled, stimulated immigration of polymorphonuclear neutrophil granulocytes (PMN) from peripheral blood into the uterine lumen. After intrauterine application of recombinant human interleukin-8 (rhIL-8) (0.5–1.25 ng in 50 ml phosphate buffered saline solution; PBS), the uteri were flushed with PBS and immigrated PMN were checked for absolute numbers, viability, morphology, phenotype, generation of reactive oxygen species (ROS) and phagocytic activity by means of quantitative and qualitative flow cytometric assays. Recombinant hIL-8 reliably attracted high numbers of viable PMN into the uterus of mares with or without degenerative endometrial changes. Compared with blood PMN, immigrated uterine neutrophils displayed significant phenotypical and functional differences: The expression of CD11 α /CD18 (LFA-1) was upregulated on uterine PMN whereas MHC class I and CD44 molecules were expressed at lower densities. The ability to phagocytose opsonised streptococci did not differ between uterine and blood PMN. However, uterine PMN displayed a higher capacity to generate ROS.

Recombinant hIL-8 was also able to attract PMN (in comparable numbers and viability) into the uteri of mares with histologically proven degenerative endometrial changes. Compared to autologous blood PMN, the uterine PMN showed on average similar phenotypical and functional characteristics as in mares with histologically healthy endometrium. However, immigrated PMN of the mares with degenerative endometrial changes displayed relatively high or exceptionally low activities to generate ROS.

Taken together, the described model system offers the possibility to characterize a multitude of functional and phenotypical characteristics of cells attracted into the uterus and, also, the action of modulating therapeutic agents. The system may be an useful instrument to answer specific questions regarding the pathogenesis of persisting endometritis in horses.

Keywords: mare, endometritis model, PMN, endometrial degeneration

Immunphänotypische und funktionelle Eigenschaften uteriner neutrophiler Granulozyten mit und ohne degenerativen Veränderungen des Endometriums

In der Arbeit wird ein neues Endometritismodell für die Stute vorgestellt. Das Modellsystem basiert auf der kontrollierten, stimulierten Immigration von polymorphkernigen neutrophilen Granulozyten (PMN) vom Blut in den Uterus. Nach intrauteriner Applikation von rekombinantem humanem Interleukin-8 (rhIL-8) (0.5–1.25 ng in 50 ml PBS) wurde der Uterus mit PBS gespült. Die immigrierten PMN wurden dann quantifiziert und anhand ihrer Vitalität, Morphologie, Immunphänotyp, Bildung reaktiver Sauerstoffspezies (ROS) und ihrer Kapazität zur Phagozytose durchflusszytometrisch charakterisiert. Rekombinantes hIL-8 lockte zuverlässig große Mengen vitaler PMN in den Uterus von Stuten mit oder ohne degenerativen Veränderungen des Endometriums. Verglichen mit Blut-PMN wiesen Uterus-PMN signifikante immunphänotypische und funktionelle Veränderungen auf. Die Expression von CD11 α /CD18 (LFA-1) war auf uterinen PMN erhöht, während MHC-Klasse I und CD44 mit geringerer Dichte exprimiert waren. Die Kapazität zur Phagozytose opsonierter Streptokokken unterschied sich bei PMN aus Blut und Uterus kaum. Dagegen zeigten uterine PMN eine erhöhte ROS-Bildung.

Rekombinantes hIL-8 induzierte auch bei Stuten mit histologisch nachgewiesenen degenerativen Veränderungen des Endometriums einen zahlenmäßig starken Influx neutrophiler Granulozyten mit hoher Vitalität. Gemessen an autologen Blut-PMN wiesen uterine PMN durchschnittlich ähnliche immunphänotypische und funktionelle Eigenschaften wie Stuten mit histologisch gesundem Endometrium auf. Die PMN der Stuten mit degenerativen Endometriumveränderungen zeigten jedoch teilweise relativ hohe oder aber außergewöhnlich niedrige Aktivitäten bei der ROS-Bildung.

Das Endometritismodellsystem bietet die Möglichkeit, eine Vielzahl immunphänotypischer und funktioneller Parameter in den Uterus gelockter Zellen sowie deren Modulation durch Medikamente zu charakterisieren. Es könnte ein nützliches Instrument bei der Beantwortung von Fragen zur Pathogenese der persistierenden Endometritis der Stute sein.

Schlüsselwörter: Stute, Endometritismodell, PMN, Endometriumsdegeneration

Introduction

Endometritis in mares results from complex interactions between opportunistic uterine bacterial pathogens and host factors. Bacterial infection during mating usually resolves within few hours or days (Evans *et al.*, 1986; Watson, 1988), and the uterus remains free of infection before the embryo enters the uterine

cavity. Some mares are susceptible to persistent endometritis with chronic inflammation, which can result in degenerative changes and reduced fertility (Hughes and Loy, 1969; Kenney, 1978). It has been suggested that inability to spontaneously eliminate bacterial infection from the uterus is associated with

an impaired local uterine immune response. The local uterine defence mechanisms that are responsible for the resolution of bacterial contamination include neutrophilic phagocytosis and antibody-mediated mechanisms (Watson, 1988). Neutrophilic phagocytic activity seems to be an important factor of uterine defence in the mare (Liu et al., 1986; Troedsson, 1999).

Several studies revealed that neutrophils obtained from the uterine lumen of mares susceptible to endometritis have an impaired functional capacity (especially phagocytic activity) compared to those obtained from blood (Cheung et al., 1985; Watson et al., 1987). In contrast, Asbury and Hansen (1987) found that the phagocytic capacity of uterine PMN collected 4 hours after infection was greater for susceptible than for resistant mares if given the right environment. In conclusion, insufficient complement in uterine secretions and impaired opsonisation were suggested to be responsible for a lower phagocytic activity of uterine PMN (Troedsson et al., 1993). However, the effect of neutrophil functional abilities on susceptibility to endometritis remains unclear.

In order to develop effective therapeutic approaches, it is necessary to understand more about the regulation of PMN migration and the modulation of their functional and phenotypic qualities during migration to the extravascular tissue. In the framework of investigations to pathogenic mechanisms of endometritis in mares, we were studying the immigration of polymorphonuclear neutrophil granulocytes (PMN) into the uterus. Using a new developed endometritis model we compared their properties with those of contemporarily harvested PMN from peripheral blood in mares with histologically proven normal endometrium or with degenerative endometrial changes.

Material and methods

Animals

Total of 15 clinically healthy mares were included into the investigations. No bacterial growth was obtained by examination of the uterine flushings before starting the experiments. Seven mares aged 3–12 years with grade I or IIa categorised endometrial biopsies represent group A. Eight mares aged 6–20 years with grade IIb or III categorised endometrial biopsies represent group B. Group B mares were barren between 1 and 3 years.

Collection and isolation of PMN

After a zero flush with 50 ml of sterile PBS, 50 ml of a solution of the chemokine recombinant human interleukin 8 (rhIL-8; 10 or 25 ng/ml PBS; Cellconcept) were introduced into the uterus using a Foley catheter. Migrated leukocytes were harvested 3, 6 and 24 h after rhIL-8 infusion by flushing the uterus with 5x50 ml PBS. Immediately after uterus flushing venous blood was taken and PMN were isolated by blood sedimentation and Percoll density gradient centrifugation. Blood and uterus cells were washed with PBS and resuspended in medium to a final concentration of 2×10^6 /ml.

Cell counts, differentiation, determination of cellular viability

Total blood and uterus leukocyte concentrations were determined in a cell counting chamber after staining with Turk's solution (Merck). Differential counts (granulocytes, lymphocytes and monocytes) were determined by flow cytometry (FACScan®, Becton Dickinson) after acquisition of at least 5,000 leukocytes. Cellular suspensions contained propidium iodide (2µg/ml, Calbiochem) for the assessment of cellular viability.

Immunophenotypical and functional characterisation of PMN

Immunophenotypical and functional characterisation of PMN were evaluated according to methods previously described for bovine PMN (Zerbe et al., 2000). The assessment of phenotypic analysis of PMN was done by flow cytometry upon indirect immunofluorescence using monoclonal antibodies: mAb CZ4 (anti MHC class I); mAb CZ11 (anti MHC class II); mAb CZ3.1 (EqCD11a/18; LFA1); mAb CZ5.15 (EqCD44); mAb 72.87 (BoCD11a; a-chain of LFA-1); mAb 25.32 (BoCD44). For the evaluation of the phagocytic capacity of PMN a nonviable streptococcus suspension (Omnisorbin®, Calbiochem) was labelled by FITC (Sigma). After opsonisation with equine serum, the bacteria were mixed with PMN suspension followed by incubation. PMN which ingested labelled bacteria displayed increased green fluorescence after flow cytometric analysis. Generation of reactive oxygen species (ROS) in Dihydro-rhodamin 123-labelled PMN (Molecular probes) was evaluated flow cytometrically after stimulation with phorbol myristate acetate (PMA; Sigma).

Results and discussion

Due to the suggestion that dysfunctional uterine PMN could be responsible for the inability to spontaneously eliminate of bacterial infections from the uterus, a new model system for the endometritis in mares was developed. In several studies, suspensions of *Streptococcus zooepidemicus* or oysterglycogen served as inducer of experimental inflammation (Liu et al., 1986; Troedsson et al., 1990). High amounts of uterine PMN could be isolated, but the effect of these substances on subsequent functional assays with uterine neutrophils remains unclear. In this work, the controlled selective immigration of PMN into the uterus was induced by the well defined chemokine rhIL-8 (10–25 ng/ml). Because of its chemoattractant properties, this physiological mediator plays an important role in neutrophil accumulation during inflammation. The amount of harvested uterine PMN (on average 6×10^7 PMN per flush in group A; PMN-purity >98%; viability >95%) and the kinetic of the neutrophilic influx with a peak at 6 hours after infusion of rhIL-8 are comparable with the results of other endometritis models in the mare (Liu et al., 1986; Williamson et al., 1987; Troedsson et al., 1990). Degenerative endometrial changes do not result in a decreased influx of neutrophils into the mares uterus. Recombinant hIL-8 was also able to attract PMN (in comparable numbers and viability) into the uteri of group B mares.

The described model system offers the possibility to characterize a multitude of functional and phenotypical characteristics of attracted cells into the uterus. When compared to autologous blood PMN, the uterine PMN showed on average comparable phenotypical and functional characteristics as in mares with or without degenerative endometrial changes. The expression of CD11a/CD18 (LFA-1) was upregulated on uterine PMN whereas MHC class I and CD44 molecules were expressed at lower densities (Tab. 1). These results conflict in part with our observations in a similar rHL-8 using endometritis model in the cow (*unpublished*). In fact bovine uterine PMN showed a reduced MHC class I expression also. On the other hand the neutrophilic expression of CD11a/CD18 was significantly downregulated in the cows uterus.

The ability to phagocytose opsonised streptococci did not differ between uterine and blood PMN (Tab. 1). This is according to previous reports, where an undisturbed phagocytosis in susceptible mares was found (Troedsson *et al.*, 1990). Uterine PMN displayed a higher capacity to generate ROS. These results agree with those obtained with our own endometritis model in the cow (*unpublished*).

Tab. 1: Immunophenotypical and functional characteristics of equine uterine PMN in comparison to PMN from blood

Immunphänotypische und funktionelle Eigenschaften equiner uteriner PMN im Vergleich zu Blut-PMN

PMN parameter	uterine PMN in comparison to blood PMN
CD11a/CD18 expression	↑
MHC class I expression	↓
CD44 expression	↓
phagocytic capacity	=
Killing capacity (ROS generation)	↑

However, in some mares with degenerative endometrial changes, immigrated PMN displayed an exceptionally low activity to generate ROS, whereas in other cases the ROS generation was relatively high. For further investigations it would be useful to compare the control animals (group A) with mares susceptible to persistent endometritis. This could show, whether these mares show an impaired killing of ingested material compared to resistant mares.

Taken together, the described model system offers the opportunity to investigate a number of functional and phenotypical characteristics of cells attracted into the uterine lumen and, also, the action of modulating therapeutic agents. The system may be an useful instrument to answer specific questions regarding the pathogenesis of persisting endometritis in horses.

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