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ORIGINAL PAPER

S100A8 and S100A9 are associated with endometrial shedding during menstruation

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Abstract

Matrix metalloproteinases (MMPs) and their major source, endometrial stromal cells (ESCs), play important roles in menstruation. However, other mechanisms in endometrial shedding may be unexplored. This study focused on four proteins: S100A8 and S100A9 (alarmins) are binding partners and induce MMPs, MMP-3 cycle-dependently plays a key role in the proteolytic cascade, and CD147, which has S100A9 as its ligand, induces MMPs. Immunostaining for these proteins was performed on 118 resected specimens. The percentage and location of each positive reaction in ESCs were measured and compared using Image J. The influence of leukocytes on S100A8 or S100A9 immunopositivity was also examined. From the premenstrual phase, S100A8 and MMP-3 began to have overlapping expressions in ESCs of the superficial layer, and ESC detachment was found within these sites. S100A9 was expressed from the late secretory phase and CD147 already from earlier. Later, the expression sites of S100A9 and CD147 included those of S100A8. Before menstruation, S100A8 or S100A9 expression was not affected by leukocytes. These results suggest that the local formation of S100A8/S100A9 complex, which occurs specifically in ESCs upon progesterone withdrawal, induces the local expression of MMP-3 and serves as a switch to the lysis phase.

Keywords S100A8 · S100A9 · Menstruation · CD147 · Metalloprotease-3 · Endometrium · Immunohistochemistry

Introduction

Menstruation is characterized by physiologic inflammation with self-programmed tissue destruction induced by progesterone withdrawal [1–5], which also has various anti-inflammatory effects [1, 2, 4], and in this respect, it may be a type of sterile inflammation [6, 7]. A critical event of endometrial shedding in menstruation is extracellular matrix degradation in the functional stromal layer [1–5], mainly in the superficial layer of the endometrium [1, 5], in which matrix metalloproteinases (MMPs) and endometrial stromal cells (ESCs, their primary source) play important roles [1–5]. However, the inflammatory process that begins during the secretory phase [1–5] remains unclear because of the complex interplay of the endocrine and immune systems [1–5]. Intermediate mechanisms in menstruation appear to be unexplored, such as unknown factors involved in the local regulation of specific MMP expression, triggering an irreversible progesterone-independent conversion to the lysis phase [1, 2]. De-coordination of various factors involved in menstruation leads to heavy menstrual bleeding and dysmenorrhea, which are not infrequent problems [1–3]. Thus, menstrual mechanisms must be further elucidated to treat menstruation-related diseases and maintain menstrual health [1, 2].

Expressions of MMPs are locally regulated by various paracrine or autocrine stimuli [8–10]. MMPs are secreted locally in an inactive form and activated by proteolysis in a chain reaction [10]. The production of MMP-3, one of the

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MMPs, is selectively and markedly increased in ESCs during the premenstrual and menstrual phases [8, 11]. MMP-3 is self-activating and plays a key role in the proteolytic cascade for MMP activation [10, 12]. In addition, MMP-3 has the ability to dissolve basement membrane matrices, such as laminin and heparin sulfate proteoglycan, which are known to increase in the stroma during the late secretory phase [13]. MMP-2 is also self-activating [14], but its increase after progesterone withdrawal is relatively small compared to that of MMP-3 [15], and whether it is cycle-dependent or not is controversial [10, 16]. MMP-9 is also self-activating [14], but its activation is rather highly dependent on MMP-3 [12]. MMP-1 is significantly increased during premenstrual and menstrual phases, but, like MMP-7, it is not capable of self-activation [10].

CD147 (an extracellular MMP inducer, EMMPRIN) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily [17]. Oligomerized CD147 can induce the production and secretion of MMPs, including MMP-3 [17, 18]. ESCs and endometrial glandular epithelium express CD147, which is involved in endometrial shedding during menstruation [18, 19]. However, after the late secretory phase, CD147 in ESCs extends to the deep layer of the endometrium [18, 19], which alone cannot explain the local regulation of MMP expression. Therefore, we focused on S100A9, one of the CD147 ligands and inflammatory mediators [20], and S100A8, a natural binding partner of S100A9 [20].

S100A8 and S100A9 belong to the superfamily of calcium-binding S100 proteins and are involved in various biological processes [21]. Both proteins are mainly expressed in neutrophils and activated macrophages [21–23]. During cellular stress, they are locally secreted extracellularly as alarmins [6, 7, 24] and contribute to inflammatory responses such as induction of cytokines and MMPs, including MMP-3, and leukocyte chemotaxis, in an autocrine or paracrine manner [21–25]. S100A8 and S100A9, which trigger inflammatory responses, have attracted attention in obstetrics for their involvement in pregnancy-related diseases such as preeclampsia and miscarriage [26]. However, to our knowledge, the involvement of S100A8 and S100A9 in endometrial shedding during the menstrual cycle has not been investigated. In this study, we examined their involvement with the aim of finding novel therapeutic targets for excessive endometrial shedding. Subsequently, during menstruation, S100A8 and S100A9 were suggested as a switch to an irreversible lytic phase.

Materials and methods

Study design

Immunostaining for S100A8, S100A9, MMP-3, and CD147 in the endometrium from the secretory phase to the menstrual phase was performed on resected uterine specimens. The percentage and location of respective positive reactions in ESCs were measured, and the expression kinetics of each protein during the menstrual cycle was compared. To determine whether the positive S100A8 or S100A9 reaction in ESCs is influenced by secretion from infiltrating leukocytes [21–25], the density of infiltrating leukocytes expressing the respective proteins in each positive and negative part for S100A8 or S100A9 was compared.

All study participants provided informed consent, and the study design was approved by the Ethics Committee of Shizuoka General Hospital (Approval no. SGHIRB#2018091/3).

Samples

Human endometrial tissues during the secretory or menstrual phase were obtained from hysterectomy specimens of 118 premenopausal patients (mean age, 44 years; range, 33–47 years). All patients had regular menstrual cycles (25–35 days) and had not received hormonal treatment in the previous 3 months. Hysterectomies were performed to treat non-endometrial diseases, i.e., uterine leiomyoma and cervical intraepithelial neoplasm, at Shizuoka General Hospital between January 2011 and December 2018. All of those resected samples were fixed in 10% neutral buffered formalin, and paraffin-embedded tissue sections were routinely stained with hematoxylin and eosin. Only histologically normal endometrial tissues were included in the study. Menstrual cycle staging, using an idealized 28-day cycle, was determined according to previously published criteria [27]: early (days 15–20, n = 11), mid- (days 21–23, n = 22), and late (days 24–26, n = 36) secretory phase, premenstrual phase (days 27–28, n = 31), and menstrual phase (day 1 of menstruation) (n = 18). For the menstrual phase, only the endometrium equivalent to day 1 of menstruation was used as a sample because various measurements become difficult when endometrial shedding progresses.

Immunohistochemistry

All antibodies used are listed in Table 1. Monoclonal antibodies against S100A8 and S100A9 showed no cross-reaction with other members of the S100 protein family [28, 29]. Immunohistochemistry was performed with serial sections from one representative tissue block from each case using Leica Bond-Max (Leica Biosystems, Melbourne, Victoria, Australia).

Evaluation of immunostaining

In the representative sections from each case, the endometrium was considered for study within a horizontal length

Table 1 Antigens used in this study

Antigen	Clone	Source	Antigen retrieval	Dilution rate
S100A8	#83	Original [Ref. 28]	ER2	1/100
S100A9	60B8	Original [Ref. 28, 29]	ER2	1/750
MMP-3	55-2A4	Kyowa Pharma Chemical	PK	1/100
CD147	MEM-M6/2a	MyBioSource.com	ER1	1/7500

ER1, pH 6.0 (Leica); ER2, pH 9.0 (Leica); *MMP* metalloproteinase; *PK* 0.2 mg/mL proteinase K (DAKO/Agilent, Carpinteria, CA, USA); *Ref* reference

of 10 mm. The expression of each protein was examined, focusing on ESCs. In the endometrium of the examined part, positive parts for each antibody in ESCs, as well as the area and thickness of the endometrium, were analyzed using Image J (version 1.52) (https://imagej.nih.gov/ij/). Cytoplasmic positivity for S100A8 and S100A9 was considered important [28, 29]. For the percentage of the positive parts, the area of the positive parts (total of the scattered positive parts) was measured and divided by the area of the endometrium. Minute positive parts with a total area of <0.3 mm^2 were considered negative. The percentage trend of positive parts for each protein during the menstrual cycle was examined. Furthermore, the percentage of positive parts during each menstrual cycle was compared between S100A8 and S100A9. For the location of the positive parts, we used the depth of their midpoint from the endometrial surface as the index. Specifically, the upper and lower depth limits for all positive parts in each case were individually measured from the endometrial surface, and the formula ([average of lower depth limits–average of upper depth limits/2] + average of upper depth limits = midpoint of positive parts) was used. If the shapes of the positive parts were irregular, the upper and lower depth limits were measured at 3–6 locations for such positive parts, depending on their shape and size, and the average of these measurements was used. The location of the midpoint of the positive parts in each case was compared based on the depth of 1/4 or 1/2 of the endometrium from the surface. In each case, the endometrial thickness was measured at four different places, and the thickness was averaged.

Density of S100A8- or S100A9-expressing infiltrating leukocytes

The density of S100A8- or S100A9-expressing infiltrating leukocytes was calculated by visually counting the total number of positive extravascular leukocytes in all positive areas in the examined endometrium and dividing by the total area of the positive parts. The density of S100A8- or S100A9-expressing infiltrating leukocytes in the negative parts was calculated by visually counting the number of such leukocytes in five random locations at × 40 objective lens with a field area of 0.307 mm^2, and the average value was taken.

Statistical analysis

For both trends in the percentage and location of each positive part in ESCs, Student's t test was used for between-group comparisons, and one-way analysis of variance (ANOVA) was used for comparisons among three or more groups. The paired t test was used to compare the percentage of each positive part for S100A8 and S100A9 during each menstrual cycle. The two-way ANOVA was used to compare the density of S100A8- or S100A9-expressing infiltrating leukocytes. The p values for multiple comparisons in the ANOVA were adjusted for Bonferroni correction. Data analysis was performed using jamovi (version 2.3.18) (https://www.jamovi.org/). A p value <0.05 was considered significant.

Results

Percentage of each protein expression in ESCs from the secretory phase to day 1 of menstruation

For all of four proteins, the percentage of their immunopositivity in ESCs significantly increased until menstruation.

S100A8

Immunopositivity was not seen during the early to late secretory phases but was found in all cases after the premenstrual phase (Table 2). The percentage of the positive parts on day 1 of menstruation was significantly greater than that during the premenstrual phase ($p<0.001$) (Fig. 1a).

S100A9

Immunopositivity was already present in some cases during the late secretory phase (16 of 36 cases, 44.44%) (Table 2). The percentage of the positive parts increased significantly until day 1 of menstruation ($p<0.001$) (Fig. 1b) and was significantly greater than that of S100A8 both during the premenstrual phase and on day 1 of menstruation (means ± standard deviations for the positive percentage of S100A9 vs. S100A8: premenstrual phase, 19.1 ± 4.62 vs. 11.2 ± 4.16, $p<0.001$; day 1 of menstruation, 51.8 ± 9.45 vs. 45.3 ± 9.59, $p=0.003$).

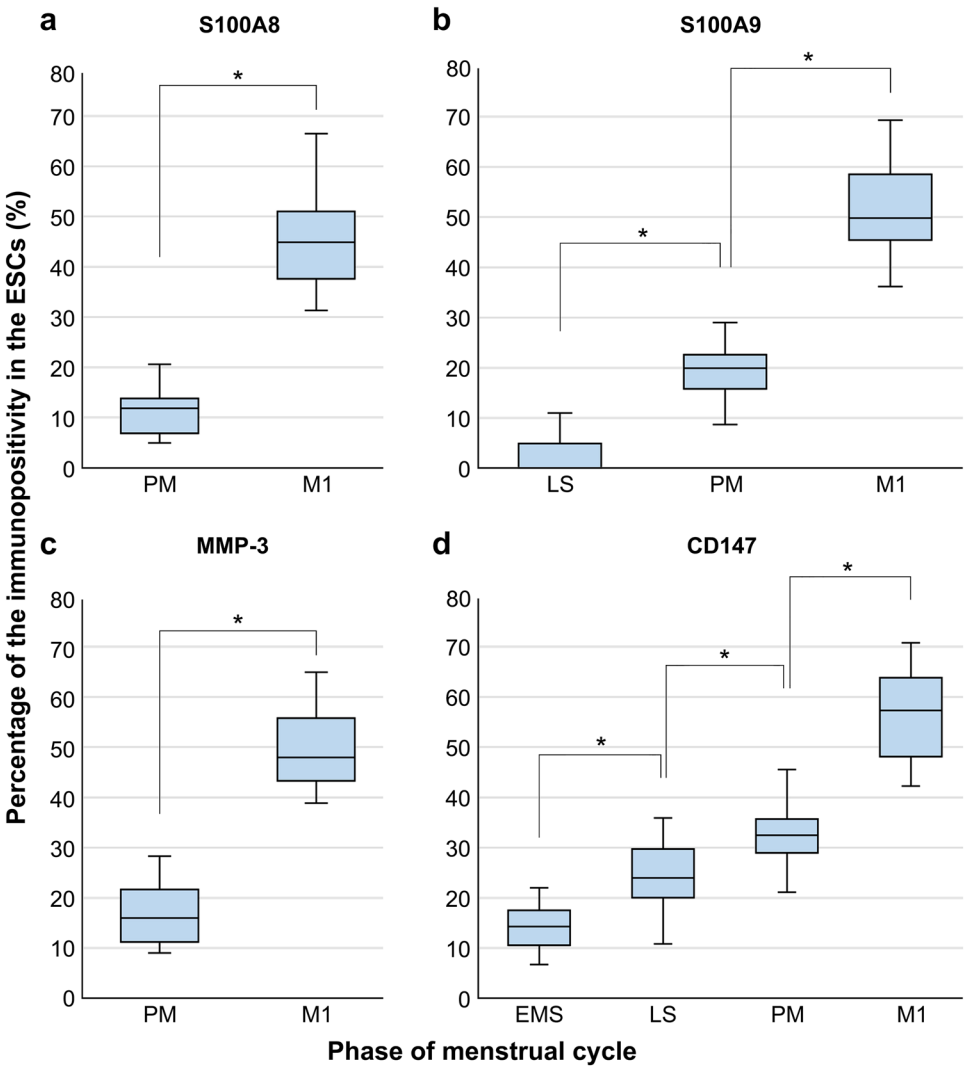

Fig. 1 Changes in the percentage of the immunopositivity of S100A8, S100A9, matrix metalloproteinase-3 (MMP-3), and CD147 in endometrial stromal cells (ESCs). For S100A8 (**a**), S100A9 (**b**), MMP-3 (**c**), and CD147 (**d**), the percentage of their immunopositivity in ESCs significantly increases until menstruation. The immunopositivity of S100A8 and MMP-3 is found after the premenstrual phase and that of S100A9 after the late secretory phase. The box-and-whisker plots indicate the median, interquartile range, minimum, and maximum values. Asterisks indicate $p < 0.001$. *EMS* early to mid-secretory phases; *ESCs* endometrial stromal cells; *LS* late secretory phase; *M1* menstruation day 1; *MMP-3* matrix metalloproteinase-3; *PM* premenstrual phase

Table 2 Expressions of S100A8, S100A9, and MMP-3 during the secretory phase to day 1 of menstruation

Menstrual cycle	S100A8	S100A9	MMP-3
ES ($n=11$)	–	–	–
MS ($n=22$)	–	–	–
LS ($n=36$)	–	16 (44.44)	–
PM ($n=31$)	31 (100)	31 (100)	31 (100)
M1 ($n=18$)	18 (100)	18 (100)	18 (100)

The number in parentheses represent the percentage of positive cases

ES early secretory phase; *LS* late secretory phase; *M1* menstruation day 1; *MS* mid-secretory phase; *PM* premenstrual phase

MMP-3

Immunopositivity was absent during the early to late secretory phases but was present in all cases after the premenstrual phase (Table 2). The percentage of the positive parts on day 1 of menstruation was significantly greater than that during the premenstrual phase ($p < 0.001$) (Fig. 1c).

CD147

Immunopositivity was already observed from the early secretory phase. The percentage of the positive parts increased significantly until day 1 of menstruation ($p < 0.001$) (Fig. 1d).

Histological findings of each immunoreactivity

In ESCs, the immunopositivity for each of the four proteins was found in the functional layer, but not in the basal layer.

S100A8

During the premenstrual phase, clusters of positively stained ESCs were sporadically distributed around the glands and

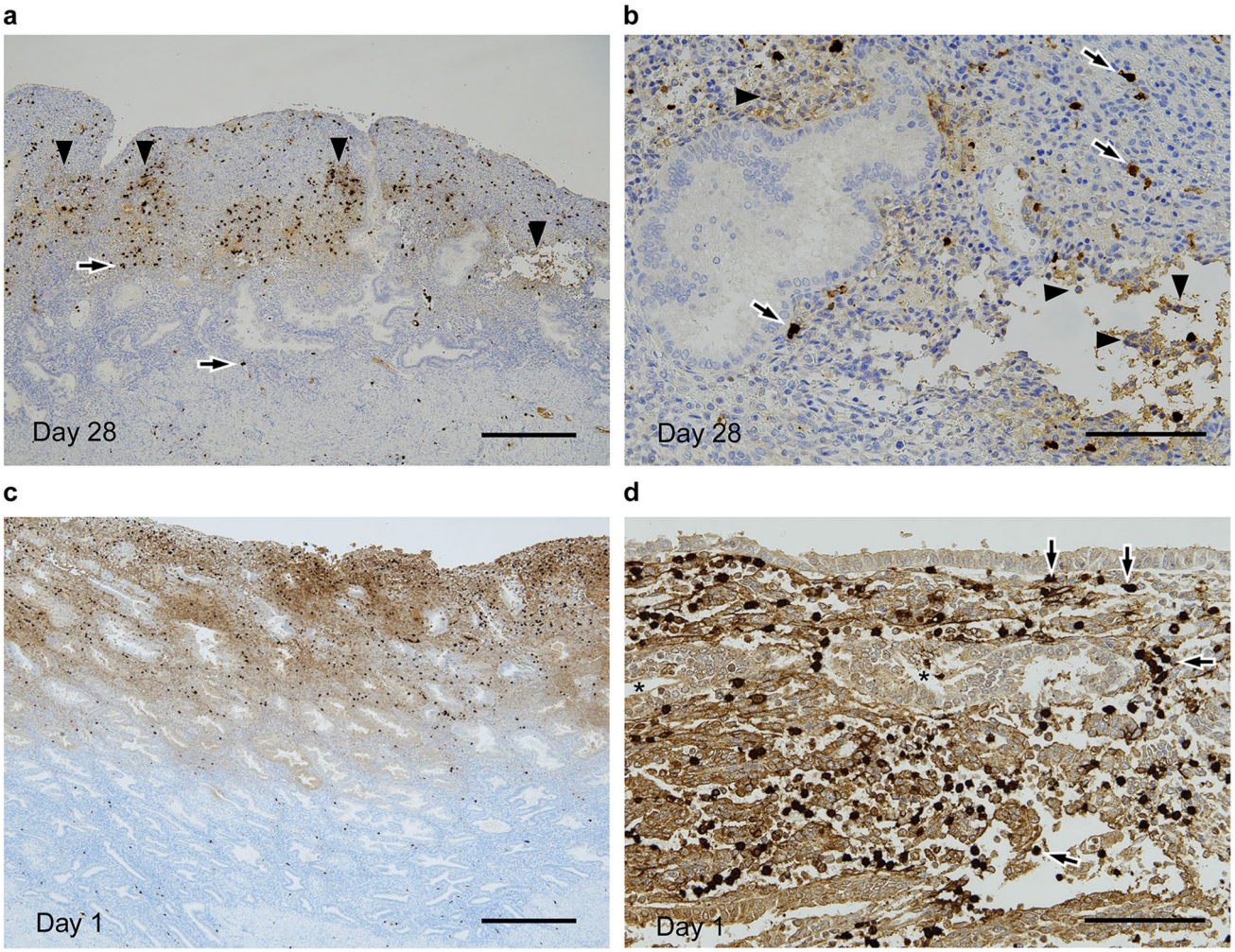

Fig. 2 Immunohistochemistry for S100A8 in the endometrium. **a** Premenstrual phase (day 28 of the menstrual cycle). Positive reactions in the stroma are scattered throughout the superficial and middle layers (arrowheads), although many of them have weak staining intensity, and some overlap with dissociation of the stroma. Scale bar: 500 µm. **b** Magnified image of **a**. Clusters of positively stained endometrial stromal cells (ESCs) are seen around a gland or blood vessel, and some of them are detached (arrowheads). Scale bar: 100 µm. **c** Day 1 of menstruation. Positive reactions in the stroma are zonally seen throughout the superficial and middle layers. Scale bar: 500 µm. **d** Day 1 of menstruation. The ESCs under the surface epithelium are diffusely detached, and almost all of them show a positive reaction. Positive glands are also seen. Asterisks indicate the lumina of the glands. Scale bar: 100 µm. In all images **a**–**d**, the positive intensity of ESCs is weaker than that of infiltrating leukocytes (arrows)

blood vessels throughout the superficial and middle layers, although many of them were weak in the staining intensity (Fig. 2a). Small detachment foci of positively stained ESCs were also seen (Fig. 2a and b). On day 1 of menstruation, positively stained ESCs were zonally distributed throughout the superficial and middle layers (Fig. 2c), where they coincided with the detachment foci of ESCs (Fig. 2d). The positive intensity was weaker than that of infiltrating leukocytes regardless of the menstrual phase (Fig. 2a–d).

S100A8 immunopositivity in the epithelium was not observed from the early secretory to the premenstrual phases. However, on day 1 of menstruation, immunopositivity was observed in some of the glandular epithelia within or near the ESC detachment foci (Fig. 2c and d).

S100A9

Clusters of positively stained ESCs were initially scattered primarily around superficial glands and vessels (Fig. 3a). After the premenstrual phase, positive parts became fused or banded, containing or overlapping S100A8-positive parts (Fig. 3b–d). As with S100A8, the positive intensity was weaker than that of infiltrating leukocytes (Fig. 3a–d). For the epithelium, on day 1 of menstruation, immunopositivity

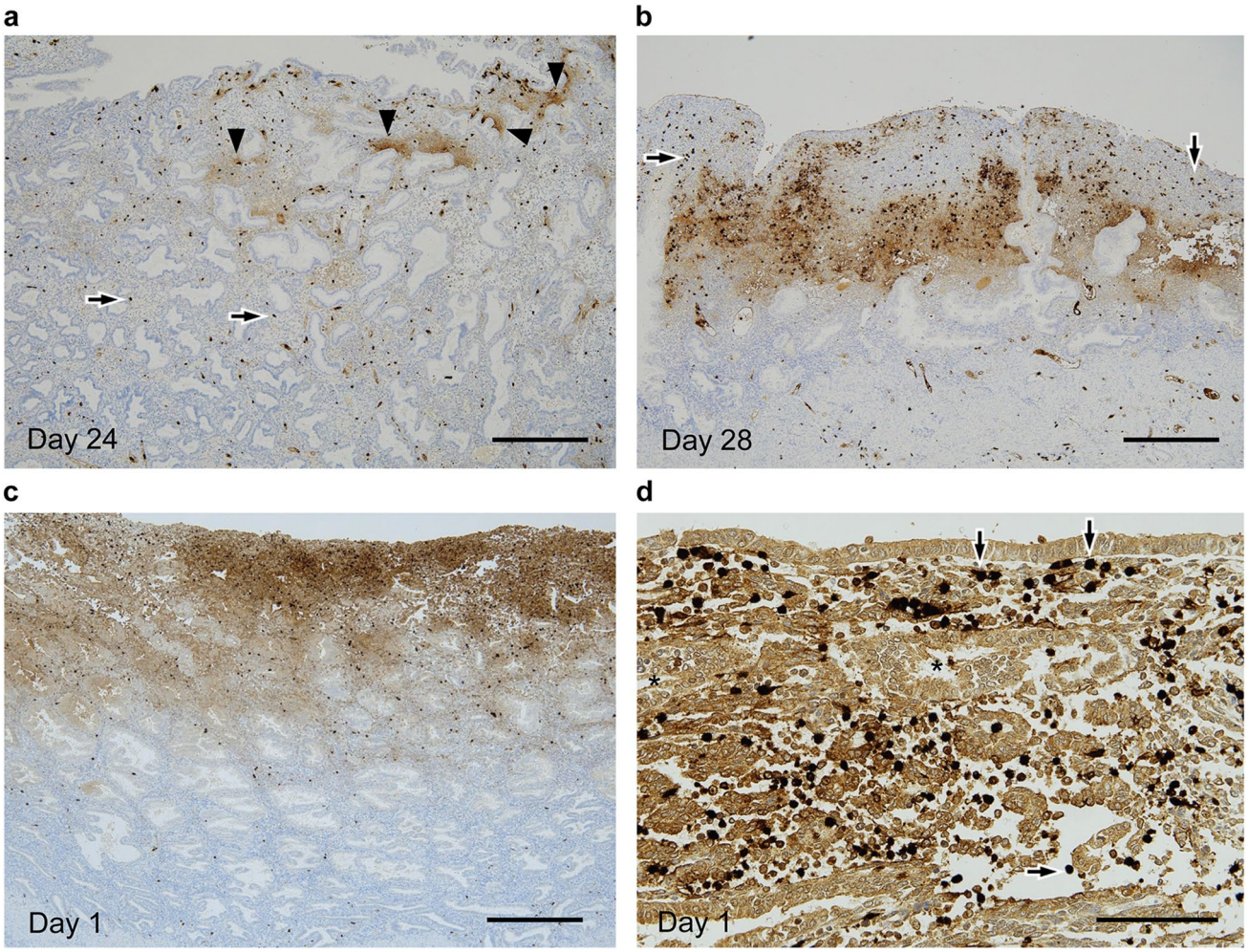

Fig. 3 Immunohistochemistry for S100A9 in the endometrium. **a** Late secretory phase (day 24 of the menstrual cycle). Positive reactions in the stroma are mainly scattered around the superficial glands or vessels (arrowheads). Scale bar: 500 µm. **b** Premenstrual phase (day 28 of the menstrual cycle). The image corresponds to Fig. 2a). Positive reactions in the stroma contain S100A8-positive parts. Scale bar: 500 µm. **c** Day 1 of menstruation. The image corresponds to Fig. 2c). Like S100A8, positive reactions in the stroma are zonally seen throughout the superficial and middle layers. Scale bar: 500 µm. **d** Day 1 of menstruation. Image corresponds to Fig. 2d. Endometrial stromal cells show a diffuse positive reaction, overlappingly with that for S100A8, and positive glands are also seen. Asterisks indicate the lumina of the glands. Scale bar: 100 µm. In all images **a**–**d**, like S100A8, the positive intensity of ESCs is weaker than that of infiltrating leukocytes (arrows)

was observed in the surface and glandular epithelia within or near the ESC detachment foci (Figs. 3c and d).

MMP-3

During the premenstrual phase, clusters of ESCs with positively stained cytoplasms were sporadically distributed around the glands and blood vessels throughout the superficial and middle layers, although many of them had weak staining intensity (Fig. 4a and b). They also overlapped with S100A8- and S100A9-positive parts (Fig. 4a), and small detachment foci were also seen (Fig. 4a and b). On day 1 of menstruation, the positive parts showed a zonal distribution and overlapped with the S100A8- and S100A9-positive parts (Fig. 4c and d). No immunopositivity for MMP-3 was seen in the epithelium.

CD147

Already during the late secretory phase, the positivity of ESCs expanded toward the deeper layer, whereas the glandular epithelium had a diffuse positivity regardless of the menstrual phase (Fig. 4e). The positivity was membranous with or without cytoplasmic.

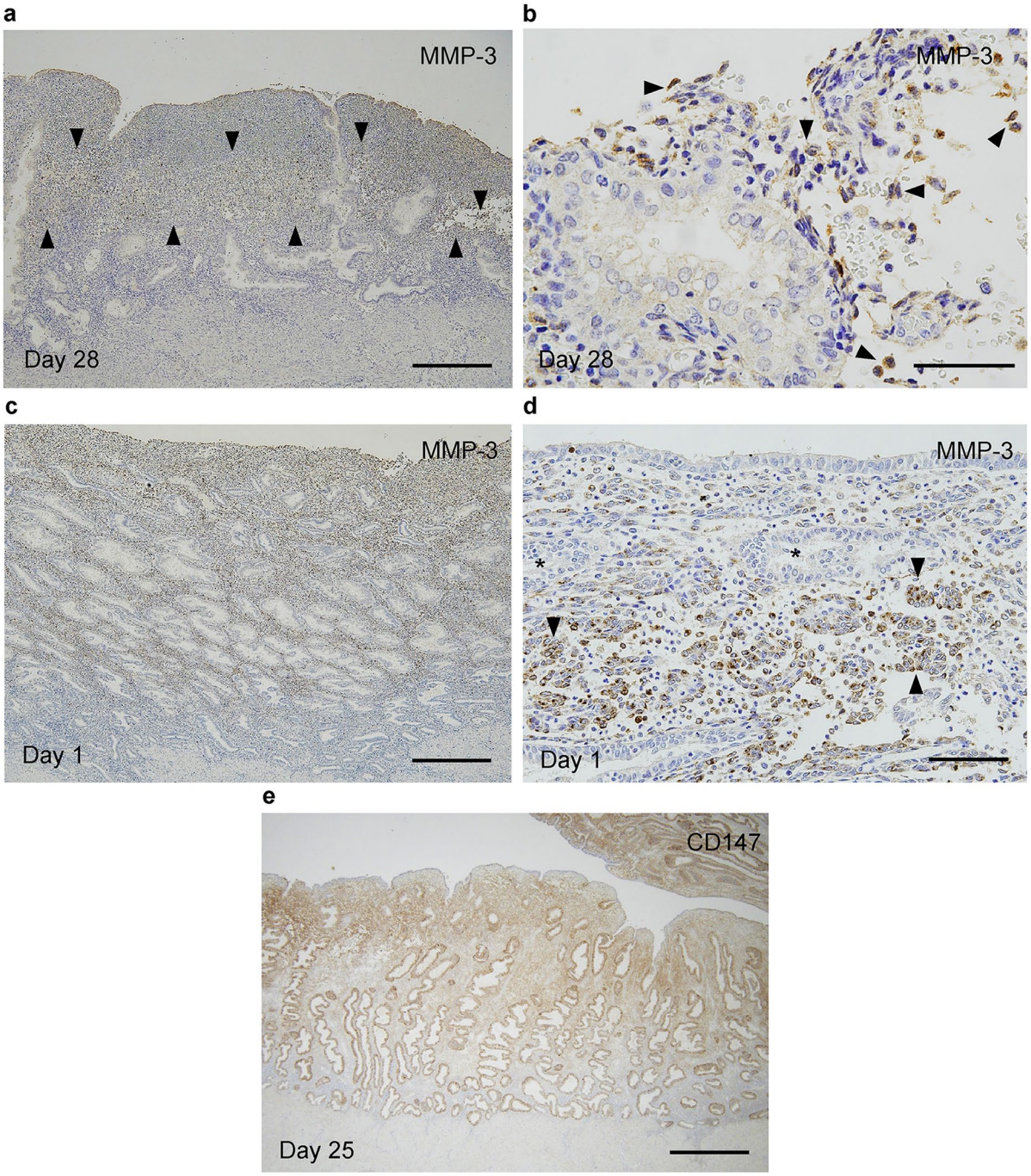

Fig. 4 Immunohistochemistry for matrix metalloproteinase-3 (MMP-3) and CD147 in the endometrium. **a** Premenstrual phase (day 28 of the menstrual cycle). The image corresponds to Figs. 2a and 3b. Positive reactions in the stroma (surrounded by arrowheads) overlapped with the S100A8- and S100A9-positive parts, including detachment foci, although many of them have weak staining intensity. MMP-3 immunostain. Scale bar: 500 µm. **b** Magnified image of **a**. Clusters of positively stained endometrial stromal cells (ESCs) are seen around a gland or vessels, and some of them are detached (arrowheads). Scale bar: 50 µm. **c** Day 1 of menstruation. The image corresponds to Fig. 2c) and 3c. Positive reactions in the stroma are zonally seen from the superficial to deep layers. MMP-3 immunostaining. Scale bar: 500 µm. **d** Day 1 of menstruation. The image corresponds to Fig. 2d and 3d. Most of the ESCs show a positive reaction, overlappingly with that for S100A8 or S100A9. The asterisks indicate the lumina of the glands. MMP-3 immunostaining. Scale bar: 100 µm. **e** Late secretory phase (day 25 of the menstrual cycle). Positive reactions in the stroma extend to the deep layer, and the glands also show a positivity. CD147 immunostaining. Scale bar: 1000 µm

Location of the midpoint of the positive parts for each protein from the secretory phase to day 1 of menstruation

S100A8

From the premenstrual phase to day 1 of menstruation, the midpoint of the positive parts significantly shifted from a depth within 1/4 from the endometrial surface to a depth between 1/4 and 1/2 ($p < 0.001$) (Fig. 5a).

S100A9

From the late secretory phase to day 1 of menstruation, the midpoint of the positive parts significantly shifted from a depth within 1/4 from the endometrial surface to a depth between 1/4 and 1/2 (late secretory phase vs. premenstrual phase, $p = 0.002$; premenstrual phase vs. day 1 of menstruation, $p < 0.001$) (Fig. 5b).

MMP-3

From the premenstrual phase to day 1 of menstruation, the midpoint of the positive parts significantly shifted from a depth within 1/4 from the endometrial surface to a depth between 1/4 and 1/2 ($p = 0.008$) (Fig. 5c).

CD147

During early to mid-secretory phases, the midpoint of the positive parts was within 1/4 depth from the endometrial surface; however, thereafter, the midpoint of the positive parts shifted significantly (early to mid-secretory phases vs. late secretory phase, $p < 0.001$; late secretory phase vs. day 1 of menstruation, $p = 0.002$) (Fig. 5d). After the premenstrual phase, the midpoint of the positive parts was between 1/4 and 1/2 depth from the endometrial surface (Fig. 5d).

Density of S100A8- or S100A9-expressing infiltrating leukocytes

The density of S100A8-expressing infiltrating leukocytes between the positive and negative parts of S100A8 was not significantly different during the premenstrual phase ($p = 0.931$) (Fig. 6a). However, the density was significantly higher in the positive parts on day 1 of menstruation ($p = 0.025$) (Fig. 6a). Similarly, the density of S100A9-expressing infiltrating leukocytes was significantly higher in the positive parts only on day 1 of menstruation ($p = 0.009$), but not significantly different during the late secretory and premenstrual phases ($p = 0.744$, $p = 0.202$, respectively) (Fig. 6b).

Discussion

In this study, S100A8 and MMP-3 were expressed synchronously in ESCs in the functional layer, with expression sites overlapping each other and ESC detachment within them. The expression of S100A8 in ESCs was slower than that of S100A9, with a time lag of 1–3 days. MMP-3 production by ESCs also involves the glandular epithelium [5, 10, 18], and CD147 was widely expressed in functional layer ESCs [13, 14]. These suggest that S100A9 alone is insufficient for CD147 to induce MMP-3 expression and that S100A8 expression is also required [5, 8–10, 18–20].

As previously reported [28, 30], S100A8 expression sites overlapped with those of S100A9 and are contained by them. S100A8 and S100A9 form a complex (S100A8/S100A9) for their stability because they are readily degraded by proteinases [31]. The overlap suggests the formation of S100A8/S100A9 [28, 30, 31]. S100A8/S100A9 is known to have unique abilities that differ from that of S100A8 or S100A9 alone [21–23]. The S100A9 subunit of S100A8/S100A9 is suggested to be involved in MMP-3 induction through CD147 [5, 8–10, 18–21, 25]. Furthermore, the local formation of S100A8/S100A9 in functional layer ESCs induce local MMP-3 expression and serve as a switch to the irreversible lytic phase [1–3, 5, 8–10, 18–21, 24, 25]. The expression sites of MMP-3 did not completely overlap with those of S100A8. This may be partly due to the paracrine effect of the secreted S100A8/S100A9 [9, 21, 22].

ESCs of the functional layer still retain progesterone receptors even during the late secretory phase [1–3, 5]. For such ESCs, progesterone withdrawal is a cellular stress [1–5] and thereby induces various proinflammatory substances, such as reactive oxygen species (ROS), interleukin (IL)-1, and tumor necrosis factor (TNF)-α [1–5, 8–10]. Some of them have also been reported to stimulate MMP-3 expression [1–5, 8, 10] and have a reciprocal production-inducing relationship with S100A8 and S100A9 [21, 23, 25, 32]. To the best of our knowledge, no study has reported that S100A9 directly induces S100A8 expression. The time lag in the expressions of both proteins was assumed to be caused by the degree or duration of cellular stress or amount of proinflammatory substances [7, 21, 23, 25, 33, 34]. For endometrial shedding in menstruation, S100A8 expression may require more intense cellular stress or proinflammatory stimuli [34].

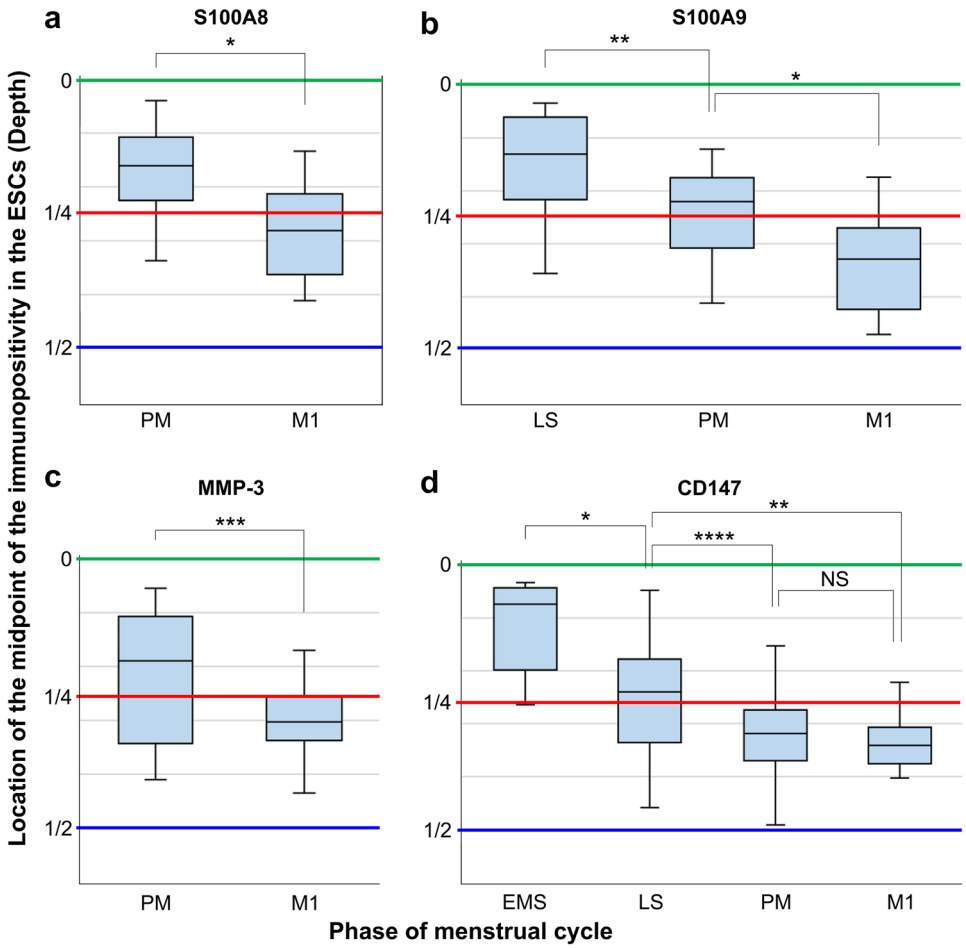

Fig. 5 Changes in the midpoint of the immunopositivity of S100A8, S100A9, matrix metalloproteinase-3 (MMP-3), and CD147 in endometrial stromal cells (ESCs). In S100A8 (**a**), S100A9 (**b**), and MMP-3 (**c**), their midpoints of immunopositivity in ESCs significantly shift to a depth between 1/4 and 1/2 from the endometrial surface until menstruation. In CD147 (**d**), the midpoint of immunopositivity in ESCs has already significantly shifted to a depth of between 1/4 and 1/2 from the endometrial surface during the premenstrual phase. The bold green line, bold red line, and bold blue line indicate the endometrial surface, 1/4 depth from the surface, and 1/2 depth from the surface, respectively. The box-and-whisker plots indicate the median, interquartile range, minimum, and maximum values. One asterisk (*), two asterisks (**), three asterisks (***), and four asterisks (****) indicate $p<0.001$, $p=0.002$, $p=0.008$, and $p=0.027$, respectively. *EMS* early to mid-secretory phases; *ESCs* endometrial stromal cells; *LS* late secretory phase; *M1* menstruation day 1; *MMP-3* matrix metalloproteinase-3; *PM* premenstrual phase

The area within 1/4 depth from the endometrial surface, where S100A8 and S100A9 began to be expressed, corresponds nearly to the superficial layer of the functional layer [1]. As previously reported [1, 5, 9, 19], this area is also the site where endometrial detachment begins, further suggesting the involvement of both proteins in endometrial detachment. Furthermore, initial S100A8 and S100A9 expressions are considered the intrinsic property of ESCs [35] in response to progesterone withdrawal and to be not affected by infiltrating leukocytes. By contrast, the expressions of S100A8 and S100A9 on day 1 of menstruation are considered influenced by a rapid increase in activated macrophage infiltrates and an initiated neutrophilic influx [1–4, 27]. The expressions of S100A8 and S100A9 in the glandular epithelium within or near ESC detachment sites are presumably caused by more intense proinflammatory stimuli [36].

Alarmins localize inflammation [6, 24, 37], which is associated with their anti-inflammatory properties [6, 37, 38]. Individual cells and proteins, involved in inflammation, have dual properties, i.e., pro- and anti-inflammatory, depending on the circumstances [6, 7, 21–23, 33, 37–41]. For the endometrium, this duality is necessary in the protection of the basal layer, termination of shedding, and subsequent scar-free repair [1–5, 38]. Recently, a study reported that menstrual fluid factors mediate endometrial repair and regeneration during and after menstruation; furthermore, S100A8

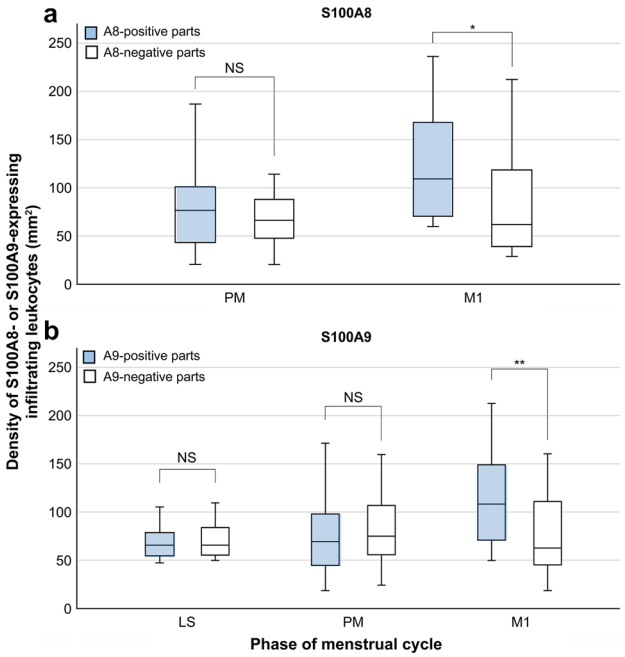

Fig. 6 Comparison of the density of S100A8- or S100A9-expressing infiltrating leukocytes in the positive and negative parts for the respective proteins. For both S100A8 (**a**) and S100A9 (**b**), no significant difference was found in the density of infiltrating leukocytes expressing the respective proteins between the positive and negative parts until the premenstrual phase. By contrast, at menstruation, the density in the positive parts is significantly higher for both proteins. The box-and-whisker plots indicate the median, interquartile range, minimum, and maximum values. One asterisk (*) and two asterisks (**) indicate $p=0.025$ and $p=0.009$, respectively. *EMS* early to mid-secretory phases; *LS* late secretory phase; *M1* menstruation day 1; *PM* premenstrual phase

and S100A9 are indicated as proteins elevated in the fluid [42]. In S100A8 and S100A9, protein modifications such as oxidation and phosphorylation, concentration, and complex types are directly related to their dual nature [37, 38, 43].

An imbalance of various menstruation-related factors, including Ca^{2+} and Zn^{2+}, could upset the balance of S100A8 and S100A9 respective dualities [2–5, 21–23, 36–38, 43]. Even during gestation, increased extracellular S100A8 and S100A9 cause certain pregnancy-related diseases [26]. The suppression of the predominant proinflammatory stimuli, including persistent neutrophil influx, may be an effective treatment for excessive endometrial shedding and certain pregnancy-related diseases [1–5, 8, 10, 26]. We believe that S100A8 and S100A9 are worthy of further investigation as targets for such treatment [6, 21, 23, 25, 26, 44–46].

This study has several limitations. First, although S100A8 and S100A9 expressions in ESCs have been genetically confirmed in cultured bovine cells [35], we have only examined them immunohistochemically. Future validation using cultured ESCs, such as the progesterone withdrawal model [47], is highly anticipated. Second, immunohistochemistry for other menstruation-related MMPs has not been performed. In this study, we targeted MMP-3, which is assumed to be the most important MMP contributing to the initial step of the cycle-dependent proteolytic cascade [10–12]. Once the MMP cascade is activated in vivo, immunohistochemistry alone may make it difficult to examine the direct relationship between S100A8 or S100A9, and the MMPs affected by MMP-3. Third, the immunohistochemical overlap between S100A8 and S100A9 has not been identified as to whether it is dimeric or tetrameric, to what extent components other than heterogeneous complexes are mixed, or whether respective proteins are phosphorylated or oxidized. Fourth, the relationship with proinflammatory substances such as ROS, IL-1 and TNF-α has not been examined. Fourth, although S100A8/S100A9 is known to have apoptotic effects [16, 18], the relationship with apoptosis, which is also considered one of the triggers in endometrial shedding [1], has not been investigated.

Conclusion

The expression of alarmins, S100A8 and S100A9, in the superficial layer ESCs is triggered by progesterone withdrawal. However, S100A9 alone was insufficient for CD147 to induce expression of MMP-3, a cycle-dependent key player in ESC detachment. S100A8 was locally co-expressed with S100A9 from the premenstrual phase, later than the initial expression of S100A9. During this phase, MMP-3 was also expressed in a synchronous and overlapping manner, and ESC detachments exist within these expression sites. These findings suggested that local formation of S100A8/S100A9 in ESCs induces local expression of MMP-3 and serves as a switch to an irreversible lysis phase, which may be mediated at least in part by the binding of the S100A9 subunit to CD147. Menstrual mechanisms must be further elucidated to treat menstruation-related diseases. We believe that S100A8 and S100A9 are worthy of further investigation as treatment targets of not only abnormal pregnancy but also excessive endometrial shedding.

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Declarations

Conflict of interest The authors declare no conflicts of interest regarding the publication of this article.

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Consent to participate and publish An opt-out approach was used to obtain informed consent from the patients, and personal information was protected during data collection.

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