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TECHNICAL NOTE

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Evaluation of mRNA Markers for the Identification of Menstrual Blood*

ABSTRACT: The detection of epithelial cells in dried bloodstains by reverse transcriptase-polymerase chain reaction is based on cell- and tissue-specific gene expression. In this paper mRNA markers suitable for the identification of menstrual blood were evaluated. RNA isolated from autopsy tissue samples including endometrium, vaginal mucosa, and blood were screened for tissue-specific expression patterns using RT-PCR with primers for hormone receptors, intermediate filaments, matrix metalloproteinases, heat shock proteins, cytokines, and growth factors. Matrix metalloproteinase (MMP) mRNA could be detected in endometrium but not in blood and other epithelia. This was confirmed in further studies with artificial menstrual bloodstains, indicating that the detection of MMP expression in bloodstains may serve as a forensic marker for menstrual blood.

KEYWORDS: forensic science, menstrual blood, RT-PCR, mRNA, matrix metalloproteinase

In the forensic examination of bloodstains it is frequently of crucial importance to determine the origin of bleeding. Alleged rape and sexual assaults, for example, which constitute a majority of cases in forensic serology, may involve the question whether bloodstains were caused by menstruation bleeding or by injury. The identification of menstrual blood was subject to several studies in the past based on the microscopic detection of cells shed from the endometrial mucosa during menstruation or on differences in isozyme patterns (1), hormone concentration (2), fibrin-fibrinogen degradation products (3), or fibrinolytic activity (4). However, these methods require sufficient quantities of blood and are sensitive to prolonged and inadequate storage. Moreover, if only stains of limited size are available, simultaneous DNA analysis often is not possible. A new approach to this problem is the detection of messenger-RNA (mRNA) exclusively expressed in endometrial tissue and not in blood or other epithelial cells. In the cell mRNA molecules are responsible for the transfer of genetic information from DNA to proteins. Therefore the mRNA pattern found in cells is determined by cell type and cell function and provides cell-specific information similar to proteins. However, due to the nucleic acid structure, very small amounts of mRNA can be amplified by reverse transcription-polymerase chain reaction (RT-PCR). Prior to amplification, RNA has to be transcribed back into DNA (complementary DNA, cDNA) using the enzyme reverse transcriptase (hence the term *reverse transcription*, RT). PCR is then performed with primers binding to different introns, which makes contamination with genomic DNA easily detectable because genomic DNA would generate longer products than RNA/cDNA due to the exons amplified. Furthermore, simultaneous DNA isolation and analysis from the same sample theoretically is possible because the extraction procedures are very similar. Isolation of mRNA suitable for RT-PCR from dried bloodstains with identification of epithelial cells was recently described by the authors (5). The study presented here was undertaken to evaluate candidate mRNAs expressed by endometrial cells for their value as a marker for the identification of menstrual blood.

Materials

Autopsy Samples

From $n = 16$ autopsies of females (mean age 28.4 $\pm$ 7.3 years, range 16 to 39 years; mean postmortem time 21.3 $\pm$ 9 h; no pregnancy, no history of rape, no disease of the reproductive system), small samples were collected from the following tissues: endometrium mucosa, vaginal mucosa, skin, oral mucosa, liver, blood, lung, muscle. In six of these individuals macroscopically visible signs of menstruation (blood clots in the uterine cavity) were present. The samples were frozen and stored at -40°C before preparation.

Bloodstains/Vaginal Swabs

Menstrual blood was collected from healthy volunteers (total number of samples $n = 66$) on commercially available tampons, partially eluted and transferred to cotton weaves, air-dried, and stored at room temperature for 1 to 30 days. Vaginal swabs and "traumatic blood" (finger pulp micropuncture) were taken from the same persons and air-dried.

Methods

Glassware and solutions were treated with diethyl-pyrocarbonate (DEPC) and autoclaved. For RNA isolation, the RNAgents-Kit

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TABLE 1—*List of selected primers used for amplification of reverse transcribed RNA from autopsy samples and menstrual bloodstains. List of primer sequences.*

Marker	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Annealing Temp.	Cycle Count	Ref.
β-actin	TCA TGT TTG AGA CCT TCA AC	CAT CTC TTG CTC GAA GTC CAG	57°C	35	(7)
MMP-7	AGT GAG CTA CAG TGG GAA CAG	CGA TCC ACT GTA ATA TGC GG	55°C	53	(8)
MMP-10	AGT TAA CAG CAG GGA CAC CG	AAC ACC CAT ATC TGT CTT CC	57°C	53	(9)
MMP-11	TCA GTA CTG GGT GTA CGA CG	CAT GGG TCT CTA GCC TGA TA	55°C	53	(10)
Luteinizing hormone receptor	CTA CCT CCC TGT CAA AGT GAT CC	GGA GAA GAC CTT CGT AAC ATC TG	55°C	35	(11)
Estrogen receptor (ER)	GGA GAC ATG AGA CTG CCA AC	CCA GCA GCA TGT CGA AGA TC	57°C	35	(12)
Cytokeratin 20 (CK 20)	GAG ACC AAG GCC CGT TAC AG	ACG ACC TTG CCA TCC ACT AC	57°C	35	(13)
Heat shock protein 70 (HSP)	AGG TGG AGA TCA TCG CCA AC	GGT AGA ATG CCT TGG TCT CC	60°C	35	(14)
HSP90α	ACA AGC ACA TAT CGC TGG ACA GCA	TTC AGT TAC AGC AGC ACT CGT ATC	60°C	35	(14)
HSP90β	TGT GAC CAG CAC CTA CGG CTG GAC	GAT CTC ATC AGG AAC TGC AGC ATT	60°C	35	(14)
HSC	CTT CCT TCG TTA TTG GAG CC	CTG CCA GCATCA TTC ACC AC	60°C	35	(14)
c-myc	CAC ATA CAG TCC TGG ATG ATG	CAC ATA CAG TCC TGG ATG ATG	57°C	35	(15)
c-jun	GGA TCA AGG CGG AGA GGA AG	GCG TTA GCA TGA GTT GGC AC	60°C	35	(15)
Leukemia inhibitory factor (LIF)	CAG CAT CAC TGA ATC ACA GAG C	CCC TGT GGG GAT GTT TCA TAC T	57°C	35	(16)
Tumor necrosis factor α (TNF α)	ACA AGC CTG TAG CCC ATG TT	AAA GTA GAC CTG CCC AGA CT	55°C	35	(17)
Endothelial growth factor (EGF)	AAG ATG ACC ACC ACT ATT CCG	AGC GTG GCG CAG TTC CCA CCA	58°C	53	(18)
Transforming growth factor β (TGF β)	ATG GTC CCC TCG GCT GGA CA	CTG CAG GTT CCA TGG AAG CA	55°C	53	(18)

from Promega was used. In this kit, all reagents for the single-step isolation according to Chomzynski and Sacchi (6) are provided in ready-to-use solutions. All reaction tubes and pipette tips were declared ribonuclease-free by the manufacturer.

The tissue samples were cut into small pieces (about 100 to 150 mg), placed into an adequate volume of denaturing solution containing guanidinium isothiocyanate, mercaptoethanol, sodium citrate, and lauroyl sarcosine, solubilized with a glass homogenizer, and homogenized by pipetting up and down through a 1-gauge syringe. After acid extraction with phenol/chloroform and precipitation with isopropanol, the precipitates were dissolved in ribonuclease-free water. The concentration was measured spectrophotometrically, and the RNA integrity was assessed by running a non-denaturing 1.2% agarose gel. Sharp and distinct bands for 28S and 18S ribosomal RNA with higher intensity of the 28S band indicated that no significant RNA degradation had occurred. One microgram of total RNA was used for DNA digestion (DNase I, Pharmacia, ten units in the RT-mix containing RT-buffer, 0.5 μg/μL oligo-dT-primer, and 5mM nucleotides; incubation at 37°C for 30 min, deoxyribonuclease inactivation at 70°C for 10 min), and reverse transcription (MMULV-reverse transcriptase, Gibco, 200 units, 37°C for 60 min and 95°C for 5 min) in a total volume of 20 μL.

RNA isolation from the dried stains (5 by 5 mm) and reverse transcription with previous DNA digestion was performed as described previously (5).

One microliter of the RT reaction was used for PCR in a total volume of 20 mL. PCR primers and amplification conditions are listed in Table 1 or in Ref 5 for glycerinaldehyde-3-phosphate dehydrogenase (GAPDH), cytokeratin 19 (CK 19), and progesterone receptor (PR). AmpliTAQ (Perkin Elmer; 0.7 U per reaction) was used as TAQ polymerase, AmpliTAQ Gold (Perkin Elmer; 0.7 units) for the hot start reactions. PCR was performed on a Perkin Elmer 2400 thermocycler and on Biometra personal cyclers.

The amplification products were visualized on 2% agarose gels stained with ethidium bromide.

Results

Autopsy Samples

Screening of endometrium and blood samples revealed that a number of genes (heat shock proteins, immediate early genes, cytokines, growth factors) were not constantly expressed in endometrium or could be detected in blood. Since this is not compatible with the function as marker for menstrual blood, no further experiments were performed with these primers.

Intermediate filament and hormone receptor mRNA was present in endometrium, menstrual blood samples, and in vaginal mucosa and other epithelia, but not in blood. Members of the matrix metalloproteinase gene family were the only genes constantly expressed in menstruating and non-menstruating endometrium but never in blood and vaginal mucosa. Inconstant expression could be found in skin and muscle tissue (Fig. 1). The results are presented in detail in Table 2.

Bloodstains/Vaginal Swabs

Menstrual blood samples, vaginal swabs, and "traumatic" bloodstains were screened for the presence of mRNA by housekeeping gene analysis. Only samples with positive amplification results for GAPDH or β-actin were considered for further investigation. MMP-11 expression could be detected in 84.8% of all samples (Fig. 2), with the lack of expression in 15.2% probably due to variations in the number of endometrial cells present in the menstrual blood; from samples taken during the first day of the menstrual phase ($n = 16$), six (37.5%) were negative for MMP-11; on Days 2 and 3 most samples (92%) were positive (Fig. 3). MMP-7 was detected in 64.1% and MMP-10 in 70.3% of all samples. In blood-

stains and vaginal swabs, no MMP expression could be found. Cytokeratin-19 mRNA was present in all MMP-11 positive samples and in 20% of MMP-11 negative samples.

Discussion

The aim of this study was to evaluate mRNA markers suitable for the identification of menstrual blood using RT-PCR. Based on the hypothesis that cells originating in the endometrial epithelium and stroma and shed during the process of tissue remodeling express a specific set of genes that makes them distinguishable from blood cells and other epithelia, 20 candidate genes were selected from studies investigating the molecular pathways involved in menstruation (19). In order to establish expression patterns for the selected genes, tissue samples obtained at autopsy were screened with primers designed for detection of corresponding cDNA. The results from these experiments indicate that a majority of mRNAs expressed in endometrium can be detected in blood and other organs and therefore cannot serve as a marker for menstrual blood. Cytokines, heat shock proteins (HSP), and other inflammation- and stress-related genes appear to be upregulated in menstruating endometrium. Since basal levels of these mRNAs are present in cir-

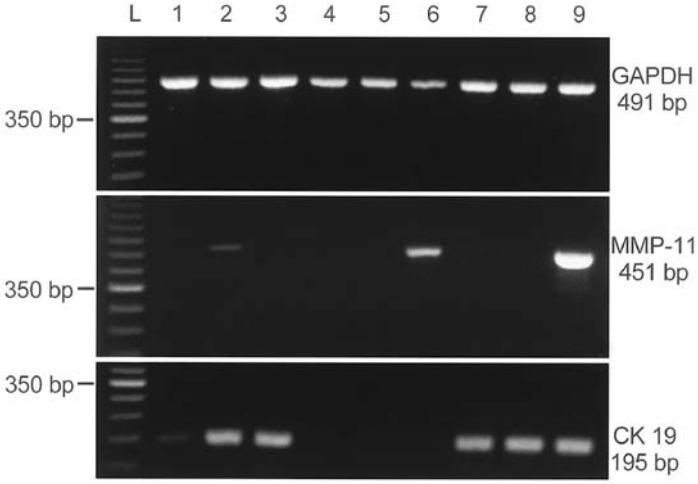

FIG. 1—*Gel photograph showing amplification results for GAPDH, CK 19, and MMP-11 from a selected autopsy case. L = ladder, 1 = lung, 2 = skin, 3 = liver, 4 = spleen, 5 = blood, 6 = muscle, 7 = oral mucosa, 8 = vaginal mucosa, 9 = endometrium.*

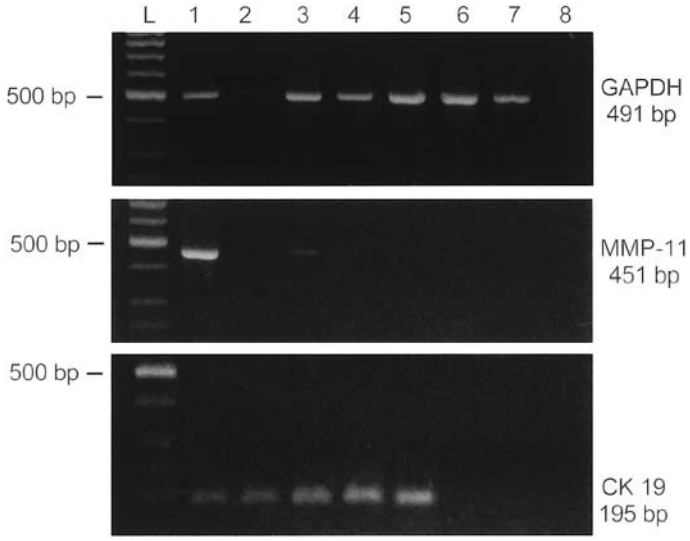

FIG. 2—*Gel photograph showing amplification results for GAPDH, CK 19, and MMP-11 from menstrual bloodstains, vaginal swabs, and "traumatic" bloodstains from three different individuals. Lanes 1, 4, and 6 and Lanes 3, 5, and 7 represent the same one person. L = ladder, 1–3 = menstrual bloodstains (Lane 2 from day one of menstruation), 4,5 = vaginal, 6,7 = bloodstains, 8 = negative control.*

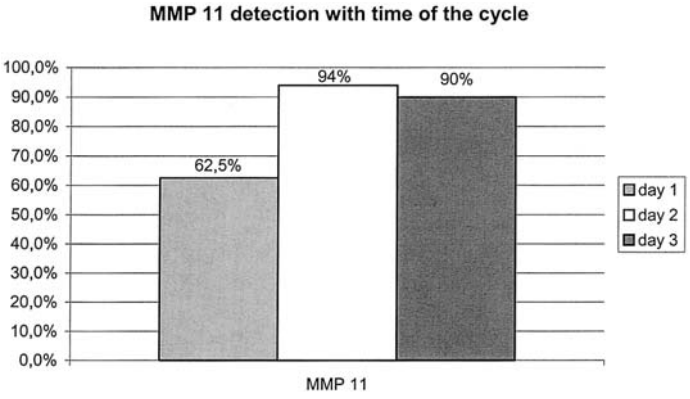

FIG. 3—*Graph showing percent positivity of MMP-11 RT-PCR with time of the menstrual cycle.*

TABLE 2—*Positive amplification results for selected primer pairs in percent. Rate of positivity of RT-PCR in various tissue samples.*

	Housekeeping Gene	Intermediate Filaments	Hormone Receptors		Matrix Metalloproteinases		
Percent	GAPDH	CK 19	PR	ER	MMP-11	MMP-7	MMP-10
Lung	100	100	0	0	0	0	0
Skin	100	100	0	0	31.3	37.8	25
Liver	100	100	0	12.5	0	0	0
Spleen	100	0	0	0	0	0	0
Blood	100	0	6.3	0	0	0	0
Muscle	100	25	81.3	25	25	31.3	25
Oral mucosa	100	100	12.5	6.3	0	0	0
Vaginal mucosa	100	100	93.8	93.8	0	13	0
Endometrium	100	100	87.5	93.8	100	87.5	81.3

culating blood cells, a quantitative RT-PCR approach would be necessary to correctly identify menstrual blood. However, since many diseases (cancer, sepsis, etc.) may affect stress gene expression in blood cells, even an elevated cytokine or HSP mRNA level would not prove the menstrual origin of bloodstains. For this reason, the use of inflammation- and stress-induced gene expression for the identification of menstrual blood currently cannot be recommended.

Some genes were expressed in endometrium and in other epithelia but not in blood (e.g., intermediate filaments). Provided that no skin or mucosa epithelia are present in blood originating from skin or mucosa injury (e.g., from the vaginal epithelium in cases of violent sexual assault), these mRNAs allow the differentiation between menstrual blood and traumatic blood. We have never found mRNA from epithelial cells in blood samples taken by micropuncture or from clothes worn by accident victims with extended lacerations (data not shown), indicating that with this method epithelial mRNA is not detectable in traumatic blood. However, genital bleedings due to injury could not be studied. As the cell junctions in mucosa epithelium are less tighter than in epidermis, the presence of epithelial cells in blood from genital injury in detectable amounts cannot be excluded, and false positive results would be obtained using primers amplifying epithelial mRNA from mucosa cells.

From all genes investigated in this study, only matrix metalloproteinases (MMP) are regularly expressed in human endometrium during the proliferative and menstrual period but not in blood and other epithelia such as vaginal mucosa. MMP-11 showed a more constant and stronger expression in menstruation than other members of the MMP gene family studied here (MMP-7, MMP-10). Weak and inconstant MMP gene expression could be further detected in skin and in muscle, which may be explained by fibroblastic activity. This should not affect the identification of menstrual blood since bloodstains from injuries as described above never displayed any MMP gene expression.

Matrix metalloproteinases are zinc-dependent endopeptidases involved in the breakdown of extracellular matrix components and expressed when tissue degradation and remodeling is required. This includes physiological conditions like menstruation (9) or development (20), as well as pathological conditions like invasive tumor growth (21) or wound healing (22). At least three distinct subsets of enzymes including the collagenases (MMP-1, -8), gelatinases (MMP-2, -7, -9), and stromelysins (MMP-3, -10, -11) with at least nine proteins are currently known. MMP gene expression is cycle-dependent with high mRNA levels for all enzymes during menstrual phase and lower or absent expression for most enzymes in the proliferative phase (9).

With this theoretical background and based on the results of the autopsy study, dried menstrual blood samples and vaginal swabs were examined by RNA isolation, and RT-PCR. MMP-11 mRNA could be detected in $>84\%$ of all menstrual blood samples but never in vaginal swabs or traumatic blood. In MMP-11 negative samples, intermediate filament mRNA (cytokeratin 19) was absent in 80%, indicating that the number of endometrial cells was low in these samples, the majority of which were taken at the beginning of the menstrual phase. The absence or very low amount of endometrial cells in menstrual bleedings at the first day of menstruation can be demonstrated by microscopy (data not shown). Samples taken after the first day of menstruation showed constant MMP-11 expression. This matrix metalloproteinase, therefore, is a reliable marker for menstrual blood. As matrix metalloproteinases are believed to be upregulated in tumors (21), after delivery (10) and during wound healing (22), false positive results might occur when the source of bleeding was a genital tract tumor, the placenta, a genital tract infection, or a recent injury. We could not study these hypotheses under forensic conditions, but in our opinion this does not interfere with the validity of the test for the identification of menstrual blood because these conditions can easily be diagnosed using the patient's medical history and performing a gynecological examination. If MMP-11 gene expression would indeed be detectable in tumor or peripartal bleedings, MMP-11 could be considered as a general marker for non-traumatic genital bleedings, even enhancing its forensic value rather than limiting it.

Regarding these results, we believe that the lack of MMP-11 expression in bloodstains points against the menstrual origin of the blood sample investigated when the successful amplification of ubiquitously and constantly expressed so-called housekeeping genes, which can be detected in every transcriptionally active cell independent of cell type and cell function, has verified RNA integrity. However, false negative results are possible if the sample is derived from the first day of menstruation with partial or complete absence of endometrial cells and if low-copy mRNAs such as MMP-11 are degraded below the RT-PCR detection limit, whereas abundant housekeeping mRNA still might be detectable. For these reasons, negative MMP-11 amplification results cannot exclude the possibility that the sample nevertheless was menstrual blood.

The successful and reliable usage of this new method in forensic casework requires some experience in RNA work and careful interpretation of results since RNA is more fragile than DNA and can be subject to degradation during analysis due to ubiquitously present ribonucleases. At least three PCRs with amplification of the following mRNA/cDNA should be performed: housekeeping gene (GAPDH or β-actin), epithelial marker (cytokeratin 19), and MMP-11. Only with positive amplification results for all three reactions the diagnosis "menstrual blood" can be established.

The age of bloodstains may affect the RT-PCR results, although data about the influence of aging on RNA analysis of dried bloodstains are not available at present. In our experience, no significant decrease in RNA content occurred after six months of storage (5). Further investigations with bloodstains stored for several years are currently performed and will be published soon. Since ribonucleases are believed to be inactive in completely dried material, we expect that RNA aging is comparable to DNA. For this reason, the method should work well with aged stains.

In contrast to our studies with artificial bloodstains (5), here it was not possible to determine the blood volume or cell number used for RNA isolation. Since the number of endometrial cells and the RNA degradation in menstrual bloodstains may vary, the sensitivity of the method cannot exactly be predicted. For standardization in our experiments, 5 by 5-mm cotton pieces with dried blood were used for RT-PCR, but smaller sample sizes may be sufficient (up to 1 by 1 mm; data not shown) since the method is capable of detecting less than 1000 epithelial cells in dried bloodstains (5).

Conclusions

Matrix metalloproteinase mRNA is preferentially expressed in endometrium and cannot be found in blood and vaginal mucosa. The detection of MMP-11 (stromelysin 3) gene expression in bloodstains proves the presence of endometrial cells and therefore can be used as a marker for the menstrual origin of blood in the absence of conditions listed above. Although there are limitations of the method concerning the interpretation of MMP-11 negative results, RNA analysis by RT-PCR can be a powerful tool for the

identification of menstrual blood provided the menstrual blood was produced after the first day of menses.

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