Cytokine array comparisons of plasma from cycling fertile women on cycle day 5 and ovulation

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Abstract

Problem—To identify plasma immuno-regulatory molecules up or down regulated between the follicular phase and ovulation of the human menstrual cycle.

Method of study—RayBio® cytokine arrays were used to screen 174 immuno-regulatory molecules in plasma collected during the follicular phase at menstrual cycle day 5 and at ovulation from 5 healthy, non-smoking, fertile women of reproductive age not using hormonal contraception.

Results—A total of 23 differentially expressed molecules were found: 10 molecules were differentially up-regulated and 13 down-regulated at ovulation compared to the follicular phase ($\alpha=0.05$, false discovery rate of 0.45).

Conclusion—Circulating immuno-regulatory molecules fluctuate over the menstrual cycle in healthy women. The combination of differentially expressed molecules suggests roles in cyclical regulation of angiogenesis of and immune cell trafficking.

Keywords

cytokine array; menstrual cycle; plasma

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Introduction

The immune system responds to pregnancy in dynamic ways. Although much attention has been given to the immunology of the fetal-maternal interface, systemic maternal immune responses also occur during pregnancy.¹ For example, men and non-pregnant women have a systemic cytokine profile that is type 1 biased (pro-inflammatory). However during late second trimester of a normal pregnancy, women switch to an overall type 2 biased (anti-inflammatory) cytokine profile.²

The immune system is also regulated hormonally by the menstrual cycle.³ Many investigators have considered whether potential dysregulation of blood cytokines might participate in female infertility. We previously reported that, in fertile women, the trafficking potential of the minor subset of blood Natural Killer (NK) cells, CD56^{Bright} cells, for egress to the uterus had menstrual cyclical variation. Trafficking potential, as detected in our *in vitro* cell adhesion assay to frozen decidual tissue sections under shear force, peaked at ovulation. Adhesion detected changes in endothelial cells within the section tissue and depended upon L-selectin (SELL) and α 4-integrin (ITGA4)-based changes in the lymphocytes.⁴⁻⁷ In women who successfully conceived from controlled hormonal ovulation and transfer of fresh embryos or transfer of frozen embryos in a monitored natural cycle, *in vitro* NK cell trafficking potential was dynamic and peaked on the day of ovulation.⁷ This cyclical pattern of blood NK cell behavior was not found in menstrual cycles of women receiving frozen embryos that did not implant.⁷

Lymphocytes from male blood incubated in plasma collected from fertile women at ovulation also showed gains in adhesive function of CD56^{Bright} NK cells in comparison to cells from the same blood sample incubated in plasma collected at cycle day 5.⁸ From this observation, we hypothesized that at ovulation circulating immune cells respond to menstrual cycle-regulated immuno-regulatory signals in plasma to egress from vessels and into endometrium. To advance this hypothesis, a screening study of 174 soluble cytokines, chemokines, growth factors and angiogenic molecules was undertaken. Test plasma was donated twice, during the follicular phase and at ovulation by each of five healthy, fertile women of reproductive age. Statistical analyses were performed to look for differentially expressed molecules during the menstrual cycle.

Materials and Methods

Blood collection

Five healthy, non-smoking, fertile adult women with proven fertility (conceived at least one healthy child within the past two years without medical intervention) with normal menstrual cycles who were seronegative for Hepatitis B and C and HIV and had not used any form of hormonal contraception within the past year were recruited. Ten mL of blood were collected by venipuncture into evacuated tubes on menstrual cycle day 5 and on the day of ovulation. The menstrual cycle day 5 blood sample will hereafter be referred to as the follicular phase sample. The first day of menses was designated cycle day 1. The day of ovulation was determined using an ovulation detection kit (Ovulation Indicator, Life Brand Shopper's Drug Mart; Toronto, Ontario, Canada). Participants were instructed to urinate on the

ovulation detection strips in the morning and in the evening beginning on cycle day 12. Blood was collected on the morning of a positive detection, or the morning following a positive evening detection. All participants provided informed written consent approved by the Human Research Ethic Board, University of Western Ontario.

Cytokine array

Blood was layered over Histopaque 1077 (Sigma; Oakville, Ontario, Canada) and centrifuged (400×g 4°C, 30 min). The resulting plasma supernatant was aspirated and stored at -80°C. Using the RayBio® Human Cytokine Antibody Array 6, 7 and 8 (catalogue numbers AAH-CYT-6, AAH-CYT-7 and AAH-CYT-8, respectively, RayBiotech Inc.; Norcross, Georgia, U.S.A.), a total of 174 immuno-regulatory molecules were simultaneously screened for each plasma sample. These molecules are listed in Table I. The membranes are pre-coated with antibodies against target peptides anchored onto the membrane for sandwich-ELISA detection. Each unique target molecule was assessed in duplicate. Manufacturer's directions were followed. Briefly, membranes were individually placed in chambers of 8-well tissue culture plates and blocked with the kit's blocking buffer. Following blocking, plasma diluted 1:10 in blocking buffer was added. After 2 hr of incubation, membranes were repeatedly washed and then incubated with biotin-conjugated antibodies for 2 hr. Following a further set of washes, horse radish peroxidase conjugated streptavidin was added and incubated for 1 hr followed by a final set of washes. The membranes were then placed in the manufacturer's chemiluminescence detection buffer and incubated for 2 min. Membranes were sandwiched between clean plastic sheets and excess detection buffer was carefully squeezed out before being exposed to Kodak x-omat AR film (MarketLINK Scientific; Burlington, Ontario, Canada). The film was then developed and scanned using a high resolution scanner (Hewlett Packard HP PSC1315; Mississauga, Ontario, Canada) and saved digitally. The densitometric value of each locus on the array was measured using ImageJ software (National Institutes of Health; Bethesda, Maryland, U.S.A.). Figure 1 is a representation of one of the array membranes used to collect data.

Statistical analysis

Statistical analyses were performed to interpret 174 different cytokine readings at two time points. Normalization was performed to account for any variation between one membrane and another to permit valid cross membrane comparisons. Briefly, the mean value of the manufacturer's positive control replicates on each membrane, called the loading control densitometric value (LCDV), was determined. The LCDV value for each membrane was subtracted from all experimental cytokine densitometric values on that membrane. The resulting value is referred to as the loading control normalized densitometric value (LCNDV) of the cytokine. To reduce variation between membranes within a plasma sample, all three membranes (comprising all 174 cytokines) were processed simultaneously. Hence, a second normalization was done for each plasma sample by taking the mean densitometric value of all 174 experimental cytokines for that sample and subtracting it from LCNDV. The resulting value is the globally normalized densitometric value (GNDV) of the loci. Because each membrane detected unique cytokines in duplicate, the GNDV values of the duplicates (i.e. two loci detecting the same molecule) were averaged and used for all of our analyses.

This value will be referred to hereafter as the mean densitometric value (MDV) of each cytokine, and was the value used for interpretation of data.

To look for differential expression between menstrual cycle days, significance analysis of microarrays (SAM, Microsoft Excel; Mississauga, Ontario, Canada) analysis was performed using one class response format for comparison between menstrual cycle days. For SAM analysis, cutoff values for significance were chosen based on the false discovery rate (FDR). The application, advantages and prevalence of the use of FDR to analyze array data, such as microarray, have been described by others.^{9;10} Use of FDR is also applicable for analyzing membrane based protein arrays.¹¹

Interaction map and functional annotation

An interaction map was created to show the relationship between the differentially expressed molecules. Gene symbols of the up- and down-regulated proteins were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins analysis platform (STRING; <http://string.embl.de/>)¹² and an interaction map was created by using STRING's high confidence setting (score=0.7) and by setting the maximum number of predicted interactors to 20. Gene ontology functional annotation was also performed using the differentially expressed molecules to characterize the biological processes regulated. Annotation was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>).¹³

Results

Using FDR-based SAM analysis, we discovered 23 significant results at a FDR cut-off of 45%. The appropriate FDR cut-off value was chosen using SAM analysis and was based on how distributed the molecules were in terms of their individual FDR values (not shown). Table II summarizes the differentially up/down-regulated cytokines found in our arrays using SAM analysis. There were 10 differentially up-regulated and 13 down-regulated molecules that were identified on the day of ovulation compared to the follicular phase (cycle day 5). Figure 2 shows the STRING generated predicted interaction map of these 23 molecules with predicted interacting partners. Table III summarizes the gene ontology functional annotation results using DAVID. Functional annotation revealed that the differentially expressed molecules participated in biological processes listed in DAVID as having relationship with "immune system processes", "cell communication", "growth factor activity", "locomotory behavior" and "positive regulation of cell proliferation".

Discussion

The molecules potentially up-regulated in fertile women at ovulation can be grouped functionally. Three molecules, Angiopoietin 2 (ANGPT2), VEGFA and c-fos induced growth factor (FIGF, also known as VEGFD) strongly promote angiogenesis through their actions on endothelium. Three molecules, chemokines ligands 1, 11 and 19 (XCL1, CXCL11 and CCL19, respectively) would be expected to increase cell homing under pro-inflammatory conditions. The latter two are specifically active on CD56^{Bright} blood NK cells.^{14;15} The structurally-related molecules oncostatin M (OSM) and granulocyte colony

stimulating factor 3 (CSF3) are cytokines of the leukemia inhibitory factor/interleukin 6 (LIF/IL6) family that support the viability of primitive cell types.¹⁶ The former is a cytokine-induced early response gene.¹⁷ Neurotrophin 4 (NTF4; formally called NTF5) is a product of pre-implantation trophoblast and is detected in mouse embryos from the 2 cell stage. In mice, both the oviduct and uterus secrete Ntf5 (now called Ntf4).¹⁸ The remaining molecule, from the tumor necrosis superfamily, is a decoy molecule that blocks the death-promoting functions of TRAIL. It is also known as CD261, Apo2, TRAIL-R1, DR4 or under the approved name of TNFRSF10A (tumor necrosis factor receptor superfamily, member 10a). Immune tissues such as spleen, thymus and blood leukocytes, particularly activated T cells express this molecule.¹⁹

Some of the 13 molecules differentially down-regulated have actions that could compliment and work synergistically with above molecules to alter circulating immune cell behavior. CD56^{Bright} NK cells have receptors for chemokine ligands 10 and 12 (CXCR3 and CXCR4, respectively).²⁰ These data may explain how a subset of CD56^{Bright} NK cells could become activated and egress from the circulation to the uterus. Activated leukocyte cell adhesion molecule (ALCAM or CD166) is likely being detected in its soluble form in our plasma samples. Soluble ALCAM enhances endothelial cell migration, inhibits endothelial tube formation and disrupts ALCAM-ALCAM homodimer tethering of a cell's actin cytoskeleton. Platelet-derived growth factor alpha (PDGFA) functions through a VEGF homology region.²¹ It also disrupts actin filaments permitting membrane ruffling and cell migration. Cells expressing PDGFRA would be expected to be more adhesive with PDGFA reduction and endothelial cells (rather than hematopoietic cells) would be more likely affected.²¹ Chemokine ligand 16 (CXCL16) mediates both chemotaxis and adhesion and is expressed by NKT cells and activated Th1 cells.²² First trimester human trophoblast also secretes CXCL16 which, through CXCR6, recruits T cells, including gamma/delta T cells and monocytes but not NKT cells or NK cell subsets.²³ The down regulation of CD14, colony stimulating factor 1 receptor (CSF1R), sialic acid binding immunoglobulin-like lectin 5 (SIGLEC5) and inhibin beta A (INHBA) suggests attenuated functions of monocyte/macrophages.²⁴⁻²⁷ The decrease in Fas ligand (FASLG) would reduce cell death and compliment the gain in circulating TNFRSF10A. Decreased circulating leukemia inhibitory factor (LIF) may be linked with the gains in CSF3 and OSM. SELL is the lymphocyte receptor we previously identified as a key molecule involved in CD56^{Bright} NK cell binding to decidual endothelium in *in vitro* adhesion assays under shear forces.⁴⁻⁷ Like ALCAM, it is shed from the cell surface and the soluble form would be detected in this assay. SELL shedding occurs during transendothelial migration by leukocytes. A reduction in soluble SELL would mean there is less competition for binding of the SELL receptors on endothelium by circulating lymphocytes, thus increased likelihood of CD56^{Bright} NK cell extravasation at activated endothelial sites.

When the differentially detected molecules were used as seed nodes to create an interaction map, CD16 and SIGLEC5 were left unpartnered. Molecules ALCAM and INHBA had limited predicted interacting partners. Some of these molecules may have limited or no interaction partners, possibly because they are false positives that were included because of FDR. The other differentially detected molecules were part of an extensive interaction relationship. Given that our statistical analysis detected differentially expressed molecules

with the inclusion of false positives, future work on molecules that showed extensive interaction with other molecules we differentially detected, such as VEGFA, may possibly be more fruitful than molecules with little relationship, such as CD14.

Conclusions

The array data we report highlight the dynamic changes of immunological consequence that occur in women over the menstrual cycle. Our results identify immuno-regulatory molecules that are potentially involved in the extravasation of CD56^{Bright} NK cells for egress to the uterus. The data also highlights potential relationships between some of these molecules that may merit further investigation.

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Figure 1. Representation of an individual membrane array after development. For each blood donor, 6 membranes (3 for each time point) were used to screen 174 molecules. A total of 30 membranes were processed to generate data used in our analyses.

Figure 2. First level interaction map of differentially expressed molecules, as well as predicted interacting partners. The map was created using seven lines of evidence selected provided by STRING: “neighborhood”, “gene fusion”, “cooccurrence”, “coexpression”, “experiments”, “databases” and “textmining”.

Table I
Official gene symbols of screened plasma immuno-regulatory molecules

Gene symbols of screened cytokines are listed alphabetically. A total of 174 molecules were screened. The gene names listed are Human Genome Organization (HUGO) Gene Nomenclature Committee approved symbols that may not necessarily correspond to symbols used by the manufacturer.

<i>RayBio® Human Cytokine Antibody Array 6</i>			
ADAM11	CCL23	GDNF	IL10
ANG	CCL24	IFNG	IL13
BDNF	CCL26	IGF1	IL15
BMP4	CNTF	IGFBP1	IL16
BMP6	CSF1	IGFBP2	KITLG
CCL1	CSF2	IGFBP4	LEP
CCL2	CX3CL1	IL1A	LTA
CCL5	CXCL6	IL1B	NAP1L4
CCL7	CXCL9	IL1R1	NTF3
CCL8	CXCL12	IL2	PDGFB
CCL11	CXCL13	IL3	PPBP
CCL13	EGF	IL4	TGFB1
CCL15	FGF6	IL5	TGFB3
CCL17	FGF7	IL6	TNF
CCL18	FLT3LG	IL7	TNFSF14
<i>RayBio® Human Cytokine Antibody Array 7</i>			
ADIPOQ	CXCL1,2,3	IL2RA	THPO
AGRP	CXCL5	IL6R	TIMP1
ANGPT2	CXCL11	IL6ST	TIMP2
AREG	EGFR	IL8	TMPRSS13
AXL	FGF4	IL11	TNFRSF1A
BTC	FGF9	IL12A	TNFRSF1B
CCL3	FIGF	IL12B	TNFRSF6
CCL4	HGF	IL17	TNFRSF10C
CCL16	ICAM1	MIF	TNFRSF10D
CCL19	ICAM3	NGF	TNFRSF11B
CCL25	IGF1R	NTF4	TNFRSF18
CCL27	IGFBP3	NUDT6	TNFSR18
CCL28	IGFBP6	OSM	TYRO3
CSF3	IL1R1	PGF	VEGFA
CXCL1	IL1RL1	PLAUR	XC1
<i>RayBio® Human Cytokine Antibody Array 8</i>			
ALCAM	FASLG	INHBA	PDGFRB
BMP5	FLT4	KDR	PECAM1
BMP7	ICAM2	KIT	PRL
CCL23	IGF2	LAP3	SELE

CD14	IL1R2	LEPR	SELL
CD80	IL2RA	LIF	SIGLEC5
CDH5	IL2RB	MMP1	TEK
CSF1R	IL5RA	MMP3	TGFA
CTF1	IL9	MMP9	TGFB2
CXCL10	IL10RB	MMP13	TIE1
CXCL12	IL13RA2	NGFR	TIMP4
CXCL16	IL18BP	PDGFA	TNFRSF21
ENG	IL18RAP	PDGFA/B	
ERBB3	IL21R	PDGFRA	

A total of 23 differentially expressed molecules (13%) were detected out of the 174 tested. Of these, 10 were differentially up-regulated molecules and 13 were differentially down-regulated molecules at ovulation compared to menstrual cycle day 5.

Table II
Differentially expressed plasma molecules at $\alpha=0.05$ with FDR cutoff of 0.45

Up-regulated at ovulation compared to cd5 (10)			Down-regulated at ovulation compared to cd5 (13)		
Symbol	Fold change	FDR	Symbol	Fold change	FDR
VEGFA	2.63	0.180077	SIGLEC5	-2.95	0
CFS3	2.16	0.180077	CXCL12	-2.76	0
CCL19	2.15	0.180077	ALCAM	-2.08	0.405172
FIGF	2.09	0.190070	LIF	-1.84	0.405172
ANGPT2	1.91	0.210609	CD14	-1.76	0.405172
TNFRSF10D	1.88	0.210609	KIT	-1.71	0.405172
OSM	1.83	0.210609	INHBA	-1.64	0.405172
XCL1	1.72	0.210609	FASLG	-1.64	0.405172
CXCL11	1.59	0.417450	CXCL16	-1.57	0.405172
NTF4	1.57	0.417450	CXCL10	-1.54	0.405172
			PDGFA	-1.53	0.405172
			SELL	-1.51	0.405172
			CSF1R	-1.39	0.446271

Table III
Database for Annotation, Visualization and Integrated Discovery (DAVID) generated gene ontology functional annotation analysis

Gene ontology functional analysis revealed that many of the significantly detected molecules were classified under a number of biological processes. Significance values were determined by DAVID.

Biological process	Count	Percent	Molecules	Significance (Benjamini)
Immune system process	12	52%	CCL19, CD14, CSF3, CXCL10, CXCL11, CXCL16, FASLG, IHNBA, KIT, LIF, OSM, XCL1	3.1×10^{-6}
Cell communication	19	83%	ALCAM, ANGPT2, CCL19, CD14, CSF1R, CSF3, CXCL10, CXCL11, LIF, KIT, FASLG, FIGF, IHNBA, NTF4, OSM, PDGFA, TNFRSF10D, VEGFA, XCL1	2.5×10^{-5}
Growth factor activity	7	30%	CSF3, FIGF, IHNBA, LIF, NTF4, PDGFA, VEGFA,	6.1×10^{-5}
Locomotor behavior	7	30%	CCL19, CXCL10, CXCL11, CXCL16, NTF4, VEGFA, XCL1	7.4×10^{-5}
Positive regulation of cell proliferation	7	30%	CSF3, CXCL10, FIGF, KIT, LIF, PDGFA, VEGFA	6.2×10^{-4}