Published in final edited form as:
Cytokine. 2011 March ; 53(3): 282–285. doi:10.1016/j.cyto.2010.11.014.

Increased plasma levels of adipokines and inflammatory markers in older women with persistent HPV infection

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Abstract

We observed diminished lymphoproliferation to multiple stimuli in older women with persistent cervical human papillomavirus (HPV) infection. Adipokines are a class of inflammatory cytokines that are altered in some persistent infections. The objective was to compare the level of adipokines and inflammatory cytokines in heparinized plasma from women with persistent HPV cervical infection (Cases, N=50, oversampled for their weak lymphoproliferation responses) with women with no evidence of persistent HPV cervical infection (Controls, N=50, oversampled for their strong lymphoproliferation responses). Plasma samples were analyzed with multiplex assays for adipokines and inflammatory cytokines. Cases had significantly elevated plasma levels of resistin ($p < 0.0001$) and sFas ($p = 0.0038$) as compared to controls. Risk of persistent HPV infection increased significantly with increasing levels of resistin and sFas. This is the first study to demonstrate elevated levels of resistin and sFas in HPV persistently infected, older women with decreased immune function expanding the understanding of the systemic inflammation and immune alterations in individuals persistently infected with HPV. Further studies within a larger cohort are needed to define the generalities of these findings and any role adipokines have in persistent HPV infection.

Keywords

HPV; persistent infection; adipokines; inflammation

1. Introduction

Adipokines are a class of cytokines originally characterized as being produced by adipose tissue. However, their production is more broad than originally thought and have been

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detected at the mRNA level in muscle, pancreatic and gastrointestinal tissues [1]. Adipokines are associated with inflammation and are thought to be involved in the etiology of metabolic diseases. They have also been shown to be deregulated in the context of chronic infections, such as HBV and HCV [2] and in the context of gynecological carcinomas [3]. Some adipokines, for example resistin, are transcribed at increased levels in PBMCs after culturing with inflammatory cytokines including TNF-α, IL-1 and IL-6 as well as TLR ligands such as LPS [4]. In turn, treatment of PBMCs with the adipokines, leptin or resistin, leads to increased production of TNF-α, IL-6, and IL-12 [5–6] indicating that there may be some level of cross talk between adipokines and hallmark cytokines of inflammation. Adipokines also alter cellular trafficking by increasing the expression of adhesion molecules including ICAM-1, VCAM-1, and PECAM by endothelial cells [7–8].

We have previously observed increased levels of circulating inflammatory cytokines (TNF-α and IL-8) in older women persistently infected with HPV [9].

To better characterize the profiles of inflammatory markers associated with persistent HPV infection and decreased immune responses in older women, we sought to determine if adipokines levels were associated with the systemic inflammation observed in a weighted subpopulation of older women with evidence of persistent HPV infection and similarly aged women without HPV infection. One adipokine, resistin, was observed to be significantly increased in persistently infected individuals. The death molecule, sFas, was also significantly increased albeit not as dramatically as resistin. We further show that the likelihood of HPV persistence is greatest in those individuals with the highest levels of resistin or the inflammatory cytokines IL-8 and TNF-α. This is the first study to report on the relationship between adipokines, specifically resistin, and HPV persistent infection.

2. MATERIALS AND METHODS

2.1 Subjects

Women included in the present study were participants in a population-based cohort study of HPV and cervical neoplasia initiated in 1993 in the province of Guanacaste, Costa Rica. Details of the cohort recruitment [10] and follow-up [11] have previously been described.

In this study, we selected a weighted subpopulation of women based on their lymphoproliferative responses (assessed with PBMCs collected at the final visit, ~9th year after enrollment) from a nested case-control study of a group of women older than 45 within the Costa Rican natural history cohort[12]. Details of the selection criteria for the weighted subpopulation have been described[9]. Briefly, cases (n=50) were selected based on the following criteria: weak lymphoproliferation response to PHA or HPV16 L1 VLP and presence of a type-specific persistent HPV infection that was detected by PCR-based testing at enrollment, at the 5–6 year follow up visit, and at the final visit ~9th year after enrollment; controls (n=50) were selected based on the following criteria: strong lymphoproliferation response to PHA or HPV16 L1 VLP, no evidence of HPV infection by PCR-based testing at the 5–6 year follow up visit and at the final visit ~9th year after enrollment. Controls from the original nested case-control study were frequency matched to cases on age and time since study enrollment. Non-fasting heparinized blood was collected ~9th year after enrollment, processed for PBMCs (lymphoproliferation study[12]) and plasma, aliquoted, and stored in vapor phase of liquid nitrogen (PBMC) or at −80° C (Plasma) until testing. The study was approved by Costa Rica and NCI IRB, informed consent was obtained from subjects and IRB guidelines were followed.

2.2 HPV detection and genotyping

As previously reported [12], HPV DNA testing results from three study visits, enrollment, follow up (5–7th year visit after enrollment) and final visit (~9th year after enrollment) were used to classify participants into analytical groups. HPV DNA positive women were grouped into either an oncogenic "High Risk" group (positive for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 or 68) or non-oncogenic "Low Risk" (positive for HPV types 6, 11, 26, 32, 34, 40, 42, 53, 54, 55, 57, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85 or 89). Those individuals infected with multiple HPV strains were grouped in the "High Risk" subpopulation if one of the infecting viruses was of the oncogenic type. Persistence was defined as having sequential type-specific HPV DNA positive results. Specifically, short-term persistence (n = 29; median = 19 months) was defined as being HPV DNA negative at enrollment and positive for the same HPV type at their follow up and final visits. Long-term persistence (n=21; median, 108 months) was defined as being positive for the same HPV type at all three visits.

2.3 Multiplex Cytokine Assays

Eleven analytes were measured in heparin plasma, collected at the final visit (~9th year after enrollment), according to manufacturer's instructions using a multiplex bead assay (Millipore Linco Research, St. Charles, MO, USA). Samples were blindly tested in 3 batches and data was analyzed using Bio-Plex Manager 3.0 software (Bio-Rad, Hercules, CA). The 11 analytes were divided over three panels: Adipokines Panel A (adiponectin, resistin, tPAI-1; samples tested at 1:400), Adipokines Panel B (HGF, TNF-α, Leptin, IL-8; samples tested undiluted), and Apoptosis/Sepsis Panel (sVCAM-1, sICAM-1, sFas, and MIF; samples tested at 1:10). In addition, CRP was measured using a MesoScale Discovery multi-array platform (Gaithersburg, MD, USA). The multiplex assay was evaluated in a pre-study validation process. The plasma from healthy donors (n = 10) was used to assess coefficients of variation (CV %) for duplicate wells (between wells), duplicates plates ran one day (between plates), and identical plates ran different days (between days) and between different technicians. These results concluded overall CV values for the various analytes of <20.0%, except for HGF (31.8%) and PAI-1 (31.6%). The ICC values ranged from 67.4% to 98.9% for all analytes. In addition, for each batch of samples, 2 healthy, normal controls were included to track the variability in the assay during the actual study. The results are summarized in Table 1. MIF, HGF and PAI-1 were not included in the final analysis because MIF was not detectable in the healthy, normal donors and HGF as well as PAI-1 were associated with considerable variability. TNF-α and IL-8 had been previously measured within this subset of individuals in our lab and inclusion of these markers in this study served as confirmation of assay performance as indicated by the significant spearman rank correlation coefficient between studies (TNF-α: $\rho=0.95$, $p<0.0001$ and IL-8: $\rho=0.97$, $p<0.0001$).

2.4 Statistical Analysis

Statistical analyses were preformed using JMP®7.0.2 (SAS Institute Inc. Cary, NC). Non-parametric Mann-Whitney tests were used for comparisons of the analytes levels between infected women and uninfected controls. Assay components of variability (CV % between wells, plates, days, and ICC) were determined with SAS System for Windows 9.0 (SAS Institute, Cary, NC) as previously described [13]. Odds ratios and P_{trend} values were calculated using SAS.

3. RESULTS

Women persistently infected with HPV had significant increases in the plasma levels of two biomarkers evaluated in this study: Resistin (89.2 vs. 24.6 ng/ml; $p < 0.0001$) and sFas (7.2

vs. 6.4 ng/ml; p = 0.0038) (Table 2). Data regarding the HPV DNA history of the participants allowed for more detailed elucidation of factors that may further differentiate groups in the persistently infected cohort. The infecting virus of each persistently infected individual was classified as either a low risk or high risk type in terms of its potential to cause cervical cancer. No significant difference existed between individuals grouped based on the oncogenic risk of the infecting virus (Resistin, p = 0.90; sFas, p = 0.50). The persistently infected cohort was also divided into those who had short-term persistence (2 positive HPV DNA tests; N=29; median = 19 months) or long-term persistence (3 positive HPV DNA tests; N=21; median = 108 months). The levels of the biomarkers did not significantly differ between these two groups (Resistin, p = 0.91; sFas, p = 0.37).

To further evaluate the association between biomarkers whose median levels significantly varied between the HPV persistence and the control groups in this weighted population, we evaluated the association between stratified levels of resistin or sFas and HPV persistence (Table 3). For both resistin and sFas, risk of HPV persistence was found to increase as the levels in patient samples increased. The third tertile (highest biomarker levels) showed remarkably high OR values, which was also evident, albeit at lower levels, in the second tertile. Significant P_{trend} values were observed for all the altered biomarkers identified (Resistin, $P_{trend} < 0.0001$; sFas, $P_{trend} < 0.0025$ IL-8, $P_{trend} < 0.0001$; TNF-Alpha, $P_{trend} < 0.0001$).

4. DISCUSSION

Adipokines are cytokines of high interest in relation to generalized inflammation and have been observed to be increased in the context of chronic or persistent infections and cancer (2–3). The goal of this study was to identify adipokines, the soluble trafficking molecules they influence, and other circulating markers of inflammation that may be differentially expressed in the context of an HPV persistent infection. Our results indicate that plasma levels of resistin and sFas are significantly increased in women, who have a persistent HPV infection and poor immune responses compared to women, who have no detectable HPV infection and robust immune responses. Resistin has a plethora of effects in the body including increased expression of adhesion [7–8] and inflammatory molecules [5–6]. The mechanism(s) underlying the increased levels of resistin and sFas in the context of HPV persistence is unknown. These results further the understanding of the previously observed association between HPV persistence and immune dysregulation and studies of these markers in a larger sample population are justifiable in the future.

Increased levels of resistin have been implicated in a variety of diseases and additional studies investigating its association in the context of persistent HPV infection would be highly informative. Resistin levels are increased in cases of cardiovascular disease [14–15]. TNF-α expression is increased by resistin (5–6). Interestingly, sFas is induced by exposure to TNF-α [16] and has also been implicated as a marker of vascular disease [17]. All three of these molecules were significantly increased in the plasma of individuals persistently infected with HPV. Higher levels of circulating resistin have been suggested to be related to obesity and insulin resistance [18–19]. While obesity may not significantly increase the risk of cervical cancer [20]; it will be insightful to understand if persistent HPV infection is associated with either obesity or insulin resistance.

This is the first study to suggest alterations in systemic levels of adipokines and sFas in the context of cervical persistent HPV infection and immune dysfunction using a reproducible multiplex assay. Further investigation within a larger cohort of persistently infected women as well as similar studies in younger cohorts are warranted to better define any links between inflammation, HPV persistence, immune status and age. Future studies using samples

collected longitudinally will be helpful in determining the predictive role of the peripheral immune alterations reported here in HPV persistence and cervical cancer and whether these alterations were present prior to HPV infection or developed following persistent HPV infection.

Acknowledgments

We thank Helen Rager of the SAIC-Frederick, Inc., Core Immunology Lab, for her assistance with MesoScale assay, Yuanji Pan for statistical analyses and Roni Falk for statistical advice. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Table 1

Multiplex analyte reproducibility assessed in plasma from healthy, control blood donors

	Coefficent of Variation (%)		
Analyte	**Between Well**	**Between Day**	**Overall**
Adiponectin	2.6	14.5	14.7
Resistin	6.9	20.3	21.5
PAI-1	9.2	30.3	31.6
IL-8	11.4	12.2	16.8
Leptin	8.2	0.0	8.2
TNF-a	12.4	6.7	14.1
HGF	9.1	37.5	38.6
sVCAM-1	9.2	15.2	17.8
sICAM-1	4.8	6.1	7.8
sFas	9.1	14.0	16.7
MIF[a]	n/a	n/a	n/a
CRP	4.9	0.7	5.0

[a] CVs could not be calculated because levels were undetectable in all samples tested

Table 2

Circulating levels of adipokines and other inflammatory markers in women with HPV persistent infection and HPV-uninfected women.

	HPV DNA Negative (n = 50)				HPV Persistent (n = 50)					
Analytes	**Mean**	**Median**	**SD**	**95% CI**	**Mean**	**Median**	**SD**	**95% CI**	**Fold Change[a]**	**p-value[b]**
Adiponectin (μg/ml)	21.0	20.0	11.0	17.8–24.1	23.6	20.9	10.0	20.6–26.5	1.0	0.074
Resistin (ng/ml)	32.6	24.6	25.3	25.4–39.8	147.8	89.2	356.8	46.4–249.2	3.6	<0.0001
Leptin (ng/ml)	26.5	21.1	19.7	21.0–32.1	37.6	27.5	39.9	26.3–49.0	1.3	0.29
sVCAM-1 (ng/ml)	545.1	463.5	275.3	466.8–623.3	608.1	569.2	259.0	534.5–681.7	1.2	0.058
sICAM-1 (ng/ml)	206.1	196.7	63.1	188.1–224.0	223.6	205.8	80.1	200.9–246.4	1.0	0.25
sFas (ng/ml)	6.9	6.4	1.9	6.3–7.4	9.3	7.2	11.5	6.0–12.6	1.1	0.0038
CRP (ng/ml)	4170.0	3363.0	3886.0	3066–5274	5033.0	2549.0	8709.0	2558–7508	0.8	0.92
IL-8 (pg/ml)	121.0	9.8	235.0	54.1–187.4	517.0	324.2	544.0	362.0–671.0	33.1	<0.0001
TNF-α (pg/ml)	28.0	4.8	63.0	9.7–46.0	88.0	62.0	77.0	66.0–109.0	12.8	<0.0001

[a] Fold change was calculated based on the median values of cases compared to controls

[b] Calculated using Mann-Whitney test

Abbreviations: SD, Standard Deviation; CI, Confidence Interval

Cytokine. Author manuscript; available in PMC 2012 March 1.

Table 3

Risk of HPV persistent infection associated with elevated levels of circulating adipokines and cytokines.

		Sample Type				
	Tertile Ranges	**Control**	**HPV Persistent**	**OR**	**95%CI**	**P_{trend}**
Resistin	<30	30	3	1.0		
	30–75	17	16	9.4	2.4–37.1	
	>75	3	31	103.3	19.3–552.8	<0.0001
sFas	<6.25	23	10	1.0		
	6.25–7.50	15	18	2.8	1.0–7.6	
	>7.50	12	22	4.2	1.5–11.7	0.0025
IL-8	<30	31	2	1.0		
	30–300	12	21	27.1	5.5–133.8	
	>300	7	27	59.8	11.4–312.5	<0.0001
TNF-a	<8.0	30	3	1.0		
	8.0–60.4	13	20	15.4	3.88–61.0	
	>60.4	7	27	38.6	9.1–164.3	<0.0001

Abbreviations: OR, Odd Ratios; CI, Confidence Interval