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Increased TNF-alpha and sTNFR2 levels are associated with high-grade anal squamous intraepithelial lesions in HIV-positive patients with low CD4 level

Takeshi Haga[a,b,*], Jimmy T. Efird[c,d], Sharof Tugizov[b], Joel M. Palefsky[b]

[a] Division of Infection Control and Disease Prevention, Department of Veterinary Medical Science, University of Tokyo, Tokyo 113-8657, Japan
[b] Department of Medicine, University of California, San Francisco, CA 94143, USA
[c] Center for Health Disparities and College of Nursing, East Carolina University, Greenville, NC 27858, USA
[d] School of Medicine and Public Health, University of Newcastle, Newcastle, NSW 2308, Australia

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ABSTRACT

Compared with HIV-negative individuals, HIV-positive individuals have a higher prevalence of anogenital human papillomavirus (HPV) infection, the causative agent of anogenital cancer. TNF-alpha is a major proinflammatory cytokine. sTNFR2 is the soluble form of one of its receptors and is strongly expressed on stimulated lymphocytes. To further understand the role of TNF-alpha, sTNFR2 and other cytokines in the pathogenesis in HPV-related neoplasia, the profiles of serum cytokines in high-risk patients were analyzed for association with anal lesion status. Patients were categorized into 4 groups based on HIV status (HIV-negative vs. HIV-positive with a CD4+ level < 200/uL) and anal lesion status [no lesion, low-grade anal squamous intraepithelial lesion (LSIL) vs. high-grade squamous intraepithelial lesion (HSIL)] based on high resolution anoscopy-guided biopsy. Following adjustment for multiplicity, HIV-negative men with HSIL had lower levels of sTNFR2 than HIV-positive men with low CD4 level and HSIL (p=0.02). HIV-positive men with HSIL had higher levels of TNF-alpha than HIV-negative men with HSIL (p < 0.001), as well as HIV-positive men with no lesion or LSIL (p=0.03). The levels of other factors, including IL-1beta, IL-2, IL-4, IL-8, IFN-gamma, GM-CSF, sTNFR1 and DR5, were not significantly different between groups. Although the sample size was small, these results suggest that systemic activation of TNF-alpha/sTNFR2 in HIV-positive patients with a low CD4 level may promote the development of HSIL and possibly anal cancer.

1. Introduction

Compared with HIV-negative individuals, HIV-positive individuals have a higher prevalence of anogenital HPV infection, the causative agent of anogenital cancer. Anal intraepithelial neoplasia (AIN) 3 and p16-positive AIN 2 is the precursor to anal cancer, just as cervical HSIL is the precursor lesion to cervical cancer. Oncogenic (high-risk) HPV infection is necessary, but insufficient for development of HSIL, and additional factors are required. Immune suppression in HIV-infected individuals as measured by CD4 level plays a prominent role in the earlier stages of HPV-associated disease, up to and including incident HSIL [1,2]. Its role in progression from HSIL to cancer is less clear. Genetic changes may play an important role in progression from anal squamous intraepithelial lesions (ASIL) to invasive anal cancer. These have been observed as early as AIN 2, especially in HIV-positive patients [3] and development of cancer may result from the cumulative effect of HPV-associated genetic instability and the resulting host genetic changes [4].

Cytokines mediate important immunoregulatory functions, and changes in their relative levels play key roles in the immune response to pathogens. TNF-alpha is a major pro-inflammatory cytokine that is implicated in several infectious diseases and cancer [5]. The level of expression of TNF-alpha has been suggested to be involved in the elimination of HPV [6] or development of HPV-related cancers [7]. This cytokine binds two cell surface receptors, TNF receptor 1 (TNFR1) and receptor 2 (TNFR2). TNFR2 lacks the death domain, and is mainly expressed on activated T cells [8]. The soluble form of TNFR2 (sTNFR2) is present in body fluids. Circulating sTNRF2 is increased in association with infection, including infection with HIV and HPV, and higher levels may be associated with immune activation against the pathogen [9–11]. Increased systemic levels of GM-CSF and IL-8, both of which are proinflammatory cytokines, have been observed in older

Abbreviations: LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

* Corresponding author at: Division of Infection Control and Disease Prevention, Department of Veterinary Medical Science, University of Tokyo, Tokyo 113-8657, Japan.

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women with persistent cervical HPV infection, and have been suggested to be associated with immune defects in lymphoproliferative responses to mitogens/antigens [12]. The increase of IL-10, which is a typical Th2 type cytokine, in early cervical lesions may inhibit immune responses against HPV clearance, thereby allowing persistent infection [13]. A shift from Th1 to Th2, especially an increase in the level of IL-10, has also been reported during the development of high risk HPV-positive cervical malignancy [14]. Therefore, we sought to investigate the cytokines as possible additional factors involved in development of HPV malignancies in the patients.

Multiplex bead array assays are powerful tools for profiling soluble factors in serum, and provide a method to measure circulating levels of cytokines and other factors [15] that may play a role in pathogenesis of HSIL and anal cancer. In this study we compared the profiles of serum cytokine and other factors in HIV- negative vs. HIV-positive patients with oncogenic anal HPV infection among those with or without anal HSIL.

Thirteen cytokines including 6 proinflammatory (IL-1beta, IL-6, IL7, IL-8, GM-CSF, and TNF-alpha) and 7 immunoregulatory (IFN-gamma, IL-2, IL-4, IL-5, IL-10, IL-12, and IL-13) cytokines were measured to assess the Th1/Th2 patterns or inflammatory factors. In addition, 3 soluble forms of TNF receptor superfamily (TNFRS) [16] [sTNFR1, sTNFR2 and DR5 (death receptor 5)] were measured to assess cell death related factors. HIV/AIDS disease is associated with upregulation of proinflammatory cytokines and the increased circulation level of soluble forms of TNFRS [11,17]. Furthermore, these factors may be up-regulated in HPV-infected lesions [6,18–20]. Thus, both HIV- and HPV-associated upregulation of these factors may contribute to the progression of HPV-associated cancer [10,21,22]. Accordingly, these cytokines and factors were investigated.

As our results indicate, increased serum levels of TNF-alpha and sTNFR2 were found to be significantly associated with HSIL in HIV-positive MSM with low CD4+ levels. Therefore, it is suggested that the systemic activation of TNF-alpha/sTNFR2 in HIV-positive patients with a low CD4 level may promote the development of HSIL and possibly anal cancer.

2. Material and methods

2.1. Sample description and study approval

This study was conducted with the approval of the Committee for Human Research of the University of California, San Francisco, USA. Participants in the study were men who have sex with men (MSM) who were studied at baseline for prevalent anal HPV infection and ASIL, and followed every 6 months for incident anal HPV infection and ASIL, with the study endpoint being prevalent or incident HSIL [1,23]. At each visit, a swab was obtained for anal cytology and HPV DNA testing, followed by high resolution anoscopy (HRA) with biopsy of visible lesions. Cytology was interpreted using the Bethesda criteria, and biopsies were graded as AIN 1, AIN 2 and AIN 3 using standard histologic criteria. Although p16 positivity was not tested in this study, AIN 2 and AIN 3 were grouped together as HSIL according to the Lower Anogenital Squamous Terminology (LAST) project [24]. If no lesions were seen, no biopsies were obtained and the result was recorded as normal. The highest grade or cytology or biopsy was recorded for the purpose of data analysis.

Serum samples collected between 1995 and 1997 were selected from among those who were positive for at least one oncogenic HPV type. Samples were included from HIV-positive MSM only if their CD4+ level was < 200/uL, because patients with CD4 < 200 may be at high risk for developing HPV-related malignancies [25]. One serum sample from each patient was chosen. As indicated in Table 1, among the patients with CD4 < 200/uL, 2 patients who donated tissues were not on HAART, 8 patients were on HAART, and HAART status was unknown for 2 patients. The groups were classified as follows for analysis: Group A: HIV-negative with anal lesion status of normal or LSIL; B, HIV-negative with anal lesion status of HSIL; C, HIV-positive with anal lesion status of normal or LSIL; and D, HIV-positive with anal lesion status of HSIL.

2.2. HPV testing and typing

Approximately 10 ng DNA from each swab was used to determine the presence or absence of HPV DNA. HPV DNA was analyzed by polymerase chain reaction (PCR) using MY09/MY11 consensus HPV-L1 primers. Samples positive with the consensus probes were then probed by dot-blotting using 29 different HPV type-specific probes as described previously [26]. Primers for amplification of the human beta-globin gene were used as a positive control to evaluate the adequacy of the specimen DNA for amplification.

2.3. Cytokine analysis

Serum cytokine levels were determined using a bead array system. The High Sensitivity cytokine 13-plex antibody beads kit (HSCYTO-60SK-13; Linco, St. Charles, Mo) was used to measure IL-1beta, IL-2, IL-4, IL-8, IL-10, IFN-gamma, GM-CSF and TNF-alpha levels, according to the manufacturer's instructions. The sensitivity of the assays (minimum detectable concentrations), as reported by the manufacturer, were (cytokine, concentration): IL-1beta, 0.06 pg/ml; IL-2, 0.16 pg/ml; IL-4, 0.13 pg/ml; IL-8, 0.11 pg/ml; IL-10, 0.11 pg/ml; IFN-gamma, 0.29 pg/ml; GM-CSF, 0.46 pg/ml; TNF-alpha, 0.05 pg/ml. Serum levels of sTNFR1, sTNFR2, and DR5 were measured using the human death receptor 3-plex antibody bead kit (LHC0006; BioSource International, Camarillo, CA) according to the manufacturer's instructions. Fluorescence data were acquired using a Luminex 100 instrument (Luminex Corporation, Austin, TX). The concentrations of the cytokines and other factors, expressed in picograms per milliliter, were obtained by interpolation from a standard curve run on each plate using stepwise dilutions of protein standards included with each kit. Five-parameter logistic curve fits were performed using MasterPlex QT software (MiraiBio, Inc., Alameda, CA).

2.4. Statistical analysis

The Wilcoxon rank-sum test (also known as the Mann-Whitney U test) was used to test for statistical differences between sample distributions. P values were adjusted for multiplicity using a step-up Bonferroni procedure [27].

3. Results

3.1. Participant information

As shown in Table 1, there were 11 HIV-negative MSM with no anal lesion or LSIL (Group A), 10 HIV-negative MSM with anal HSIL (Group B), 4 HIV-positive MSM with no anal lesion or LSIL (Group C), and 9 HIV-positive MSM with anal HSIL (Group D). To control for HPV status in this study, only samples having high-risk HPV were selected. With the exception of 4 participants (B07, D01, D06, D07), all had HPV 16 DNA in their anal swab sample.

3.2. Cytokine and soluble factor analysis

Of 13 cytokines from the Linco kit, 8 cytokines passed quality control, including IL-1beta, IL-2, IL-4, IL-8, IL-10, IFN-gamma, GM-CSF and TNF-alpha. The data on the other 5 cytokines (IL-5, IL-6, IL-7, IL-12p70, IL-13) were not included in the analysis. The mean and standard deviations as well as the median of these measured factors in each group are summarized in Table 2. After the adjustment for multiplicity, HIV-positive MSM with HSIL [Group D] had significantly

Table 1
HIV status, CD4 level, anal squamous intraepithelial lesion status, and anal HPV status of participants.

Group	Subject serial number	HIV status	CD4 count	HAART status (Months after)	AIN grade		HPV type in anal swab
					Histology	Cytology	
A: HIV-negative/Normal or LSIL	A 01	HIV-			No results	Normal	16
	A 02	HIV-			No results	Normal	16
	A 03	HIV-			No results	Normal	16, 58
	A 04	HIV-			No results	Normal	16
	A 05	HIV-			No results	Normal	16, 66
	A 06	HIV-			Normal	Normal	16
	A 07	HIV-			No results	Normal	16
	A 08	HIV-			No results	Normal	16
	A 09	HIV-			No results	Normal	16, 58
	A 10	HIV-			No results	Normal	16
	A 11	HIV-			No results	Normal	16
B: HIV-negative/HSIL	B 01	HIV-			AIN 2	HSIL	16, 54, 84
	B 02	HIV-			NORMAL	HSIL	16
	B 03	HIV-			AIN 2	HSIL	16, 31, 59
	B 04	HIV-			AIN 1	HSIL	16, 31, 52, 53, 58
	B 05	HIV-			AIN 2	HSIL	16, 66
	B 06	HIV-			AIN 1	HSIL	16, 68, 69
	B 07	HIV-			AIN 2	HSIL	06, 11, 31, 66
	B 08	HIV-			AIN 3	HSIL	16, 18, 70
	B 09	HIV-			AIN 2	HSIL	16
	B 10	HIV-			AIN 2	Normal	16, 70
C: HIV-positive/Normal or LSIL	C 01	HIV+	cd4 < 200	12	AIN 1	Normal	06, 16, 33, 56
	C 02	HIV+	cd4 < 200	1	No result	Normal	16, 52
	C 03	HIV+	cd4 < 200	Before	AIN 1	Normal	06, 16
	C 04	HIV+	cd4 < 200	4	No results	Normal	16, 31, 33, 54, 58
D: HIV-positive/HSIL	D 01	HIV+	cd4 < 200	Unknown	Condyloma	HSIL	06, 39, 68
	D 02	HIV+	cd4 < 200	Before	AIN 2	HSIL	16, 54, 58, 68
	D 03	HIV+	cd4 < 200	Unknown	AIN 2	HSIL	16
	D 04	HIV+	cd4 < 200	11	AIN 1	HSIL	06, 16, 51, 52, 54, 68, 70
	D 05	HIV+	cd4 < 200	12	AIN 1	HSIL	16, 33, 53
	D 06	HIV+	cd4 < 200	12	AIN 1	HSIL	11, 32, 33, 45, 52
	D 07	HIV+	cd4 < 200	Unknown	AIN 2	HSIL	11, 31, 45, 53, 54, 68
	D 08	HIV+	cd4 < 200	12	Condyloma	HSIL	06, 16, 52
	D 09	HIV+	cd4 < 200	39	AIN 1	HSIL	16, 18, 52, 54, 84

LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion.

Table 2
Serum levels of cytokines and soluble factors in each group.

Serum factor	(pg/ml)	A: HIV-/Normal or LSIL	B: HIV-/HSIL	C: HIV+/Normal or LSIL	D: HIV+/HSIL
IL-1beta	**mean ± SD**	1.46 ± 1.40	5.68 ± 9.48	1.62 ± 2.53	5.51 ± 12.8
	median	0.7	2.05	0.58	0.74
IL-2	**mean ± SD**	6.09 ± 7.70	14.4 ± 25.3	3.48 ± 4.74	19.2 ± 53.4
	median	2.38	5.06	1.72	1.39
IL-4	**mean ± SD**	67.6 ± 92.4	159 ± 338	2.53 ± 2.98	155 ± 307
	median	17.6	7.59	2.19	1.8
IL-8	**mean ± SD**	11.0 ± 5.5	23.4 ± 35.1	6.50 ± 1.43	20.5 ± 22.2
	median	8.9	8.67	6.16	9.73
IL-10	**mean ± SD**	5.99 ± 4.09	10.4 ± 16.8	4.68 ± 3.72	12.9 ± 8.08
	median	5.62	4.9	3.31	11.1
IFN-gamma	**mean ± SD**	6.07 ± 14.9	16.4 ± 31.9	2.26 ± 3.29	3.64 ± 6.55
	median	0.18	4.6	1.03	0.01
GM-CSF	**mean ± SD**	6.61 ± 14.3	7.62 ± 10.1	1.05 ± 0.89	13.1 ± 34.6
	median	2.2	4	0.94	0.8
TNF-alpha	**mean ± SD**	5.95 ± 2.91	5.07 ± 1.69	7.83 ± 0.35	19.8 ± 26.0
	median	5.22	5.1	7.85	11.5
sTNFR1	**mean ± SD**	2.39 ± 1.91	3.01 ± 1.68	1.95 ± 0.40	3.44 ± 1.39
	median	2.02	2.5	1.88	2.98
sTNFR2	**mean ± SD**	1.24 ± 0.67	1.41 ± 0.66	2.18 ± 0.65	3.22 ± 1.10
	median	1.44	1.34	2.32	3.36
DR5	**mean ± SD**	0.95 ± 1.24	0.83 ± 0.48	0.69 ± 0.11	0.97 ± 0.53
	median	0.64	0.74	0.71	0.82

Average ± SD and median is shown for each group.

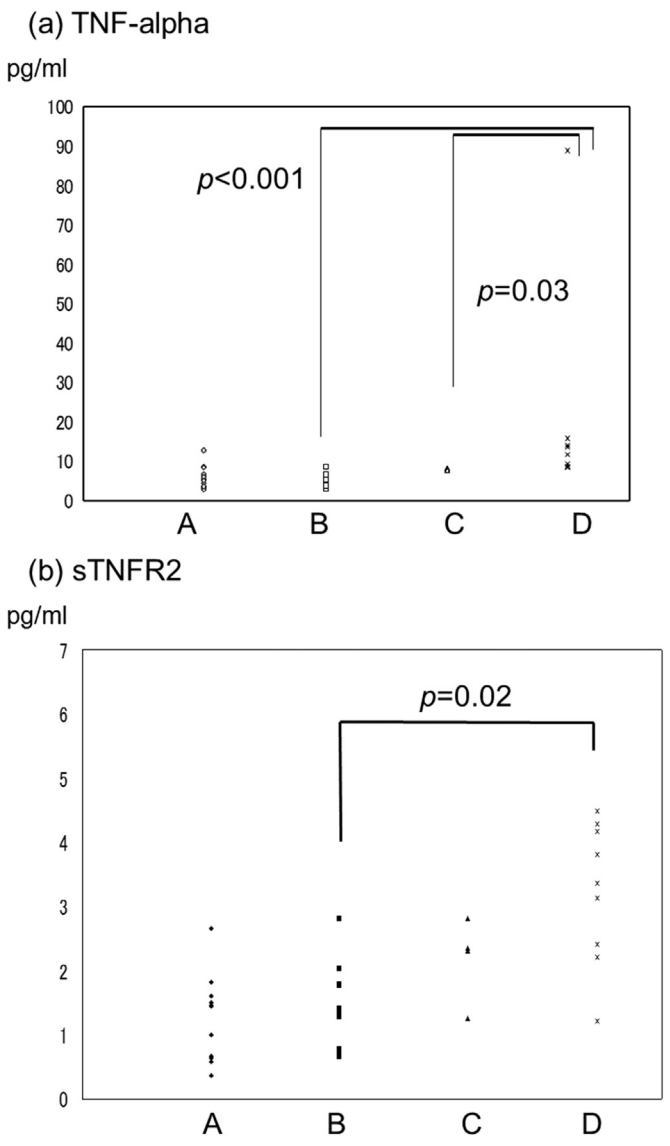

Fig. 1. : Activation of the TNF-alpha/sTNFR2 system with HSIL in HIV-positive patients. a) Serum level for TNF-alpha (Fig. 1a), and for sTNFR2 (Fig. 1b). Each dot represents a single subject. Patients were classified into 4 groups: A, HIV-negative/Normal or LSIL; B, HIV-negative /HSIL; C, HIV-positive/Normal or LSIL; and D, HIV-positive/HSIL. Serum values were determined using a multiplex beads array system as described in the Materials and Methods section. Individual values for factors whose differences were statistically significant between groups are presented. ** between solid lines represents statistical significance of $p < 0.05$ using a multiplicity adjusted p-value.

higher levels of sTNFR2 than HIV-negative men with HSIL [Group B] (p=0.02) (Fig. 1). HIV-positive men with HSIL [Group D] had significantly higher levels of TNF-alpha than HIV-negative men with HSIL [Group B] ($p < 0.001$), as well as HIV-positive men with no lesion or LSIL [Group C] (p=0.03). sTNFR2 levels in the normal or LSIL group tended to be higher in the HIV-infected group [Group C] than in the HIV-uninfected group [Group A], but the difference was not significant after the adjustment (unadjusted p=0.078, adjusted $p > 0.1$). However, the p-value for the univariable result was less than 0.1.

Other pertinent findings are worth noting. IL-8 levels in HIV-infected patients tended to be higher in the HSIL group [Group D] than in the normal or LSIL group [Group C] (unadjusted p=0.006, adjusted p=0.056), and IL-8 levels in the normal or LSIL patients [Group C] were lower in the HIV-infected group than in the HIV-uninfected group [Group A] (unadjusted p=0.056, adjusted $p > 0.1$). IL-10 levels in the HIV-infected patients were higher in the HISL group [Group D] than in the normal or LSIL group [Group C] (unadjusted p=0.034, adjusted $p > 0.1$), and IL-10 levels in the HISL patients were higher in the HIV-infected group [Group D] than in the HIV-uninfected group [Group B] (unadjusted p=0.068, adjusted $p > 0.1$). GM-CSF levels in HISL patients were higher in the HIV-infected group [Group D] than in the HIV-uninfected group [Group B] (unadjusted p=0.051, adjusted $p > 0.1$).

The levels of other factors, including IL-1beta, IL-2, IFN-gamma, sTNFR1 and DR5, were not significantly different between groups.

Some of the HIV-positive MSM in our study were on HAART at the time of serum collection. In our results, there was not a clear difference of TNF-alpha/sTNFR2 levels in patients with HAART.

4. Discussion

To our knowledge, this is the first report on the relationship between serum cytokines and soluble factors and anal lesion status among HIV-negative and HIV-positive men known to have high risk anal HPV infection.

The most striking results of this study were the differences in TNF-alpha and sTNFR2 levels between the different groups. After adjustment for multiplicity, the serum level of TNF-alpha was significantly higher in HIV-positive patients regardless of anal lesion status. The serum levels of sTNFR2 were also higher in the HIV-positive group with HSIL compared HIV- negative MSM with HSIL.

TNF-alpha is a pro-inflammatory cytokine with pluripotent physiological functions. TNF-alpha plays an important role in the progression of HIV infection to AIDS [28]. The high levels of circulating TNF-alpha likely reflects systemic HIV infection rather than local HPV infection of epithelial cells since we selected MSM with CD4 less than 200 for this analysis. Our data suggest that TNF-alpha may be playing a role in ASIL pathogenesis since these levels were higher among HIV-positive men with anal HSIL than HIV-positive men with no lesion or LSIL and we did not find differences between these groups for other inflammatory cytokines known to be increased in advanced HIV infection.

The level of TNF receptors may also be important in HPV-associated disease pathogenesis. Serum levels of sTNFR1 and sTNFR2 have been shown to be significantly increased in patients with HPV16- or −18-associated cervical carcinomas and perianal Bowen's disease [9]. The authors concluded that these soluble receptors may facilitate the growth of lesions. sTNFRs bind TNF-alpha with high affinity and may act either as inhibitors of TNF-alpha or as carriers of this cytokine, and may prolong its biological effect [29].

sTNFR2 is also described as a prognostic marker for AIDS outcome during the course of HIV infection. Increased serum levels of sTNFR2 have been reported in patients with HIV infection [30] and has been reported to correlate with HIV RNA copy number in HIV-positive patients [31]. It has been suggested that the TNF-alpha/TNFR system is turned on before and during HIV infection, raising the possibility that sTNFR2 may be important in HIV pathogenesis. Thus, while the increased levels of sTNFR2 in the circulating blood of HIV-positive patients may reflect the degree of activation of the TNF system it is also possible that the increased sTNFR2 observed in this study may also facilitate the growth of lesions in HIV-positive patients.

TNF-alpha may have different effects depending on stage of infection or disease induced by HIV and HPV. It may protect against development of disease by inducing cellular immunity and contributing to elimination of viruses, but once viral infection is established it may potentiate disease development and may be a tumor promoter [10,32]. Clinically, chronic, low-level TNF-alpha exposure is reported to be linked with the acquisition of a pro-malignant phenotype such as increased growth, invasion and metastasis [5]. TNF-alpha is also involved in potentiates epithelial–mesenchymal transition, which is associated with tumor invasion and angiogenesiss [33].

TNF-alpha inhibits the growth of normal cervical keratinocytes but stimulates the proliferation of HPV-immortalized and cervical carcinoma-derived cell lines when mitogens such as epidermal growth

factor (EGF) or serum are depleted [34]. Activation of the TNF-alpha system may provide a selective growth advantage for HPV-immortalized epithelia, possibly through transactivation of the epidermal growth factor receptor (EGFR) [35], which is also up-regulated in anal carcinoma [36],

TNF-alpha promotes HPV E6/E7 RNA expression [37] and cyclin-dependent kinase activity in HPV-immortalized keratinocytes by a ras-dependent pathway [34]. Inhibition of TNF-alpha/cycloheximide induced apoptosis in human laryngeal cancer cells by the HPV 16 E6 and E7 oncoproteins has been reported [38]. This inhibition may allow the accumulation of chromosomal abnormalities [3], resulting in a malignant outcome in ASIL and anal cancer. It is suggested that the acquisition of TNF resistance may constitute an important step in HPV-mediated carcinogenesis [10]. The role of TNF is indicated as 'double-edged swords' [16], because TNF was originally found to be cytotoxic to tumor cells, but once TNF resistant cells appear, TNF may function as tumor premotor.

Our study has several limitations. The sample size is small, and further study is required to confirm our results. Other risk factors such as smoking may influence the interpretation of results. Circulating levels of cytokines and soluble factors were measured in serum. Consequently, the microenvironment in anal lesions may not be well reflected by the serum level of these soluble factors, and further analysis of local immunity in anal lesions is needed. The effect of HAART-induced HIV suppression on circulating markers of inflammation and immune activation was recently reported. Although several markers of T-cell activation in the patients with HAART-induced HIV suppression returned to normal levels, the markers for residual immune activation, particularly monocyte/macrophage activation, including TNF-alpha and sTNFR2, remained increased after HIV suppression [39]. Given the increased importance of HPV as the cause of non-AIDS-related cancer among HIV-positive individuals [40], further study is required to elucidate the effect of HAART on HPV oncogenesis.

5. Conclusion

Activation of the TNF-alpha/sTNFR2 system was found to be significantly associated with HSIL in HIV-positive MSM with low CD4+ levels. These results suggest that systemic activation of TNF-alpha/sTNFR2 may play a role in the pathogenesis of anal cancer. A possible scenario of the activation of TNF-alpha/sTNFR2 in HPV-related carcinogenesis is as follows: 1) TNF-alpha levels are increased in HIV-positive patients. 2) This may lead to up-regulation of HPV E6/E7 expression in HPV-infected cells. 3) Up-regulation of E6 and E7 may lead to genetic instability of HPV-infected cells. 4) Increased mutation may allow the appearance of TNF-resistant HPV-infected cells, which acquire the ability of increased growth, invasion and metastasis. The increased sTNFR2 observed in this study may facilitate the growth of lesions in HIV-positive patients by prolonging biological effect of TNF-alpha, while it may just reflect the degree of TNF activation.

Given the increased importance of HPV-related malignancy especially in HIV-positive patients in HAART era, further studies are necessary to elucidate the pathophysisological significance of increased sTNFR2 and TNF-alpha levels in HSIL and/or HIV-positive patients. These may further help in the development of improved prognostic markers and better therapeutic applications.

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