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The Role of Macrophages in Angiogenesis. Comparison Between HIV + and HIV – Populations with Anal Dysplasia and Anal Cancer

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Abstract. *Background: While macrophages (CD68+) have been associated with angiogenesis in some inflammatory and neoplastic processes by increasing the release of vascular endothelial growth factor (VEGF), their role in anal intraepithelial neoplasia (AIN) and anal squamous cell carcinoma has not been established. This study records macrophage infiltration in anal pre-invasive and invasive lesions in HIV+ and HIV– populations, and determines their relationship with angiogenesis. Materials and Methods: Sixty patients (31 HIV+) with AIN and anal SCC were studied. Paraffin sections were stained for CD68, VEGF and von Willebrand factor. The density of CD68 cells, the expression of VEGF and angiogenesis were quantified, and compared amongst groups and between HIV+ and HIV– populations. Results: All three parameters increased linearly as the lesions became more dysplastic, in HIV+ and HIV– groups. The CD68 count was statistically lower in HIV+ ($p<0.005$) compared with HIV– groups, while the differences in VEGF expression and in angiogenesis were not significant between HIV+ and HIV– populations. Conclusion: There was a significant decrease of macrophage infiltrate in the HIV+ group. The relative increase in VEGF expression and angiogenesis in the face of lower macrophage infiltration in HIV+ patients may be explained either by a greater release of angiogenic factors by macrophages, or by VEGF expression not being solely dependent on macrophage activation.*

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The incidence of anal warts and the progression to anal cancer has increased from less than 1/100,000 to over 30/100,000 since the AIDS outbreak in the late 1980's (1-3). The incidence is now particularly high in homosexual males(4), while before the AIDS era, the disease was more common in females(5). Anal cancer can arise from dysplastic warts (anal intraepithelial neoplasia – AIN) originally caused by the human papillomavirus (HPV), particularly the HPV 16 and 18 carcinogenic serotypes (6, 7).

HIV+ populations, partly due to their inherent immunosuppression but also because of their sexual lifestyle, have an increased risk of developing anal warts and of these progressing to dysplasia and cancer (8). The reason for this increased risk is still unclear, but seems to be related to the host's inability to clear the HPV infection.

Macrophages are involved in the development of malignant lesions at several levels: protease release (9), extracellular matrix digestion (10), coagulation and angiogenesis (11). An increase in macrophage infiltration associated with angiogenesis has been reported in a number of inflammatory processes (12-15). Macrophages are known to release angiogenic cytokines, including TNFα, IL-1β and IL-6 (16), a process enhanced by the protein E7 of carcinogenic HPV subtypes. The release of tissue factor by monocytes and macrophages (17) is of particular interest in the pro-angiogenic activity of these cells, as tissue factor increases the production of VEGF by 1000-fold (18, 19). Two studies have shown an increase in angiogenesis in AIN (20, 21), but the involvement of macrophages in HPV-related pre-invasive lesions has not yet been fully determined.

Materials and Methods

Materials. Sixty patients (31 HIV+) were included in the study. Following revision of the slides stained with hematoxylin and eosin (FD), each case was graded as: warts (n=13), LG-AIN (12), HG-AIN (22) and anal SCC (13). Six specimens of normal anal skin

Table I. *List of antibodies used for immunohistochemistry staining, at their optimal dilutions and incubation times used. The control column refers to the tissue used as positive control in each staining.*

Antibody	Source	Clone	Ag enhancement	Dilution	Incb'n time	Control
vWF	DAKO	A0082	Press. cooker (citrate)	1/1000	60'	Placenta
CD68	DAKO	MO876	Press. cooker (citrate)	1/50	60'	Tonsil
VEGF-A	R&D	26503	Protease digestion	1/100	45'	Placenta

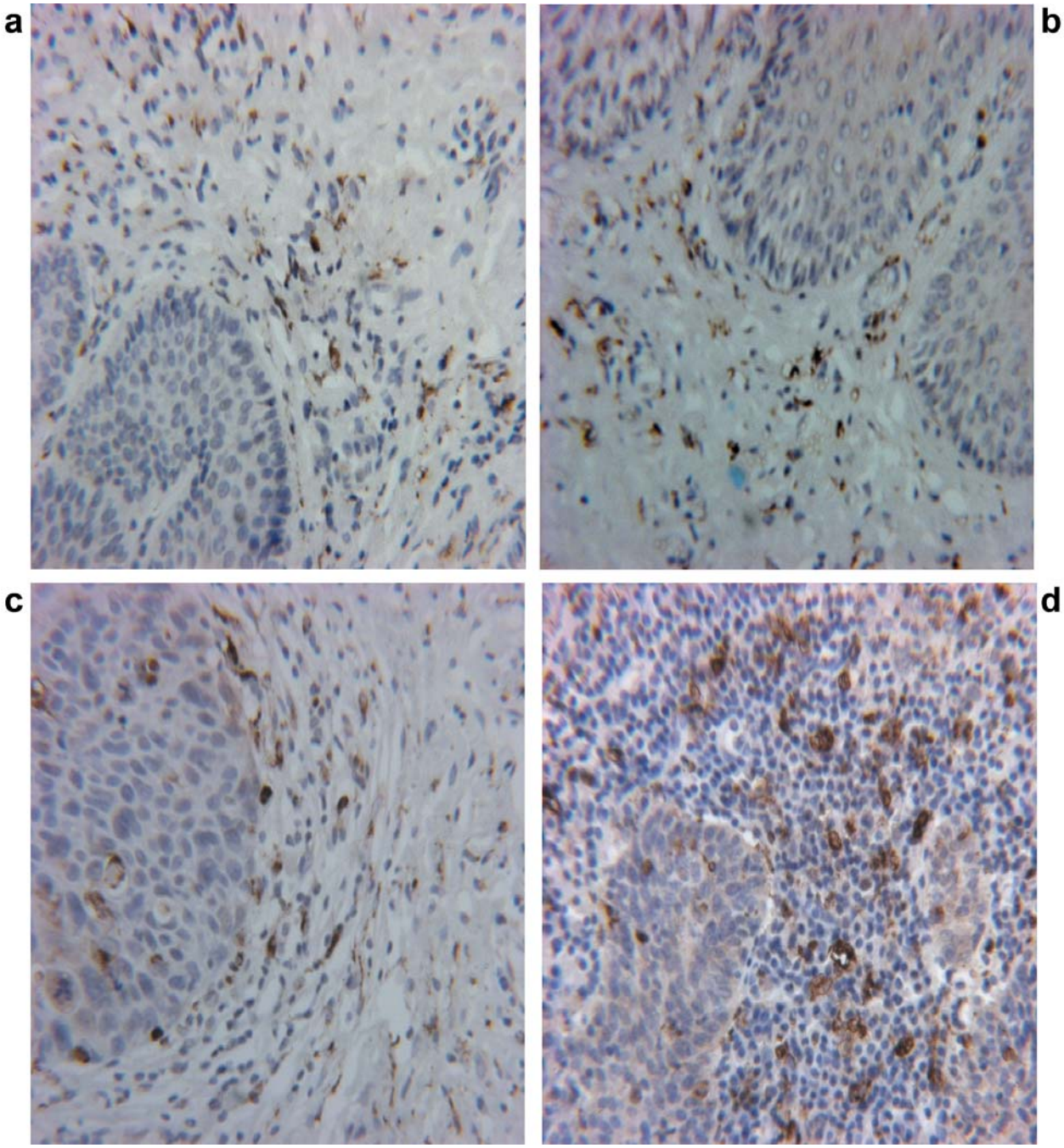

Figure 1. *Photomicrographs of macrophage infiltration in wart (1a), LG AIN (1b), HG AIN (1c) and anal SCC (1d). Original magnification x250.*

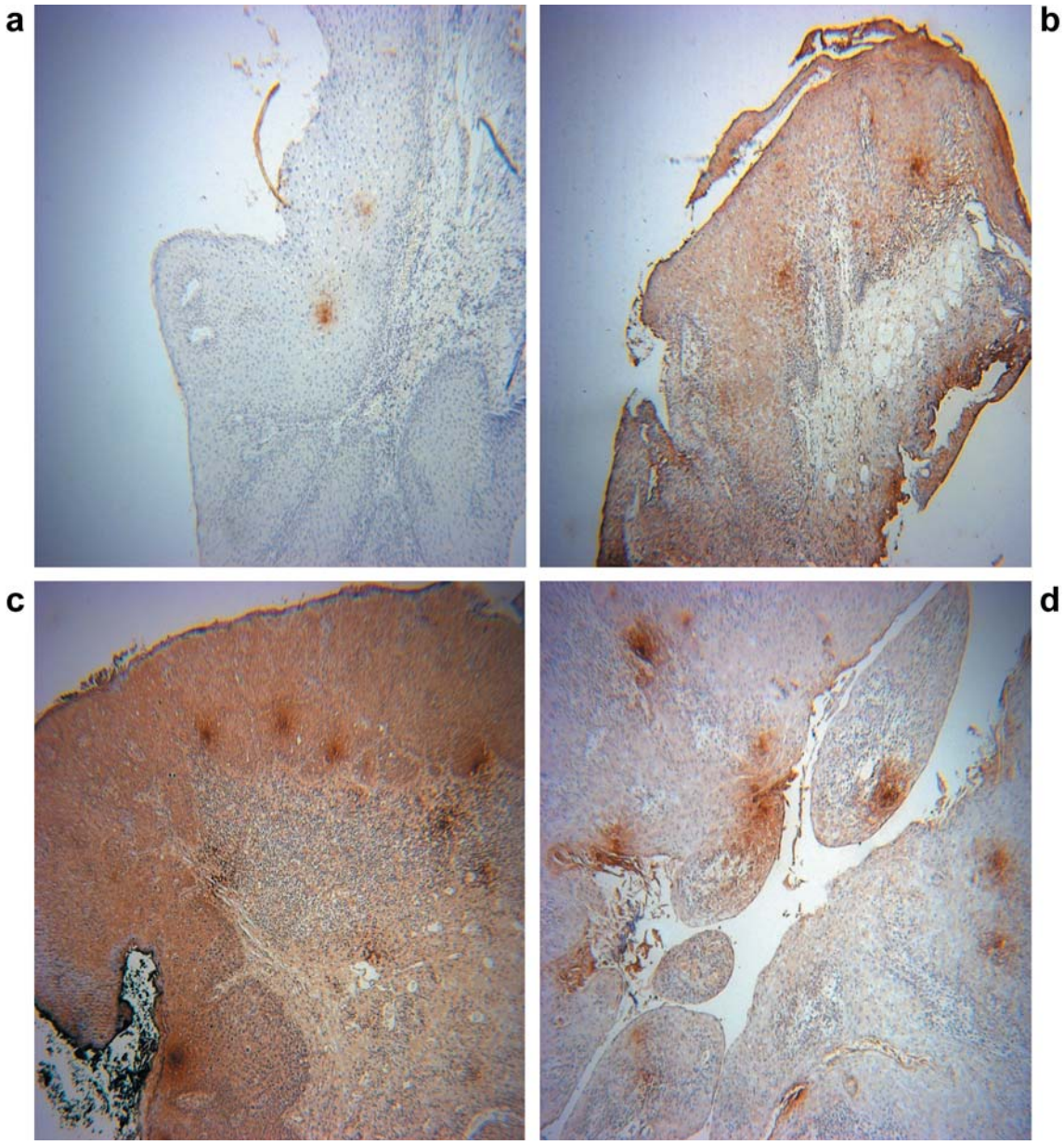

Figure 2. *Photomicrographs of VEGF expression in wart (2a), LG AIN (2b), HG AIN (2c) and anal SCC (2d). Original magnification x40*

were used as controls. We subdivided the dysplastic lesions into low-grade (LG) AIN (AIN I) and high-grade (HG) AIN (AIN II and AIN III) according to the Bethesda system (22).

Immunohistochemistry. Consecutive 3 mcm sections were cut from the original paraffin-embedded surgical blocks and stained with CD68 (DAKO, Ely, Cambridgeshire, UK), anti-VEGF-A (R&D Systems Europe Ltd., Oxon, UK) and anti-vWF (DAKO) to evaluate the macrophage infiltration, angiogenic cytokine expression and angiogenesis, respectively. The staining method has been described in detail elsewhere (21) but, briefly, the sections were deparaffinised using xylene and alcohol, the endogenous peroxidase was blocked and the antigen was made available. The primary antibody was applied as described in Table I. The slides were subsequently stained with a standard dilution of DAB as a chromagen, followed by counterstaining solutions. Placenta and tonsil tissues were used as positive controls and Tris-buffered saline (TBS) was used instead of the primary antibody to serve as a negative control.

Assessment of staining patterns. All stained sections were reviewed by two of the authors (JM and FD). In all cases there was <5% variation in quantification between observers. The density of the infiltrate was assessed using a computerised image acquisition and analysis system (Axiovision 3.0, Carl Zeiss Vision GmbH, Munich, Germany).

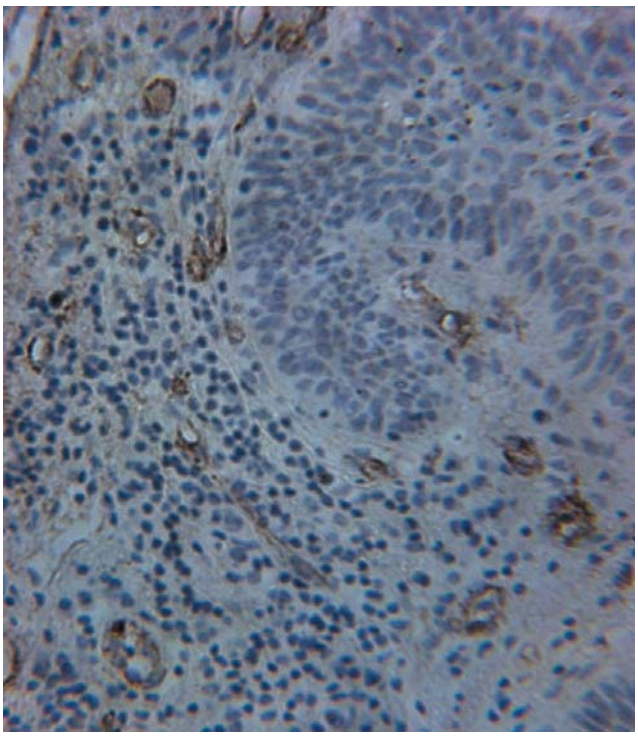

Figure 3. *Photomicrograph of microvessels stained with vWF. Original magnification x250.*

Macrophages. Macrophage infiltrate was identified as CD68 positively-stained cells in the stroma (Figure 1). A manual count of stained cells was performed on no less than 4 representative areas of stroma within 200mcm of the basal membrane at x250 magnification. In invasive lesions, an area within 200mcm of the original epithelium was selected for assessment.

VEGF-A. VEGF was quantified by manual counting of 'focal areas' (areas where VEGF-A was expressed). These were mainly found in the squamous cell epithelium of the lesions (Figure 2). A consecutive section of tissue was stained with mouse IgG_{2b} isotype control (R&D Systems), a specific antibody designed as a negative control for VEGF-A, using the same procedure and compared to minimise false-positive scoring. This method of VEGF expression has been described in a previous work (23).

Angiogenesis. A microvessel was defined as a group of endothelial cells positively-stained with vWF, smaller than 8mcm in diameter, with or without lumen. The angiogenesis assessment was carried out following Weidner's microvessel density (MVD) counting method (24). Briefly, Weidner's method consists of selecting stromal areas with a high number of microvessels per optical field ('hot-spots'), and selecting 3-5 hot-spots at x100 magnification in the stroma of the lesions, within 200mcm of the basal membrane (Figure 1a,b). Within these fields, the number of microvessels is then counted at x250 magnification (Figure 3).

Statistics. The data was analysed using GraphPad Prism 3.00 software. The Kruskal-Wallis test was used to analyse the results in each group, a non-parametric correlation test (Spearman) was used to compare groups of 2 parameters, and a non-parametric test (ANOVA) was used to compare the difference in distribution between multiple groups.

Results

Macrophages. All samples from both HIV+ and HIV– groups showed a degree of macrophage infiltrate compared to normal anal skin (Figure 4). There was a progressive increase in macrophage infiltrate in the stroma as the lesions progressed to AIN and anal SCC. In the HIV– group, the increase was statistically significant in HG-AIN (17.32±1.40, mean±SE) ($p<0.01$) and anal SCC (23.27±2.89) ($p<0.001$), both compared with anal skin (6.70±1.17). In the HIV+ group, the density of macrophage infiltration again increased as the lesions progressed from anal warts (1.23±0.54) to LG-AIN (4.52±0.89), yet, in these samples the increase was not statistically significant. In HG-AIN the number of macrophages was significantly greater (7.52±1.58) ($p<0.01$). When LG-AIN and HG-AIN samples from HIV+ and HIV– groups were compared, the macrophage infiltrate was much higher in the HIV– samples ($p<0.0001$) in both cases.

VEGF expression. The VEGF expression was similar in HIV+ and HIV– groups (Figure 5). In both groups there was a gradual increase in the VEGF expression as lesions progressed to HG-AIN. In anal SCC lesions from HIV– subjects, no further increase in VEGF expression was seen over that recorded in the HG-AIN samples. The increases over normal skin (0.21±0.10, mean±STE) in all cases were significant: in samples of warts (1.95±0.70) ($p<0.001$); in samples of LG-AIN (3.25±1.25) ($p<0.001$); in samples of HG-AIN (7.58±1.16) ($p<0.001$); in samples of anal SCC (7.65±1.99) ($p<0.001$) (see Methods for statistical analysis). In lesions from all groups, the expression of VEGF was mostly localised in the epithelium, both in samples derived from HIV+ and HIV – subjects.

Angiogenesis. Angiogenesis occurred in the stroma, in a close relationship with the macrophage infiltrate (Figure 6). In neither samples from HIV+ or HIV – individuals did the presence of warts (10.90±1.27 and 5.25±0.96, HIV – and HIV+, respectively) or LG-AIN (11.20±1.74 and 10±2.12, HIV – and HIV+, respectively) cause any significant increase in the level of angiogenesis over that recorded in normal skin (10.16±1.51) (Figure 6). When HG-AIN biopsies were studied from both HIV – (18.91±1.24)) and HIV+ (21.58±2.21), a significant increase in the level of angiogenesis was observed over the data recorded in the LG-AIN samples ($p<0.01$, in both cases). In samples from anal SCC derived from HIV – subjects, a further increase in angiogenesis was recorded (27±1.98). However, this increase was not statistically significant (Figure 6).

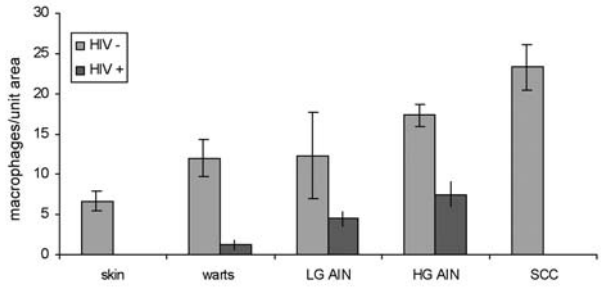

Figure 4. *Graph showing the macrophage infiltrate in HIV – and HIV + groups. Columns show the mean macrophage infiltration per unit area, with standard error values.*

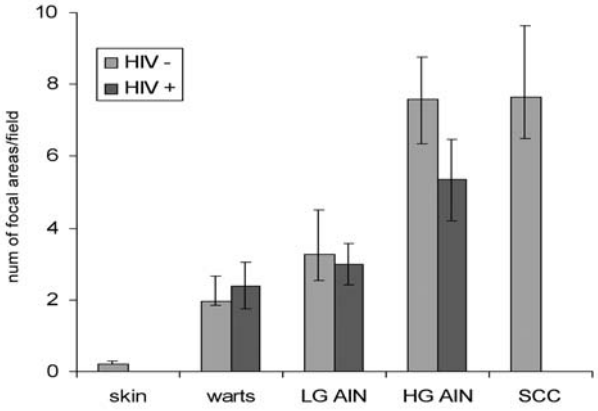

Figure 5. *Graph showing VEGF expression in HIV – and HIV + groups. Columns show the mean number of focal areas, with standard error values.*

Correlations. Data was analysed to determine whether a relationship existed between the level of macrophage infiltration and the expression of VEGF (Spearman r=0.54), and between the macrophage infiltration and angiogenesis (Spearman r=0.53). In both cases, a significant correlation was observed. When these relationships were investigated in the HIV+ and HIV– separately, no relationship could be detected in the data obtained from the HIV+ subjects, however, when the samples from the HIV– individuals were analysed, the correlations observed within the whole group held ($p<0.0001$).

Discussion

Angiogenesis is a vital process in the progression of malignant disease (25). Several cells and molecules present in inflammatory and neoplastic processes have been linked with angiogenesis (26). Amongst these, there is much evidence that macrophages may play a key role both in cancer progression and angiogenesis. Different studies have reported that macrophage release of thymidine phosphorylase factor (9, 27), tissue factor synthesis (28) and the production of VEGF (29) may all be linked to the promotion of angiogenesis in the context of neoplasia. To extend this body of knowledge, the current study shows, for the first time in cases of anal neoplasia, a highly significant relationship existing between the development of macrophage infiltration, the release of VEGF and an increase in angiogenesis. As with previous studies of cervical neoplasia, by examining a selection of resection specimens from cases of anal warts through to anal SCC, it has been possible to investigate these parameters in groups of subjects recognised as representing progressive severity within one disease process. Such investigation has allowed us to confirm that there is an increase in macrophage infiltration related to an increased severity of the lesions. This observation is true in both HIV– and HIV+ subjects. Most interestingly, the level of macrophage infiltration was consistently lower in

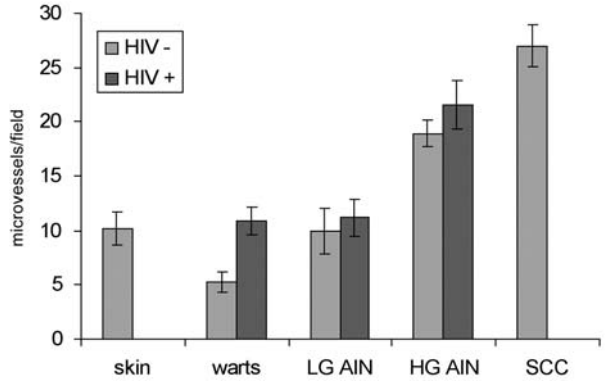

Figure 6. *Graph showing the microvessel density in HIV – and HIV + groups. Columns show the average microvessel density for each group, with standard error values.*

resection specimens from the HIV+ subjects compared to the HIV–. This may relate to the severe compromise of macrophage function in HIV+ subjects as a consequence of infection of these cells with the virus (30). Further, as the ability of HIV+ subjects to mount an effective T cell response is obviously compromised, the reduced level of macrophage infiltration may be a consequence of reduced T cell reactivity to the HPV-related changes of the local tissue. This could be parallel to the reduced release of IFNγ in HIV+ patients (31). IFNγ normally plays a leading role in macrophage activation (32).

Increases in the local expression of VEGF also seemed to occur consistently with the severity of the lesions from which the biopsies were derived, and this increase is shown to correlate with the level of macrophage infiltration. However, any conclusions drawn from this relationship should be made

with caution, as the correlations between macrophage numbers and VEGF, while present for the total number of specimens studied, failed to show statistical significance when only the HIV+ specimens were investigated. These, of course, were those with the lower levels of macrophage infiltration (see above). Such an observation would suggest that there may be other factors contributing to the levels of VEGF expression within the tissue, not observed in this study. It has already been shown that other cells and molecules (33-35) may have an effect on VEGF secretion. Such regulation would also be consistant with the observation that, while levels of macrophage infiltration continue to increase between the HG-AIN and anal SCC samples, no increase in the expression of VEGF between these two samples was observed within the HIV – group.

There is no doubt, however, that relationships exist between the levels of VEGF and MVD since increased angiogenesis was observed throughout the spectrum of HPV lesions, particularly in the HG-AIN and anal SCC samples. This, of course, is not a surprising finding, as such relationships have been reported previously in a variety of situations, both *in vitro* and *in vivo* (36).

Overall, however, the results of this study lead to the conclusion that the increase in numbers of macrophages in the subjacent stroma of these lesions may contribute to the development of malignant disease by the promotion of increased angiogenesis. This, to our knowledge, is the first time data supporting such a relationship to be presented.

Conversely, the fact that reduced levels of macrophage infiltration occur in samples from HIV+ subjects, while this patient group are well known to show an increased incidence of progression to AIN and anal SCC(8), could argue in favour of a positive role for the macrophage population within the context of an immune response against the developing dysplasia and beyond. Further work is required to determine the full functional capacity of macrophages within this situation.

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