Immunological and Viral Factors Associated with the Response of Vulval Intraepithelial Neoplasia to Photodynamic Therapy

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ABSTRACT

Topical 5-aminolevulinic acid-based photodynamic therapy (PDT) has produced complete response rates of >90% for nonmelanoma skin carcinomas, which are mostly human papillomavirus (HPV) negative. Using a similar treatment protocol, we observed a short-term response in only one third (10 of 32) of high-grade vulval intraepithelial neoplasia (VIN 2–3) lesions. Unifocal lesions were found more responsive than multifocal and pigmented lesions. Animal model studies have suggested that long-term PDT response involves an immune reaction in which CTLs play a crucial role. In this study, we have assessed: (a) HPV infection; (b) HLA expression; and (c) immune infiltrating cells in VIN biopsies from responders and nonresponders to determine whether these factors may limit response to topical 5-aminolevulinic acid-based PDT. Tissues from normal vulva (n = 9), vulval carcinoma (n = 11), and VIN (32 patients from which 19 pre- and 43 post-PDT biopsies were taken) were investigated for immunohistostaining and HPV infection by immunohistochemistry and HPV infection by PCR. There was a greater likelihood of HPV positivity associated with a lack of response of VIN to PDT (P = 0.002), and VIN nonresponders were more likely to show HLA class I loss compared with responders (P = 0.030). HLA class I down-regulation was significantly greater in the carcinomas (82%, total loss) than the VIN (28%, 19%, total loss; and 9%, allele loss; P = 0.004). None of the cases with class I down-regulation responded to PDT, whereas 3 of 6 (50%) of cases that showed total class I loss subsequently developed superficial invasion. Compared with normal vulval skin, VIN lesions showed increased infiltration by CD4 (T-helper) and CD68 (macrophages) but not CD1a (Langerhans cells) or CD8 (CTLs). There was, however, a significant increase of CD8 infiltration in posttreatment VIN responders compared with nonresponders (P = 0.0001). These data clearly support the contention that high-risk HPV infection and lack of cell-mediated immunity may play a role in the observed poor response of lower genital lesions to topical PDT.

INTRODUCTION

The detection rate of VIN1 has doubled over the last 20 years, and the multifocal presentation, together with an uncertain risk of progression to malignant disease, makes it a difficult disease to treat, with a high rate of recurrence (1–3). Conventional treatment often involves repeated local excision or laser ablation, which can be painful and mutilating. Thus it is clear that new forms of treatment are required for the management of VIN, and a simple means of identifying those women most at risk of progression to vulval carcinoma would also be of great benefit.

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4 The abbreviations used are: VIN, vulval intraepithelial neoplasia; ALA, 5-aminolevulinic acid; PDT, photodynamic therapy; HPV, human papillomavirus; CD1a, Langerhans cells; CD4, helper T cells; CD8, cytotoxic T cells; CD68, macrophage cells; LP34, anti-cytokeratin.

PDT, a promising new treatment for many cancers (4), uses the interaction between a tumor-localizing photosensitizer and light of an appropriate wavelength to bring about molecular oxygen-induced cell death (5). ALA-based PDT is particularly attractive because this drug is activated by conversion to protoporphyrin IX in rapidly growing cells thus reducing incidental damage to surrounding normal tissues (6). PDT-mediated tumor destruction involves not only direct tumor cell killing and damage to the vascular stroma but also immune mechanisms (7). The latter result from induction of local inflammation, recruitment of immune effectors (8, 9), and the release of a plethora of immunoregulators (10–12). Indeed, there is evidence from various animal models that tumor-associated macrophages (13, 14) and CTLs (5, 8, 15–17) have important roles in the long-term control of disease after ALA-based PDT.

Two previous studies (18, 19) have shown that topical ALA-PDT treatment of multifocal high-grade VIN produced a short-term response in only 37 and 27% of these lesions. It was also reported that lower grades (VIN 1) and monofocal and bifocal high grades (VIN 2–3) are much more responsive to topical ALA-PDT than multifocal VIN 2–3 (19). The reasons for this poor response are not clear, especially when compared with >90% complete cures obtained with ALA-PDT treatment of Bowen disease and other nonmelanoma skin lesions (20).

The role of high-risk HPV infection in the etiology of cervical and warty/basaloid types of vulval neoplasia is well established (21), whereas the vast majority of Bowen skin carcinomas are HPV negative (22). Cellular immunity is important in the control of high-risk HPV-associated lower genital tract neoplasia because immune suppression increases the prevalence of these lesions (23). A possible immune escape mechanism, present in cervical neoplasia, is indicated by the frequency of HLA class I down-regulation in progressive genital lesions (24).

The present study correlates the incidence of high-risk HPV infection, HLA class I loss, and numbers of infiltrating immune cells with the clinical response of VIN patients treated with topical ALA-based PDT.

MATERIALS AND METHODS

Patients. Cases of VIN enrolled in this study were recruited from the colposcopy clinic at St. Mary’s Hospital in Manchester (The study had ethical approval from Central Manchester Ethics Committee). Only high-grade VIN 2–3 lesions were selected for this study, and in all of the cases the condition was diagnosed after biopsy. To standardize the diagnosis, all of the biopsies were examined by the same consultant pathologist before and after therapy. Lesions were categorized as either unifocal or multifocal according to their number. No exclusion criteria apart from pregnancy were adopted for selection of cases for this study. Three women were under systemic immunosuppressive therapy, two for renal failure and one for Crohn disease. Another 3 women used topical steroids to alleviate their pruritus vulvae, and the remaining 26 women had no concurrent diseases and received no regular medication. The median age and SD of women with VIN was 37.5 ± 11 (range 18–70 years). All of the patients except one were white Caucasians.

Photodynamic Treatment. The light source used in this study was a nonlaser lamp (Paterson Lamp). Red light at 630 nm was used, and the dose of light was escalated from 50 J/cm² for the first 10 patients to 100 J/cm² for all...
of the other patients. Two grams of 20% ALA cream (w/v; Sigma Aldrich Company Ltd., Gillingham, Dorset, United Kingdom) were applied topically to the affected area. The cream was kept in place by Tegaderm adhesive plaster for 5 h. Premedication with local anesthetics was necessary in all of the cases to reduce pain during and after the application of light. At 12 weeks after PDT, all of the patients were evaluated both clinically and histopathologically. The case was considered to be responding to treatment if both the clinical and the histopathological reports declared the absence of abnormal cells.

**Clinical Material.** Biopsies were obtained before and after PDT from high-grade VIN in women that participated in the above-described clinical trial. Posttreatment VIN biopsies were taken at 3 months after PDT, and the response to treatment was measured by restoration of normal tissue histology in the biopsy area. Biopsies from vulval carcinomas and associated normal skin were also available from frozen archival material at Saint Mary’s Hospital tumor bank. All of the biopsies were snap frozen and stored in liquid nitrogen before being processed for immunohistochemistry and PCR.

**Immunohistochemistry.** Cryostat sections on coated slides were studied by an indirect immunoperoxidase method that used DAKO EnVision+ System, Peroxidase (diaminobenzidine) Code No. K4006 (Dako Ltd., Cambridge, United Kingdom) according to the manufacturer’s guidelines. Sections were allowed to come to room temperature before being fixed in acetone, air dried, and rehydrated with PBS. Blocking for endogenous peroxidase and nonspecific proteins was performed using Dako peroxidase block and 10% casein, respectively. Incubation with primary mouse monoclonal antibodies or isotype-matched irrelevant monoclonal antibody as a negative control was followed by secondary goat antimouse antibody for 30 min. Each incubation was followed by three washes in PBS. Diaminobenzidine chromogen solution was applied with Gills X1 Hematoxylin, dehydrated, and mounted.

Monoclonal antibodies used were: monomorphic HLA class I expression, W6/32 (anti-HLA-A2, A28; American Type Culture Collection); BM63 (anti-β2 microglobulin; Sigma Aldrich Co. Ltd., Poole, United Kingdom); 116.5.28 and 126.39 (anti-HLA-Bw4 and -Bw6, respectively; K. Gelsthorpe, Sheffield Blood Transfusion Service, United Kingdom); monomorphic HLA class II expression, CR3/43 (anti-HLA class II; Dako); positive control, LP34 (anti-cytoketatin; Dako); infiltrating immune cells, anti-CD1a (Langerhans cells; Dako); CD4 (T helper/inducer; Dako); CD8 (T cytotoxic/suppressor; Dako); and CD68 (macrophage specific marker; Dako).

Immunohistochemical staining was analyzed independently in a blinded fashion by two observers (E-S. A-H. and P. L. S.) and, in cases of disagreement, by the two observers together. The epithelial cells were identified using monoclonal antibody LP34 staining, and HLA expression was determined by comparison with the stroma by the criteria of Keating et al. (25). Quantification of immune infiltrating cells used a 10 × 10 eyepiece graticule and a 40 × objective lens as described by Bishop et al. (26). CD1a positive cells were counted in the epidermis, whereas CD4, CD8, and CD68 positive cells were counted in the upper dermis close to the epidermis. The total number of cells/three high power fields was counted.

**Statistical Analysis.** Significance was calculated using the Mann-Whitney (nonparametric) test. Other comparisons between HLA and HPV in different groups used Fisher’s exact and Kruskal-Wallis tests.

**PCR.** To control for possible contamination between samples, sections of human tissue (20 μm), preceded and followed by sections from mouse liver, were analyzed by PCR-HPV typing. DNA was extracted by proteinase K digestion and then amplified with β-globin primers, as described by Bauer et al. (27). All of the specimens were positive for β-globin, and HPV DNA was detected using the consensus primers GP5+/GP6+, as described by de Roda Husman et al. (28). Additional PCR genotyping was performed using type-specific primers for types 6/11, 16, 18, 31, and 33. Amplification products were resolved on 2% agarose and visualized by ethidium bromide staining.

### Table 1 Clinical response, HLA expression and HPV DNA in 32 high-grade (VIN 2–3) cases treated with topical ALA-PDT

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<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>No. of lesions</th>
<th>Histology</th>
<th>Associated features</th>
<th>Biopsy pre- and post-treatment</th>
<th>Response to treatment</th>
<th>HPV type detection in pre- and post-treatment biopsies</th>
<th>HLA expression</th>
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<td>W</td>
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<td>6/11 and 16</td>
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</tbody>
</table>

* M, multifocal lesions; U, unifocal lesions; W, warty; B, basaloid; D, differentiated VIN; SCH, squamous cell hyperplasia; LS, lichen sclerosis; TS, topical steroids; RT, renal failure; RT, renal transplant; Pigmen., pigmentation; R, responding to topical ALA-PDT at 3-month biopsy; NR, nonresponding to topical ALA-PDT at 3-month biopsy.

HPV +ve means that the generic primer was positive, but the type-specific primers were negative.

Cases that progressed to microinvasion during follow-up for 1 year.

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Table 1. Clinical response, HLA expression and HPV DNA in 32 high-grade (VIN 2–3) cases treated with topical ALA-PDT.
RESULTS

Clinical Response to ALA-PDT. The first 10 women were treated with a light dose of 50 J/cm², and only 2 women (20%) showed short-term response to PDT. Subsequently, the light dose was increased to 100 J/cm², and 8 of 22 (36.36%) patients were reported to have normal histology at 12-week biopsy. Small unifocal lesions (usually recurrent after previous surgical excision) were most responsive to PDT, whereas hyperkeratinized, pigmented, and scarred lesions were least responsive as shown in Table 1. Two differentiated unifocal VIN lesions (patients 27 and 28 in Table 1) both associated with lichen sclerosis failed to respond to PDT, perhaps because of the concomitant use of topical steroids by these patients. The histopathological type of the lesion did not affect the outcome after PDT because the responders were six warty, three warty/basaloid, and one differentiated VIN (see Fig. 1). Because the number of responders was relatively small, it is difficult to correlate the response to specific clinical variables.

HPV DNA in Vulval Neoplasia. Pretreatment high-grade VIN biopsies were obtained from 19 of 32 patients, whereas in the other 13 cases, diagnosis had been made at a referring hospital, and biopsy material was not available. All of the 32 patients were biopsied at 3 months after PDT, and 11 cases had a second PDT after failure of the first treatment. HPV DNA was detected in 23 of 32 (72%) of one or more of the individual patient VIN biopsies (Table 1). However, if the three differentiated VIN lesions (patients 10, 27, and 28 in Table 1), which are not pathobiologically related to HPV, are excluded, the incidence of HPV infection would go up to close to 80%. HPV infection with high-risk types (16, 16 and 33, or 31) was detected in six lesions. HPV 16 was the most prevalent and persistent infection both in the pretreatment (9 of 19; 47%) and after the first (15 of 32; 47%) and second treatments (6 of 11; 55%). Of the 10 women who showed normal histology at the treatment site, after the first or second PDT (responders), 4 were HPV negative before treatment, 5 became HPV negative after treatment, and 1 remained positive. By contrast, of 22 nonresponders, 17 had persistent high-risk HPV infection of the type detected before or after the first and second PDT treatments. Thus, VIN lesions that failed to respond to PDT were more likely to have detectable HPV compared with the responders ($P = 0.002$; Table 2).

Table 2. HPV and HLA in vulval carcinoma and in VIN (responders and nonresponders) treated with PDT

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>HPV +ve</th>
<th>HLA normal</th>
<th>HLA loss</th>
<th>$P$</th>
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<tbody>
<tr>
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<td>Low risk</td>
<td>HPV +ve</td>
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<tr>
<td>VIN (32)</td>
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<td>21</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>PDT responder (10)</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>0.002*</td>
</tr>
<tr>
<td>PDT nonresponder (22)</td>
<td>17</td>
<td>1</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

* Significant $P$ comparing +ve versus -ve groups on Fisher’s exact test.

In vulval carcinomas, only 3 of 11 (27%) had detectable high-risk HPV DNA (type 16, 16 and 33; type 16, 18 and 33). Therefore, there is a greater prevalence of HPV infection associated with VIN compared with that of vulval cancer ($P = 0.014$).

HLA Expression in Normal and Neoplastic Vulva. Monomorphic HLA class I expression was analyzed by staining with W6/32 anti-$\beta$-2m. A limited number of allele-specific, monoclonal antibodies were used to analyze expression of HLA A and B loci, as indicated in “Materials and Methods,” providing information on appropriately genetically positive individuals indicated by labeling of the stromal tissue. Normal HLA class I expression was seen as homogeneous, with staining of both epithelial cells (epidermis) and stroma (dermis) giving a positive result. HLA class II expression in normal vulval skin was limited to the stroma, whereas the epithelium was negative (data not shown). There were, however, Langerhans (dendritic) and immune infiltrating cells forming islands of class II positivity within vulval skin.

Of the 11 vulval carcinomas studied, 9 showed total HLA class I down-regulation confirmed by allele/locus-specific reagents in genetically positive cases. HLA class II expression was not up-regulated in these carcinomas. Two HLA class I positive tumors were associated with lichen sclerosis and were HPV negative. Three of the remaining carcinomas were found to be positive for high risk HPV (Table 2).

Table 1 summarizes the HLA phenotypes of the 32 high-grade VIN lesions studied. Nine (28%) of VIN biopsies showed HLA class I loss, of which 6 (19%) showed total and 3 of 32 (9%) allelic down-regulation (Table 2 and Figs. 2 and 3). HLA expression was shown to be stable with no variation between pretreatment and posttreatment biopsies; A, H&E staining of pretreatment warty type VIN 3; B, H&E staining of pretreatment basaloid VIN 3; C, H&E staining of after PDT responding case showing normal vulval skin; D, immunohistochemical staining of posttreatment CD4 cells present mainly in the underlying stroma of a HPV16-positive, nonresponsive lesion; E, immunohistochemical staining of posttreatment CD8 cells present mainly in the epithelium of a HPV16-negative responding lesion.

Fig. 1. Histological appearance of pretreatment warty and basaloid types of VIN and immunohistochemical staining of T-cell subsets in PDT responding and nonresponding biopsies; A, H&E staining of pretreatment warty VIN 3; B, H&E staining of pretreatment basaloid VIN 3; C, H&E staining of after PDT responding case showing normal vulval skin; D, immunohistochemical staining of posttreatment CD4 cells present mainly in the underlying stroma of a HPV16-positive, nonresponsive lesion; E, immunohistochemical staining of posttreatment CD8 cells present mainly in the epithelium of a HPV16-negative responding lesion.
I down-regulation, whereas all of the cases of VIN that showed either total or allele loss failed to respond to ALA-PDT, and all but one had a high-risk HPV infection at some stage. Indeed, of six VIN patients with total HLA class I loss, three (50%) developed microinvasive disease during a follow-up period of 1 year. However, more cases and longer periods of follow-up are needed to confirm that HLA class I loss can increase the risk of progression of VIN to carcinoma. Clearly, HLA class I down-regulation was significantly more common in vulval carcinomas (9 of 11) than in VIN (9 of 32; \( P = 0.004 \); Table 2).

**DISCUSSION**

This study is the first demonstration, using ex vivo patient material, that ALA-PDT produces an immunological reaction at the treated site in which CTLs (CD8) are associated with a curative response, and it is entirely consistent with previous animal-based work (16, 17). Furthermore, the observed correlation among HLA class I loss, HPV infection, and CD8 cell count, with the different responses of VIN patients to ALA-PDT, indicates a role for both immunological and viral factors in the treatment response of this disease. Moreover, this is not a simple relationship because these factors are clearly related.

The short-term response of a proportion of the VIN lesions to PDT seems to be associated with anatomical, viral, and immunological factors. We observed that unifocal lesions were more responsive than multifocal VIN 3, and increased pigmentation scarring and hyperkeratosis were associated with the least response. The same observations were also reported by Hillemanns et al. (19), which included patients with VIN grades 1–3 and used laser light and 20% ALA solution rather than a cream-based application. In our study, the clinical response was found to improve with absent high-risk HPV infection. On the other hand, the lesions that showed no evidence of response to PDT were associated with a higher prevalence of high-risk HPV infection and loss of HLA class I expression; here there may be a lack of relevant immunity or an escape of the target cells. In contrast to HPV infection, HLA class I loss was a consistent feature of all of the follow-up biopsies analyzed from patients with this phenotype.

It has been reported previously (29, 30) that genital warts, which were nonresponsive to IFN treatment, were markedly depleted in CD8 levels and showed high HPV copy numbers when compared with
responder lesions. The study of Al-Saleh et al. (31) has indicated that high-risk HPV infection of the cervix can suppress cytotoxic T-cell activity by producing infiltration with higher than normal levels of CD4 T-helper 2 cells. Our data are consistent with this hypothesis because the CD4 count was significantly higher (P = 0.003) in VIN than in normal vulva (Fig. 1 and Table 3). However, other factors may complicate this explanation. Inspection of data for infiltration of CD8 T cells in individual tissue samples, with regard to other clinical observations, highlights the complexity of the contributing immunological factors. For example, three patients (patients 27–29 in Table 1) with lesions that showed relatively low CD8 counts at the different biopsy points had been receiving topical steroid therapy for their pruritus vulvae which would compromise the function of CD8 effector cells in VIN.

The observed progression to microinvasive disease that occurred in 50% of VIN patients with total HLA class I loss indicates that HLA phenotype may prove to be a valuable prognostic marker for the management of this difficult condition; however, more cases and longer periods of follow-up are needed. Indeed, total HLA class I loss was found in 82% of the vulvar carcinomas studied, which suggests this may represent a common feature between progressive VIN and invasive vulvar carcinoma. The relationship between vulvar carcinoma, HPV infection, and HLA class I loss is complicated because it is believed that there are two subsets of vulval carcinoma: (a) non-HPV-associated carcinomas, which are prevalent in older women; and (b) HPV-related tumors that often occur in younger patients and are associated with warty/basaloid VIN (32–34). Because the detection of HPV-related VIN has increased over the last 20 years, it might be predicted that the incidence of HPV-positive vulvar carcinoma could also increase in the next decade. Thus there is a real need for an effective treatment for VIN, particularly in younger women.

ALA-PDT used in our clinical study may offer only short-term resolution of VIN, and there will probably be persistence of HPV below detection levels in some cases. Nevertheless, the implication is that PDT can restore normal histology of some VIN lesions with no scarring (18) and reduce the viral load. PDT has also been shown to be effective in eradicating both HPV infection and cervical intraepithelial neoplasia (35). Clearly, the effectiveness of this treatment is mediated at least in part by immune factors. Thus, the possibility remains to combine PDT with a suitable means of modulating the immune system to increase the curative response.

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Immunological and Viral Factors Associated with the Response of Vulval Intraepithelial Neoplasia to Photodynamic Therapy


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