Cervical response to vaccination against HPV16 E7 in case of severe dysplasia

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Abstract

Objective: To evaluate the tolerance to vaccination against human papillomavirus (HPV)16 E7 (in SB adjuvant ASO2B) and its histological and immunohistological effects on HPV16 associated high-grade cervical dysplasias associated with HPV16. Study design: Five patients with histologically demonstrated severe cervical dysplasia (CIN3) HPV16 positive were injected three times before conization was performed 2 months after the first injection. We studied cytological, histological, proliferative pattern and immune profile before and after vaccination. The slides were compared with those obtained from non-injected patients. Results: The injections were well tolerated and the specimens displayed a limited regression of the lesions. Nevertheless, massive CD4 and CD8 T cell lymphocytic infiltration was noticed after vaccination. Discussion: We conclude that the vaccination we used provides an obvious immune histological reaction in the HPV infected cervix and that the 2 months delay before the final step (conization) is done is probably too short.

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1. Introduction

Human papillomaviruses (HPVs) are responsible for 10% of the worldwide cancer incidence. They are the primary cause of cervical cancer, and they are expressed and detected in more than 99% of these cancers. HPVs are also linked to 50% of vulvar, vaginal, penile, anal and perianal cancers and to 20% of oral, laryngeal and nasal cancers. The virus types associated with malignancy belong to the high risk genital group that includes HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52 and HPV56, with HPV16 found in approximately 50% of cancer lesions [1]. Although HPV infection is wide spread, infected cells rarely evolve to invasive lesions. Progression of localized intraepithelial dysplastic high-grade cervical intraepithelial neoplasia into an invasive carcinoma is a slow multi-step process. The host’s cell-mediated immune response is thought to control HPV-induced lesions. This hypothesis is supported by the high rate of T cell-mediated spontaneous regression of low-grade HPV-induced lesions and the increased incidence of HPV-associated lesions in immunosuppressed patients [2].

Based on the observation that all HPV-induced neoplasms express virally-encoded oncoproteins, many studies have evaluated the therapeutic potential of E6- and E7-based vaccines [3]. These oncoproteins, continually expressed by HPV16-induced tumor cells, are recognized by murine and human T cell lines and clones. Moreover, E6 and E7 HPV16-derived proteins have been shown to be tumor rejection antigens in mice [3,4]. Preclinical studies have shown that vaccination based on E6 and E7 proteins or peptides combined with adjuvant, presented by dendritic cells, or expressed in recombinant viruses, protects and cures mice from syngeneic HPV16 positive tumors [3,5–7]. These promising results have already led to the design and implementation of some HPV vaccine Phase I/II clinical trials using HPV16 or HPV18 E6 and E7 antigens [8–11]. Most of these trials were performed with patients presenting late stage cervical cancer associated with reduced immunocompetence and consequently have not allowed to conclude on vaccine efficacy. Potentially therapeutic vaccines should ideally be tested before HPV induced lesions became invasive.
CIN1 is not the most appropriate indication to assess the efficacy of such therapeutic vaccines in a clinical trial, since their high rate of spontaneous remission requesting a large cohort of patients to display a significative effect [12], their rare natural progression and the high efficacy of classical surgical treatments.

CIN3 cases on the other hand are appropriate models to test the efficacy of HPV-based therapeutic vaccines, due to their relatively high rate of progression. within a relative short period of time. Local surgical therapies (conization) of such CIN3 which are currently available lead to the total clearance of the dysplastic lesions in ca. 95% of the cases, however this surgical approach is not always without complication for the patient and her future pregnancies. A vaccine inducing viral clearance and rejection of infected dysplastic cells would represent a great medical benefit for these patients.

The major aims of the present clinical study were to test the safety/tolerability and efficacy of a vaccine based on a mutant form of HPV16 E7 protein in patients presenting HPV16-associated CIN3 cervical lesions with high viral DNA copy number and to study both the colposcopic and histologic response of the cervix after such a therapy.

In order to increase the immunogenicity of the vaccine, the mutated HPV16 E7 is expressed in E. coli as a fusion protein with part of the protein D of Haemophilus influenza B (Prot D HPV16/E7). The vaccine is reconstituted in the SB immunostimulant preparation ASO2B which contains a delipidated monophosphoryl A (MPL) from Salmonella Minnesota, a saponin extract of the tree Quilaja saponaria (QS-21) and an oil in water emulsion (SB 62, GlaxoSmithKline Beecham Biological Immunotherapeutics sa (Rixensart, Belgium). They are observed for two hours after the injection and any secondary effect are reported. They all undergo a conization (laser or cold knife) at 56 ± 2 days.

Blood samplings for chemistry and evaluation of immunity, pregnancy test colposcopy and cervical cytology are performed prior to each of the injections, at the time of cervical surgery and every 3 months till the follow-up period (9 months post-surgery). In case of progression of the lesion during the vaccination course, a conization is performed without delay.

2. Materials and methods

The inclusion/exclusion criteria for this study are here described.

Patients are aged between 18 and 60 years, either post-menopausal or using a contraception. They are warned not intent to be pregnant during the whole duration of the study.

The patients present a high-grade squamous intra-epithelial lesion demonstrated at the single layer cytology. The colposcopy examination demonstrates exclusively exocervical dysplastic lesions with less than 3 mm involvement of the endo-cervical canal.

The clinical evaluations are performed by one of the two senior gynecologists (PS or FB). The cervical lesion covers at least 35% of the total surface of the transformation zone with absence of any other suspicious lesion. The biopsies reveal dysplastic lesion graded CIN3 (all the slides are reviewed by the senior pathologist JCN). At least 65% of the cervical lesion persists after biopsy. Only patients presenting an HPV16 infection (see below for method) are included in the study.

The patient are rejected from the study in case of any sign of infiltrating cervical carcinoma at cytological or histological examination.

The exclusion criteria also includes: drug abuse within the last 6 months, any primary or secondary immunosuppressive disorders; any systemic disease that might require major co-medication during the study. The consumption of corticoids or other immunosuppressive drugs within 1 month prior to the enrollment in the study, pregnancy or lactation are also considered as exclusion criteria.

Six patients are included in this study. They all meet the inclusion criteria here described. After giving their informed consent, the patients are treated as followed.

The patients get injected with 200 µg Prot D HPV16/E7 [13] in SB adjuvant ASO2B three times (days 0, 14 and 28). The vaccination was kindly provided by SmithKline Beecham Biological Immunotherapeutics sa (Rixensart, Belgium). They are observed for two hours after the injection and any secondary effect are reported. They all undergo a conization (laser or cold knife) at 56 ± 2 days.

Blood samplings for chemistry and evaluation of immunity, pregnancy test colposcopy and cervical cytology are performed prior to each of the injections, at the time of cervical surgery and every 3 months till the follow-up period (9 months post-surgery). In case of progression of the lesion during the vaccination course, a conization is performed without delay.

2.1. Cytology slides preparation and HPV detection

For each patient, liquid-based, ThinPrep cytology slides are prepared from PreservCyt vial according to the manufacture’s standard protocol and read by trained cytopathologists according to routine practice. Following the preparation of the ThinPrep, the PreservCyt vials are forwarded for HPV testing both by Hybrid Capture II methodology to detect oncogenic HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and PCR amplification with MY09/1 primers following by HPV16 typing by the DEIA method as previously described [14,15].

2.2. Immunohistochemistry

Biopsies specimens and conizations from vaccinated patients are immediately fixed in 4% formalin for 12 h and then embedded in paraffin. For each specimen, 10 serial sections of 4 µm are performed. The first section is stained with haematoxylin and eosin, the others sections are used for immunohistochemistry and controls. For immunohistochemistry, the antigen retrieval method was performed as previously described [16]. The characteristics and dilution of the primary antibodies are respectively:

- Mouse anti-Human Ki-67 antigen, clone MIB-1 (Immucor, Fleurus, Belgium) (dilution: 1/50). This antigen is considered as a proliferating marker and is present in nuclei during all phases of the cell cycle.
• Mouse anti-Human p16\textsuperscript{INK4a}, clone E6H4 (Dako, Glostrup, Denmark) (dilution: 1/25). This protein is a cyclin-dependent kinase (CDK) inhibitor. Recent biological studies have revealed that p16 expression is strongly influenced the status of the retinonoblastoma gene product pRB. Indeed, pRB inhibits the transcription of p16. p16 overexpression has been demonstrated in cervical cancers and cervical intraepithelial neoplasia because of the functional inactivation of pRB by oncogenic HPV E7 proteins.

• Mouse anti-Human CD4 antigen, clone 1F6 (Novocastra Laboratories Ltd., Newcastle, UK) (dilution: 1/20).

• Mouse anti-human CD8 antigen, clone C8/144B (Dako, Glostrup, Denmark) (dilution: 1/25).

• Mouse anti-human CD1a antigen, clone JPM30 (Novocastra Laboratories Ltd., Newcastle, UK). CD1a is a protein expressed on dendritic cells.

2.3. Interpretation of immunostaining

All immunostainings are reviewed by two independent observers and interpreted as follows:

• For Ki-67 by counting the percentage of positive nuclei in at least 500 epithelial cells.

• The distribution of p16 is scored on a semi-quantitative scale, as follows: negative (<1% of cells were positive), sporadic (isolated cells are positive, but <5%), focal (small cell clusters, but <25% of the cells are positive) and diffuse (>25% of the cells are stained).

• For CD4, CD8 and CD1a by counting the number of positive cells in 1 mm\(^2\) of tissue including both the dysplastic epithelium and the underlying connective tissue.

Control: Six conizations are evaluated in the same manner as those after vaccination to evaluate the potential immunological reaction of the cervical tissue 2 months after a colposcopy guided biopsy is performed.

3. Results

Five patients are included in the study. Their mean age is 36.5 years (20–51). One of these patients is retrieved from the study after the first injection; the colposcopic examination prior to the second vaccination revealing a lesion that was not described at the time of inclusion. All the biopsies performed before inclusion demonstrate a severe dysplasia (CIN3). The cytology at the time of the conization demonstrates a

Table 1
Injection related symptoms as related by the patients during the course of their vaccination

<table>
<thead>
<tr>
<th>Patient</th>
<th>Injection 1</th>
<th>Injection 2</th>
<th>Injection 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Local pain 36 h</td>
<td>Local pain 36 h</td>
<td>Local pain 36 h</td>
</tr>
<tr>
<td>Patient 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>Local pain 48 h, pyrexia 38.2 °C, 24 h, fatigue 48 h</td>
<td>Local pain 48 h</td>
<td>Local pain 12 h</td>
</tr>
<tr>
<td>Patient 3</td>
<td>Local pain 36 h, pyrexia 12 h</td>
<td>Local pain 36 h</td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>Local pain 36 h, moderate flu-like syndrome</td>
<td>Conization</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>Moderate flu-like syndrome, local pain 72 h</td>
<td>Local pain 48 h</td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Histology, virology and molecular results summary

<table>
<thead>
<tr>
<th>Patients</th>
<th>Histology</th>
<th>HPV\textsuperscript{a}</th>
<th>p16\textsuperscript{b}</th>
<th>Ki-67\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biopsy</td>
<td>Cone biopsy</td>
<td>Biopsy</td>
<td>Cone biopsy</td>
</tr>
<tr>
<td>P1</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P4</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P5</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P6</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S1</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S3</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S4</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S5</td>
<td>H-SIL</td>
<td>H-SIL</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Detection of oncogenic HPVs using Hybrid Capture II assay.

\textsuperscript{b} p16 distribution was scored on a semi-quantitative scale as follows: negative (<1% positive cells), sporadic (<5% positive isolated cell), focal (small cell clusters, but <25% positive cells) and diffuse (>25% positive cells).

\textsuperscript{c} Percentage of positive nuclei within ≥500 epithelial cells. S patients have not been vaccinated. For P patients biopsy was obtained before vaccination and cone biopsy 4 weeks after the last vaccination.
Table 3

Immunohistological characterization of infiltrating cells

<table>
<thead>
<tr>
<th>Patients</th>
<th>CD4</th>
<th>CD8</th>
<th>CD1a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biopsy</td>
<td>Cone biopsy</td>
<td>Cone biopsy/ biopsy</td>
</tr>
<tr>
<td>P1</td>
<td>NT</td>
<td>550</td>
<td>NC</td>
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<tr>
<td>P2</td>
<td>120</td>
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<tr>
<td>P4</td>
<td>125</td>
<td>600</td>
<td>4.8</td>
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<tr>
<td>P5</td>
<td>200</td>
<td>750</td>
<td>3.8</td>
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<tr>
<td>P6</td>
<td>175</td>
<td>500</td>
<td>2.9</td>
</tr>
<tr>
<td>P7</td>
<td>250</td>
<td>250</td>
<td>1.0</td>
</tr>
<tr>
<td>P8</td>
<td>200</td>
<td>300</td>
<td>1.5</td>
</tr>
<tr>
<td>P9</td>
<td>200</td>
<td>150</td>
<td>0.8</td>
</tr>
<tr>
<td>S1</td>
<td>250</td>
<td>150</td>
<td>0.6</td>
</tr>
<tr>
<td>S2</td>
<td>200</td>
<td>150</td>
<td>0.8</td>
</tr>
<tr>
<td>S3</td>
<td>250</td>
<td>250</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a Number of positive cells within 1 mm² of dysplastic epithelium and the underlying connective tissue.

b Patients that have been enrolled in the vaccination trial and to whom biopsy was taken before vaccination and cone biopsy, 4 weeks after the last vaccination.

c Patients that have not been vaccinated and to whom biopsy was obtained prior conization.

d Number of positive cells in the cone biopsy divided by the number of positive cells in the biopsy per surface unit.

high-grade lesion in all five patients and the histology of the cone obtained either by laser resection or cold knife the persistence of either moderate (CIN2) in three patients or severe (CIN3) dysplasia in two patients. A regression of the surface of the lesion superior to 20% of the initial lesion noticed on colposcopic examination was not in any case.

Our patients tolerated well the injection. Nevertheless, all of them presented pain at the injection site lasting for an average of 36 h and they all except one presented flu-like symptoms after at least one of the injections. The side-effects resulting from the injections are described in the Table 1.

The Hybrid Capture II assay after vaccination never displays (Table 2) the disappearance of HPV at the time of conization whether the patient has been vaccinated or not. P16 was neither modified after treatment.

The proliferative index Ki-67 (Table 2) does not display any significant modification (from a mean value of 32 to 31 for the vaccinated group and from 37 to 38 for the control group.

The vaccination induces an obvious lymphocytic infiltration of the cervical tissue as described in Table 3. Indeed, CD4 as well as CD8 positive cells are more numerous at the time of conization in the tissue of the vaccinated patients. The CD1a positive cells appears to be increased in the same manner in both groups.

4. Discussion

Due to the high incidence of human papilloma infections in young population and to the to potential consequences of these diseases, the need for an eradication process for those carrying the virus is obvious. In a low risk population screening, up to 20–46% of patients aged between 20 and 30 present at a given time smears indicating an HPV infection [17]. A prophylactic HPV16 vaccine has recently shown its efficacy in reducing the risk of infection by this oncogenic virus [18]. Hopefully, the majority of these patients will get free of it within the next 2 years and those who are mostly at risk for invasive cancer are probably found in the group of female carrying multiple oncogenic HPV after the age of 35 as the persistence and progression to a high-grade are more frequent at that moment [19].

Theoretically, a vaccination aiming at the shortening of the carrying phase and at the disappearance of the virus from the cervix meets several goals. Through the reduction of the number of individuals carrying the virus, it could reduce the risk of contamination for their sexual partner as it could reduce the occurrence of invasive cervical tumor. It should also diminish the size of the cervical pre invasive lesions reducing the indication of cone biopsies and their subsequent surgical and obstetrical complications. It should reduce the risk of recurrence after surgical treatment as it is established that recurrence is directly linked to the persistence of HPV after conization [20].

The vaccination that we use is directed against the E7 protein of HPV16. The choice for HPV16 results from its frequent implication in the cancerous transformation of the cervix and the vulva. It is recognized as the most common high risk HPV type in most countries [21]. E7 is an early viral protein continuously produced in infected cells, whom expression is necessary for induction and maintenance of cell transformation. Through the production of interferon alpha, E7 thus rapidly decrease the host immunity in the infected area allowing the persistence and the growth of the transformed tissue. Moreover, preclinical studies performed in mice have shown that E7-based vaccination can induce protective as well as therapeutic anti-tumor effects. For these reasons, this protein appears as a target of choice for therapeutic vaccination [9].

However, the results obtained in this study as those obtained by Muderspach et al. are disappointing. No
disappearance of lesion was observed and no obvious reduction of their size were noticed. On the opposite, the clear infiltration of the cervical stroma by T lymphocytes and dendritic cells indicates an obvious immunological reaction after the vaccination.

Several factors can explain the lack of regression observed in this study.

To date, at least 15 HPV types have been associated with high-grade CIN and cervical carcinoma (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Even if HPV16 is the most often represented papillomavirus found in dysplasia, multiple type infections are nevertheless very frequent. This could possibly explain the lack of regression noticed. The development of a vaccine directed against a panel of HPV strains should therefore be of the greatest interest. CIN3 lesions are maybe not the ideal target for clinical vaccination as conization is an adequate treatment for them. Eradication of the viral infection sooner in the process (CIN1 and/or CIN2) should probably be more concerned by this therapeutic approach.

On the other hand, the 2 months delay between the first dose of vaccine and the conization is most probably too short to allow major histologic modifications and regressions of intraepithelial lesions. This delay was chosen at a date where very few HPV-directed vaccination studies in patients demonstrating non-infiltrating lesions have been published [9,22]. We were therefore anxious of the risk of progression of the CIN3 during the course of the treatment and of a possible underestimation at the initial assessment of the lesion.

In future trials, we shall focus on using a multi-type HPV vaccination as most of the cervical and vulvar HPV infection displays not only a single HPV16 and on enlarging the delay between the vaccination and the conization to allow an improved histologic response as consequence of the immunologic reaction.

Acknowledgements

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References