Local applications of GM-CSF induce the recruitment of immune cells in cervical low-grade squamous intraepithelial lesions.

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Running title: Local GM-CSF treatment of HPV-cervical lesions

Abstract

Problem

Quantitative alterations of antigen-presenting cells (APC) in (pre)neoplastic lesions of the uterine cervix associated with human papillomavirus (HPV) infection suggest a diminished capacity to capture viral antigens and to induce a protective immune response.

Method of study

To test if a cervical application of GM-CSF could restore an immune response against HPV in women with cervical low-grade squamous intraepithelial lesions (LSIL). We performed two clinical trials with11 healthy women and 15 patients with LSIL.

Results

GM-CSF applications were well tolerated in all enrolled women and no difference in toxicity between the treated and placebo groups was observed during the follow up (until 30 months). Interestingly, in the GM-CSF treated group, a significant increased APC and cytotoxic T lymphocyte infiltration was observed in the cervical biopsies with no change in regulatory T cell numbers. All the $HPV16^{+}$ patients exhibited an immune response against HPV16 after GM-CSF applications, as shown by NK and/or T cells producing IFN-γ whereas no cellular immune response was observed before the treatment. Moreover, the anti-VLP antibody titers also increased after the treatment.

Conclusion

These encouraging results obtained from a limited number of subjects justify further study on the therapeutic effect of APC in cervical (pre)neoplastic lesions.

Key words*:* clinical trial, Granulocyte Macrophage–Colony Stimulating factor (GM-CSF), human papillomavirus, immune response, safety, uterine cervical lesion

Introduction

Even if prophylactic vaccines against human papillomavirus (HPV) are now available, morbidity and mortality caused by HPV infection still represent a major health problem with very high direct and indirect costs [1]. Furthermore, the protection is restricted to 2 HPV types involved in 30-70% of pre-cancerous lesions (squamous intraepithelial lesions, SIL). Incomplete type coverage is especially problematic in developing countries where no effective screening programs are available as an alternative approach for reducing cervical cancer risk associated with minor oncogenic HPV types. The vaccination blocks HPV primary infection by inducing high titers of neutralizing antibodies, but does not accelerate clearance of the virus in pre-existing infections [2]. This could be linked to the fact that cell-mediated immunity is critical for the clearance of HPV-associated lesions [3-5]. Moreover, the current surgical methods for treating SIL may generate complications, e.g. pregnancy complications [6] and persistence and/or recurrence of SIL occur in 5–25% of patients [7]. All these facts underlie the need for other therapeutic protocols.

Most SIL are characterized by a decreased density and function of antigen-presenting cells such as dendritic and Langerhans cells (DC/LC) in comparison with the normal paired squamous epithelium[8, 9]. However, Granulocyte Macrophage–Colony Stimulating factor (GM-CSF) can restore the colonization of DC/LC in a neoplastic epithelium transplanted *in vivo* in NOD/SCID mice [10]. This cytokine is produced by

keratinocytes and a correlation has been observed *in vivo* between the levels of GM-CSF produced by some carcinomas and the distribution/differentiation of tumor-associated DC [11]. Moreover, *in vitro* DC specifically induce apoptosis of keratinocytes transformed by HPV, whereas normal keratinocytes are not affected [12]. This apoptotic effect of DC is not restricted to HPV-transformed keratinocytes. Manna et al. [13] have also demonstrated a direct tumoricidal effect of DC on breast carcinoma cells. The safety of GM-CSF injected subcutaneously [14, 15] or intradermally [16] to patients with cancer was previously evaluated, but to our knowledge, the tolerability of topical GM-CSF applications on a mucosal surface has never been assessed.

The purpose of this study was to establish a proof of concept for a treatment based on local applications of a GM-CSF-containing gel. We therefore evaluated the safety and the ability of this gel to induce the recruitment of DC/LC and lymphocytes into an epithelium infected by HPV and to elicit an immune response against HPV-infected cells. In order to have a long follow up (more than 6 months this study was performed on patients with low-grade SIL (LSIL) to circumvent the risk of cancer development.

Materials and Methods

Patients

Eleven women without cervical lesions were enrolled for a phase Ia clinical trial. For safety reasons and to be able to have a 6-month follow up without treatment, we chose to include in phase Ib clinical trial patients with LSIL. The eligibility criteria for patients were the persistence of LSIL and/or detection of oncogenic HPV for a minimum

of three months with a median of 8.5 months for the GM-CSF group and 6 months for the placebo group (no statistical difference). One hundred ninety two patients were screened and fifteen patients with LSIL were enrolled (Fig. 1, Table 1).

Vital signs (heart rate, blood pressure and oral temperature), local symptoms (pain, redness, swelling, inflammation), serious and other adverse events were recorded in the Case Report Form (CRF) of each patient.

HPV genotyping was determined using a PapilloCheck Test Kit (gift from Greiner Bio One, Frickenhausen, Germany)[17] (Table 1) and the viral load of oncogenic HPV was estimated by Hybrid Capture II (Digene, Venlo, Netherlands) at the first (visit A) or the second visit (B) and at the end of the clinical protocol.

These clinical protocols were approved by the local ethics committee of the University Hospital of Liège.

Clinical protocol

Patients received 1 (healthy women) or 4 (patients with LSIL) applications of 3-4 ml GM-CSF (or placebo) in polycarbophil gel separated by 3 days. They were followed for one week or for a period of 6-7 months for the phase Ia and the phase Ib clinical trial, respectively. A late visit (26-30 months) was also performed for the phase Ib clinical trial.

The polycarbophil gel (1.0% w/w) was prepared by dispersing Noveon AA1 (Noveon, Brussels, Belgium) in purified water. The mixture was stirred until thickening occurred and was then neutralized by drop wise addition of 40% (w/w) tromethamol, until a transparent gel appeared. The quantity of tromethamol was adjusted to achieve a gel of pH 5.5 [10].

For the active formulation, GM-CSF (Amoytop Biotech, Xiamen, China) was incorporated into the hydrogel at a concentration of 400,000 IU/ml, 800,000 IU/ml or 1,000,000 IU/ml for the phase Ia clinical trial and 800,000 IU/ml for the phase Ib clinical trial. The first administration of the gel was made by a senior gynecologist. The other applications were self-administered at bedtime with a polystyrene applicator (Besins Healthcare, Belgium).

At visit A (week -35 to -5) and B (week -21 to -1) a colposcopy and sampling for viral detection and DNA load determination were performed. GM-CSF or the placebo gel was applied at visit C (Week 0). Follow up was performed at visit D (week 2), visit E (week 6 to 15) and visit F (week 23 to 42).

Keratinocyte proliferation assay

Normal keratinocytes (KN), HaCat cell line and HPV-transformed keratinocyte cell lines (CK2, CaSki, SiHa and KT1F4) were cultured ($5X10^3$ cells/well) in 96-well plates (Nunclon Surface, NUNC) with or without GM-CSF (63µg/ml-800 000 U/ml). Proliferation was measured after 48h at 37°C, following an 18 h incubation with 0.4µCi/well of [^{3}H]thymidine (6.7Ci/mmol, Moravek Biochemicals, Brea, CA). DNA was harvested by an automated sample harvester (Packar, Canberra, Tilburg, The Netherlands) and thymidine incorporation was analyzed using a liquid scintillation counter (Top Count, Packard, Canberra).

The assessment of cell proliferation in vivo was performed on biopsies by using an anti-Ki-67 antigen antibody (clone MIB-1 from Dako) following a previously described procedure [8]. Ki-67 antigen staining was evaluated by analyzing the localization of positive cells and

expressing the proliferation as normal when the positive cells were present in the basal layer, low when positive cells reached the first third of the epithelium and moderate to high when the positive cells were present in the second or the last third of the epithelium.

Immunostaining and systemic immune response

The density of immune cells in biopsies was assessed by immunoperoxidase detection with anti-CD1a (Novocastra, Newcastle, UK) and anti-CD8 (Dako, Glostrup, Denmark) monoclonal antibodies (mAb) for DC/LC and CTL, respectively, as described previously [18]. NK cells were detected with NKp46 mAb (R&D Systems, Oxon, Belgium) and regulatory T cells (Treg) were stained with Foxp3 mAb (eBiosience, UK). The results were expressed as ratios by dividing the values obtained after treatment by the values before treatment.

The cellular immune response was evaluated by IFN-γ intracellular staining on PBMC stimulated with the E7 HPV16 protein (gift from GSK Biologicals, Rixensart, Belgium) and L1 HPV16 Virus-like particles (VLP) at each visit as described previously [19]. Briefly, PBMC (2 x 10^6/ml) in 5 ml round bottom polypropylene tubes containing X-vivo 20 medium (BioWhittaker, Cambrex Bio Science, Verviers, Belgium, supplemented with 20 U/ml IL-2) were cultured overnight at 37 °C with or without the E7 HPV16 protein and L1 HPV16 VLP. HPV16 VLP were produced in insect cells using a recombinant baculovirus encoding HPV 16 L1 protein and were purified as previously described[20]. Brefeldin A (10 µg/ml, Sigma, St. Louis, MO) was added during the last 4 h of culture. After stimulation, cells were stained for cell surface molecules at 4 °C for 20 min with anti-CD4-PE, anti-CD8-PerCP, anti-

CD3-PerCP, anti-CD16-PE and anti-CD56-PE (Becton Dickinson Erembodegem, Belgium). After washing with PBS, cells were fixed and permeabilized with FacsLyse and FacsPerm respectively (Becton Dickinson) according to the manufacturer's instructions. After an additional washing with PBS, the cells were stained with anti-IFN-γ-FITC (Becton Dickinson) for 20 min at room temperature. After another washing, cells were resuspended in 400 µl of 1% paraformaldehyde in PBS and acquisition was performed within 24 h on more than 100,000 events per staining with a flow cytometer (Facsvantage, Becton-Dickinson). Analysis was performed using CellQuest on an SSC/FSC gate on viable lymphocytes. Results were expressed as the percentages of cytokine positive cells in the defined cell population after background (no activator) was subtracted. Overnight supernatants from PBMC cultures were stored at –70°C and analyzed for IFN-γ content in a specific capture ELISA according to the manufacturer's instructions (R&D Systems).

Plasma samples were used for serological testing for HPV16 and 31 by ELISA assays with HPV16 and 31 VLP L1 as described previously[21].

Statistical analysis

Statistical analysis was performed using the Student's *t* test. The calculation of power gave values from 92% to 39% according to the SD (from 0.4 to 0.8; 49% for weighted SD= 0.7). All calculations were performed with SAS version 9.1 (SAS institute, Cary, NC, USA)

Results

Safety

Even if GM-CSF was already used in clinical trials [14-16], we performed pre-clinical studies because GM-CSF was described to mildly stimulate the growth of HaCat keratinocyte cell line [22]. We confirmed previous results showing the stimulation of HaCat cell proliferation and a slight increased growth of normal keratinocytes, but no significant effect was detected with 4 different HPV^+ cell lines (Fig. 2a).

The phase Ia clinical trial was performed because there was no previous information about the potential toxicity of mucosal applications of GM-CSF. The doses used were extrapolated from our data obtained in a murine model [10]. This clinical trial was conducted on 11 normal women with normal cervical cytology (Table 1) who received one application gel containing 400,000 IU/ml (4 women), 800,000 IU/ml (4 women), 1,000,000 IU/ml (3 women) of GM-CSF. Since no adverse effect was reported, we decided to compare 4 applications of the gel containing the intermediate dose (800,000 IU/ml, n=10) with a placebo gel (n=5) in a second clinical trial including patients with LSIL (Table 1). All women encountered none or only minor and transient side effects with no difference between women receiving gel with or without GM-CSF. All patients were examined by gynecologists 6 to 30 months after the second clinical trial and no side effects related to the trial were reported.

To further assess the absence of keratinocyte proliferation in the presence of topical application of GM-CSF, we also performed an anti-Ki67 labeling [23] on biopsies taken before or after treatment (visit D and F: 2 weeks and 5 to 10 months after the GM-CSF application,

respectively). No statistical significant modification in the proliferative index of epithelial cells was observed in women treated with GM-CSF in comparison with the placebo group (Fig. 2b).

Immune response

DC/LC, cytotoxic T lymphocytes (CTL) density in biopsies was monitored by CD1a (Fig. 3a) and CD8 (Fig. 3b-c) immunostaining, respectively. The different types of staining were scored as previously described [18] and the results were expressed as ratios compared to the scores obtained before treatment. Significantly higher ratio of CD1a positive cells was observed at visit F ($p=0.0197$) in GM-CSF-treated group versus placebo (Fig. 3a). GM-CSF also induced similar results for $CD8^+$ T cells, significant data were obtained for visit F in the stroma adjacent to the lesion ($p=0.040$; Fig. 3b-c). However, the scores of CD1a and CD8 staining were generally higher in the placebo group before the start of the clinical trial (data not shown). NK cells were detected with an NKp46 antibody in cervical biopsies (Fig. 3d). Since the number of $NKp46^+$ cells was low in cervical biopsies, we counted the cells instead of scoring. In the epithelium only few cells were detected (data not shown). In the stroma, an increased NK infiltration was observed at visit D ($p=0.029$) (Fig. 3d).

We also counted the number of $Foxp3^+$ Treg (Fig. 4 a-b). We were not able to obtain ratios for the infiltration in the epithelium because Treg were not detected in several cervical biopsies (Fig. 4a). Both in the epithelium and in the stroma, we did not observe a significant increase of $Foxp3^+$ Treg after treatment except for one patient in GM-CSF group

(Fig. 4 a-b). Interestingly, the patient G2 with the highest number of Foxp3$^+$ Treg cells (empty symbol in Fig. 4) showed a low score of CD8+ T cells in the epithelium (empty symbol in Fig. 3b), but not in the stroma or for NK cells (empty symbol in Fig 3c-d). The number of Treg decreased at visit F (empty symbol in Fig. 4), whereas both CD8$^+$ T and NK cells increased (empty symbol in Fig. 3b-d).

Systemic immune responses were also evaluated against HPV16 and 31. Three patients were HPV16$^+$ (all in GM-CSF group), which is in concordance with the prevalence of HPV16 in LSIL (24%) in Belgium [24] and two patients were HPV31$^+$ (one in GM-CSF and one in placebo group). No T cell response was observed before treatment. There was a substantial increase in the percentage of IFNγ^+ cells in PBMC from the 3 patients (G4, G5 and G10), who had received GM-CSF (Fig. 5a-b). Depending on the patient and the type of stimulation (E7 or L1-VLP), IFNγ producing cells were T lymphocytes (CD8$^+$ or CD4$^+$) or NK cells. In patients G4 and G5, the number of IFNγ^+ cells dropped at week 23-42 after the treatment (Fig. 5a-b). We also observed an increased secretion of IFNγ in response to E7 and L1-VLP by ELISA assay, except for patient G10 (data not shown). We tested the anti-HPV16 response in PBMC from the 12 patients negative for HPV16. A trend for a higher percentage of lymphocytes expressing IFNγ after stimulation with HPV16 L1-VLP was observed in the GM-CSF group in comparison with the placebo patients (data not shown).

Before treatment no antibodies or low titers (except for the patient HPV16+ G10) against HPV16 and HPV31 VLP were observed (data not shown). For all the HPV16$^+$ patients (G4, G5 and G10), antibodies

against HPV16 VLP were detected after treatment with GM-CSF (Fig. 5c). Interestingly, the antibody titer in the serum of patient G4 was very high and similar to those observed after vaccination with HPV16 VLP. Both HPV31^{+} patients (G4-P1) had antibodies against HPV31 VLP but the patient receiving GM-CSF (G4) had a higher concentration of antibodies than the patient (P1) in the placebo group (Fig. 5d).

Clinical response

The clinical status (grade of SIL and viral load) was also checked in the 15 patients enrolled in the phase Ib study. Despite the detection of an immune response, we were not able to observe any difference in clinical response, the proportion of patients showing regression or progression was similar in both groups (data not shown). This could be linked to the small cohort of patients, to the high spontaneous regression rate of LSIL [25] or to the fact that GM-CSF-treated patients presented, in our study, negative factors (multiple infections, higher viral load and significantly older age ($p=0.03$) [26]) than those of the placebo-group (Table 1).

Discussion

In this study, we first demonstrated that the intraepithelial recruitment of antigen-presenting cells by topical applications of GM-CSF is safe and well tolerated (Fig. 2 and Table 1). This is in contrast with other immunomodulators used topically to treat HPV-associated lesions such as imiquimod [27-31]. Moreover, GM-CSF did not induce proliferation of keratinocytes transformed by HPV both in vitro and in vivo (Fig. 2).

Interestingly, the recruitment of DC/LC in SIL was associated with an immune response against HPV in HPV16$^+$ patients. Our previous work, both in vitro and in a murine model of HPV-induced intraepithelial neoplasia, demonstrated that GM-CSF is able to stimulate the migration of DC/LC in an epithelium transformed by HPV [32]. We also observed this migration in GM-CSF-treated patient biopsies (Fig. 3 a). A higher infiltration of CD1a$^+$ cells was previously described in vulvar intraepithelial lesions in patients who responded to a therapeutic vaccine against HPV, illustrating the importance of APC in the induction of immune response against HPV [33]. Other immunotherapy protocols using tumor cells engineered to secrete GM-CSF also showed increased numbers of activated APC at the immunotherapy injection sites and in draining lymph nodes [34].

In similar clinical protocols, dense lymphocyte infiltrates were observed at injection sites in the majority of patients [35]. In our study, we observed an increased infiltration of cytotoxic cells such as NK cells and CD8+ lymphocytes (Fig. 3b-d). Interestingly, the expression of NK receptor ligands was described on cervical tumor cells [36], suggesting that NK cells can recognize and kill these cells. In a mouse model, tumor protection induced by GM-CSF was shown to be CD8 T cell dependent [37] and the persistence of HPV16 infection was associated with the lack of cytotoxic T lymphocyte response in human [38]. As already described in other immunotherapy protocols [39], we observed Foxp3$^+$ Treg cells in cervical biopsies (Fig. 4). These cells could inhibit the anti-tumoral response [40], but GM-CSF treatment did not induce a significant increase of these cells (Fig. 4).

A systemic cellular immune response against HPV16 VLP and E7 protein was observed in all HPV16+ patients after GM-CSF applications (Fig. 5 a-b). In 2 patients (G4 and G5), the number of IFNγ^+ cells dropped at week 23-42 after the treatment (Fig. 5a-b), suggesting that a larger GM-CSF therapeutic window could be beneficial in sustaining the immune response, but we could not exclude a regulatory role of T cells in this inhibition since the number of Treg Foxp3+ cells slightly increased in some patients (Fig. 4). In some HPV16 negative patients, we also observed an immune response against HPV16 VLP, but not against HPV16 E7 (data not shown). Cross-reactivity has already been reported in PBMC from HPV-16 VLP vaccine recipients who can respond to L1-VLP from heterologous HPV types, suggesting the presence of conserved T cell epitopes [41]. In contrast with VLP vaccines, which induce type-restricted immunity, the immune effect of GM-CSF is likely to be present for all the HPV genotypes. However, we were not able to confirm this statement because VLP and E7 proteins were not available for all the HPV types present in our cohort of patients.

Concerning the clinical response, we analyzed patients with LSIL because the treatment of the lesion can be postponed without danger for the patient. Due to spontaneous and rapid regression rate in these patients [25], we were unable to show an effect of the treatment in this small cohort of patients.

In conclusion, our study demonstrated the safety of our therapeutic approach and suggested that GM-CSF induces DC/LC, CTL and NK cell recruitment in the lesion and the induction of a systemic immune

response (humoral and cellular) against HPV. These results justify further analysis of this local immunotherapy protocol in a larger number of subjects.

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Figure legends

Fig. 1: Schedule of enrollment.

Fig. 2: Proliferation of normal keratinocytes (KN) and keratinocyte cell lines (means ±SD, n=3) in presence of GM-CSF (**a**). Proliferation index (Ki67 staining) in biopsies of patients treated with GM-CSF or placebo gel (means ±SE, n=10 for GM-CSF and n=5 for placebo) (**b**).

Fig. 3: Infiltration of DC/LC ($CD1a^+$), CTL ($CD8^+$) and NK cells (NKp46+) in cervical LSIL. Ratio of $CD1a^+$ cells in the epithelium (**a**), of $CD8^+$ cells in the epithelium (**b**) or in the stroma (**c**), of NKp46+ NK cells in the stroma (**d**) at visit D (week 2) and visit F (week 23-42) after application of GM-CSF or placebo gel. Empty symbols= patient G2.

Fig. 4. $Foxp3^+$ Treg cells in cervical biopsies. Number of $Foxp3^+$ cells in the epithelium before (visit A/B) or after (visit D, week 2 or visit F, week 23-42) applications of GM-CSF or placebo gel **(a)**. Ratios of $Foxp3^+$ cells in the stroma at visit D and F (**b**). Empty symbols= patient G2.

Fig. 5: IFNγ production in response to HPV16 E7 or HPV16 L1-VLP in HPV16 positive patients. PBMC were stimulated overnight with HPV16 E7 (a) or L1-VLP (b). (a) The percentage of $IFN\gamma^+$ cells was determined in $CD4^+$, $CD8^+$ or $CD56^+CD16^+CD3^-$ (NK cells) gated cell populations. Antibody titers against HPV 16 (c) and HPV 31 (d) VLP in patient plasma samples. The antibody titer was determined in ELISA assay by serial dilutions of plasma samples. The assay was performed at least in duplicate. BT= before treatment.