ORIGINAL ARTICLE

Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with MVA E2 recombinant vaccine

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Human papillomavirus (HPV) is the etiologic agent for cervical cancer. In Mexico, a women dies every 2 h, and since 1990 the statistics have shown that the numbers of deaths are increasing. We conducted a phase II clinical trial to evaluate the potential use of the MVA E2 recombinant vaccinia virus in treating high-grade lesions (CIN 2 and CIN 3) associated with oncogenic papillomavirus. Fifty-four female patients with high degree lesions were treated either with an MVA E2 therapeutic vaccine or with conization. Thirty-four women received the therapeutic vaccine, at a total of 10^7 virus particles per dose injected directly into the uterus once every week over a 6-week period. Twenty control patients were treated with conization. By colposcopy, 19 patients out of 34 showed no lesion, in three patients the lesions were reduced by 85–90%, in eight other lesions had reduced by 60%, and in four more patients, they were reduced by 25%. Histological analysis showed total elimination of high-grade lesions in 20 out of 34 patients after treatment with MVA E2. Eleven patients had a 50% reduction in lesion size. In two other patients, the lesion was reduced to CIN 2 and in one more patient the lesion was reduced to low grade (CIN 1). All patients developed antibodies against the MVA E2 vaccine, and generated a specific cytotoxic response against papilloma-transformed cells. DNA viral load was significantly reduced in MVA E2-treated patients. Conization eliminated the lesions in 80% of the patients, but patients did not develop cytotoxic activity specific against cancer cells and did not eliminate the papillomavirus. In addition, three patients treated with conization had recurrence of lesions 1 year later. These results show that therapeutic vaccination with MVA E2 proved to be very effective in stimulating the immune system against papillomavirus, and in generating regression of high-grade lesion.

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Introduction

Cervical cancer and precancerous cervical lesions constitute a major problem in women's health. Each year, 470 000 new cases of cervical cancer are diagnosed worldwide. Half of these women normally die from this disease.^{1,2} Methods like surgery, conization, radiotherapy, and chemotherapy are being used to reduce papillomas and cancer tumors. Unfortunately, these methods are not 100% effective and many deaths are still recorded each

year. Multiple approaches are being tried in the quest for an effective therapy.

Vaccines are a novel alternative against papilloma-virus-induced tumors. The vaccinia virus recombinant MVA E2 was constructed as a therapeutic vaccine against papilloma-transformed cells. MVA E2 was shown to stop human tumor growth in mice and also to induce tumor regression in tumor-bearing rabbits.^{3,4} More recently, MVA E2 was also shown to induce elimination of pre-cancerous lesions CIN 1 and CIN 2.⁵ To further evaluate the therapeutic potential of the MVA E2 recombinant vaccine on high grade (CIN 3 lesions), we undertook this Phase II clinical trial with 54 female patients.

At the end of the treatment with MVA E2, we found that 58.85% of the patients showed no lesion, while 41.18% of the patients presented up to a 60% reduction in lesion size.

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Methods

Study design and subjects

We conducted a phase II clinical protocol involving 52 female patients with CIN 3 and 2 patients with CIN 2 high degree lesions at the Instituto Mexicano del Seguro Social (IMSS), and the Hospital Santa Fe in Mexico. Patients were treated with MVA E2 virus or conization. The MVA E2 virus was injected weekly directly into the uterus in a radial clockwise fashion at points corresponding to 3, 6, 9, and 12 o'clock over a 6-week period, using 10^7 virus particles per dose. Reduction of lesions was monitored weekly by colposcopy. At the end of the protocol, a biopsy was taken exactly at the same place as the first biopsy for histological analysis. The type of immune response generated by the MVA E2 treatment was determined by measuring antibody titers against the E2 protein and the MVA E2 virus, and by quantitating cytotoxic activity against cancer cells bearing papilloma-virus DNA.

A control group, consisting of 20 patients with mainly CIN 3 lesions, was treated with conization. Patients were admitted to the study protocol if they fulfilled the relevant eligibility criteria. These criteria included presence of oncogenic human papillomavirus (HPV) type 16 or 18, age between 25 and 49 years, not to be pregnant, and to have high-grade (CIN 2 and CIN 3) lesions. The complete description of the study protocol has been published elsewhere.⁵

Virus and cells

Chicken embryo fibroblast (CEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD), 20 μ M glutamine, 50 U/ml penicillin, and 50 μ g/ml of streptomycin, and were maintained in a humidified air/5% CO₂ atmosphere at 37°C. The MVA E2 virus was titrated by end point dilution to obtain the 50% tissue-culture infectious dose (TCID₅₀). Virus-infected cells were harvested at 24 and 48 h and immediately freeze-thawed three times. The entire harvesting, titration, and purification procedure was performed under aseptic conditions according to current good manufacturing practice (cGMP) guidelines.

Collection of biopsy samples and detection of papillomavirus DNA

Biopsy samples were divided into two portions. One portion (0.2 cm) was processed for histological studies and the other portion was placed in 1 ml of Digene Specimen Transport Medium and stored at -20°C for later DNA detection.

Histological examination

Biopsies were taken during visits 2 (beginning) and 8 (end of treatment). On each occasion, the same pathologist interpreted the results and classified the samples as type CIN 1, CIN 2, or CIN 3, according to the World Health Organization (WHO) classification. Lesions CIN 1, CIN 2, and CIN 3 correspond to low, moderate, and severe

dysplasia, respectively, or low grade (CIN 1) and high grade (CIN 2, and CIN 3). Biopsy samples were processed as described.⁶

Enzyme-linked immunosorbent assay (ELISA)

The specific antibody response against the recombinant MVA E2 vaccine, and the HPV E2 protein, was evaluated as previously described.⁶

Cytotoxicity assays

Cytotoxicity assays were performed as described before.⁶

Results

Clinical and demographic characteristics of patients with high-grade (CIN 2 and CIN 3) lesions diagnosed between 2002 and 2004

Approximately 1300 female patients were screened according to the inclusion and exclusion criteria for the study. Among them, patients with high degree lesions and the presence of an oncogenic HPV were identified. The 54 patients included in the study were all Hispanic, with a mean age of 35 years. Their average height was 156 cm and average weight was 64 kg. In this study, 34 patients with high degree lesions were treated with the MVA E2 vaccine. The control group of 20 patients was treated with conization (Table 1, and data not shown).

MVA E2 therapeutic vaccine can stimulate the regression of high-grade (CIN 2 and CIN 3) lesions

A total of 34 patients with high grade were treated with MVA E2 therapeutic vaccine. The MVA E2 vaccine was injected directly into the uterus (see Methods section). At visit 9 (first evaluation), 3 weeks after treatment was completed, 56.25% of the patients with CIN 3 and two patients with CIN 2 were free of lesions, as diagnosed by histological and colposcopy analysis (Table 1, and Figure 1a and b, see patients nos. 006, 008, 014, 018, 019, 021, 023, 024, 026, 032, 034). These patients presented lesions covering from 20 to 40% of the transformation zone. In 11 patients, the lesion was reduced to 50% of the original size. In these patients, the lesion was covering the whole transformation zone (100% is considered as the total surface of the transformation zone). In two more patients, the CIN 3 lesion was reduced to CIN 2, and in another patient the CIN 3 lesion was reduced to CIN 1 (Table 1). These results show that treatment with MVA E2 efficiently stimulates the regression of high-grade lesions in the uterus. Moreover, by colposcopy, 55.8% of patients did not show the presence of papillomavirus infection and the lesion was diagnosed as having reduced by 100% (see patients nos. 001, 006, 008, 009, 012, 014, 018–024, 026, 029 031–034 in Table 1). In 32.44% of the patients, the lesion was reduced up to 60% (patients nos. 003, 005, 007, 010, 013, 016, 017, 028 in Table 1). In four more patients (11.76%), the lesion was only reduced 25% from the original size (patients nos. 002, 004, 011, 015, in Table 1). After 3 weeks, after the last clinical evaluation (visit 9), a conization was performed on all the patients in order to

Table 1 Clinical and immunological characteristics of the 34 patients with high grade lesions, diagnosed between 2002 and 2004, and treated with MVA E2

Patient number	Anti-vaccinia antibody titer	E2 protein antibody titer (1/dilution) ^a	Initial size of lesion (in %) ^b	Presence of HPV DNA ^c (% of viral load) after treatment	Elimination lesions ^d
202001	1000	256	(25)	(0)	100
202002	600	128	(100)	(50)	25
202003	300	256	(80)	(0.16)	60
202004	300	256	(100)	(3.31)	25
202005	600	256	(60)	(100)	60
202006	300	500	(40)	(21.94)	100
202007	1000	500	(60)	(100)	60
202008	1000	256	(30)	(0)	100
202009	1000	500	(30)	(0)	100
202010	1000	500	(50)	(22.24)	60
202011	1000	256	(100)	(15)	25
202012	1000	128	(25)	(0.036)	100
202013	1000	256	(50)	(24.46)	60
202014	1000	256	(30)	(0)	100
202015	1000	256	(90)	(35.9)	25
202016	1600	256	(50)	(1.15)	60
202017	1600	256	(40)	(25)	60
202018	1000	256	(30)	(0)	100
202019	1600	256	(40)	(52.45)	100
202020	1000	256	(100)	(0)	100
202021	1000	256	(30)	(0)	100
202022	1000	256	(25)	(50)	100
202023	1600	500	(30)	(0)	100
202024	1000	500	(35)	(0)	100
202025	1600	256	(30)	(11.56)	90
202026	1000	500	(25)	(0)	100
202027	500	256	(25)	(10.6)	85
202028	1000	500	(40)	(29.35)	60
202029	500	256	(30)	(10.5)	100
202030	1000	500	(40)	(20)	90
202031	1600	500	(30)	(10.34)	100
202032	1000	256	(20)	(0)	100
202033	1600	500	(20)	(1.25)	100
202034	1000	256	(30)	(0)	100

^aAntibody titer at week 7 after MVAE2 treatment.^bPercentage of the initial size of the lesion (% from the transformation zone).^cHPV-positive patients at week 7 after MVA E2 treatment.^dPercentage of lesion reduction, as diagnosed by colposcopy, at week 7 after MVA E2 treatment.

verify the efficacy of the MVA E2 therapeutic vaccine. The histological analysis from this conization revealed the same results as those obtained from the biopsy sample taken at the end of the MVA E2 treatment (visit 9) (data not shown, and Table 1). These results suggest that, at the end of treatment with MVA E2, it is sufficient to take a small biopsy sample from the place where the lesion was located, in order to verify the efficacy of the treatment. In addition, the small aceto-white spots found in patients with complete regression of lesion did not correspond to the remaining lesions as confirmed by histological analysis (Figure 1a and b). None of these patients presented recurrences after 1 year of treatment.

In the control group, 16 (80%) out of 20 patients treated with conization were free of lesions as diagnosed by colposcopy and cytologic analysis (data not shown). Four other patients remained with the CIN 3 lesion as diagnosed by colposcopy and histology analysis. These

results show that conization can eliminate the high-grade lesions induced by HPV. However, in contrast to the MVA E2-treated patients, three control patients presented recurrence of the CIN 3 lesion 1 year after the conization.

Immune response generated against the MVA E2 vaccine

Sera from all patients were collected every week in order to determine the humoral immune response generated against the MVA E2 virus. Specific antibodies against the MVA E2 virus were found in all treated patients (Table 1). Antibody titers ranged from 1:300 to 1:1600. The high antibody titer remained unchanged at week 9. In contrast, no specific antibodies were detected either in untreated patients or in the control group treated by conization (data not shown). Antibodies against the E2 protein were also detected in all treated patients. Antibody titers were

Figure 1 Colposcopy and histology of papillomavirus-induced cervical lesions from patient treated with MVA E2 therapeutic vaccine. Photographs of cervix from a representative patient (a) (patient 202006), with CIN 3 lesions before (V1-B) and after (V9-A) MVA E2 treatment. In (a), left side is cervixes with aceto-white staining and right side are cervixes with iodine staining. The aceto-white staining in the uterus reveals the presence of possible spots with papillomavirus infection (white areas). Cells infected with papillomavirus normally take acetic acid inside more easily than normal cells. The iodine staining shown in dark color probably represents areas not infected with papillomavirus. In (b), after MVA E2 treatment, the epithelium is free of koilocytes (right). Arrowhead shows the epithelium. V1 and V9 correspond to visits 1 and 9, respectively.

from 1:128 to 1:500 (Table 1). A direct correlation was observed between anti-MVA E2 antibodies and the regression of lesions. All patients with antibodies against the MVA E2 vaccine showed reduction of lesions. In patients with high-grade lesions covering 25% or less of their transformation zone, the MVA E2 treatment completely eliminated the lesions (see patient nos. 001, 006, 008, 009, 012, 014, 18–24, 26, 29, and 31–34 in Table 1). Other patients with lesions covering 35–40% of the transformation zone eliminated 90% of the lesion (see patient nos. 025, 027, 030 in Table 1). In contrast, in patients with lesions covering 100% of the transformation zone, the MVA E2 treatment showed only a partial reduction of the lesions, approximately 25% of the whole lesion (see patient nos. 002, 004, 011, 015 in Table 1). In other patients with lesions covering 50% of the transformation zone, the reduction was up to 60–70% (patient nos. 003, 005, 007, 010, 013, 016, 017, 028, Table 1). These results indicate that the generation of antibodies against the MVA E2 correlates with the reduction of lesions. However, the percentage of regression of lesions was related to the initial size of the lesion. Small lesions were completely eliminated after MVA E2 treatment (see patient nos. 001, 006, 008, 009, 012, 014, 18–24, 26, 29, and 31–34 in Table 1, whereas large lesions showed smaller reduction (see patient nos. 002, 004, 011, 015 in

Table 1). However, all patients developed antibodies against MVA and E2 protein, and the percentage in the reduction of lesion was most probably due to lesion size (Table 1).

Treatment with the MVA E2 therapeutic vaccine eliminated HPV in patients with high-grade (CIN 2 and CIN 3) lesions

Using the Hybrid Capture method, we assessed the presence of HPV in patients with CIN 3 lesions, both before and after treatment with the MVA E2 therapeutic vaccine. Twelve out of 34 patients efficiently eliminated all papillomavirus DNA (patient nos. 001, 008, 009, 014, 018, 019, 021, 023, 024, 026, 032, and 034 in Table 1). In some patients, viral load was diminished by 95% (patient nos. 003, 004, 012, 016, and 033 in Table 1). In the remaining patients, viral load was reduced between 15 and 50% (Table 1). These results showed that the MVA E2 therapeutic vaccine significantly reduced the presence of papillomavirus in the cervix. In contrast, none of the 20 control patients treated by conization eliminated the HPV (data not shown). In addition, all MVA E2-treated patients developed cytotoxic T lymphocytes (CTL) directed against tumor cells that were observed in all patients treated with the MVA E2 therapeutic vaccine (data not shown). Presence of these cytotoxic cells correlated with the elimination of lesions. In contrast,

patients before treatment with the MVA E2 vaccine or from patients treated by conization (control group) did not show cytotoxicity (data not shown).

Treatment with MVA E2 vaccine does not cause major adverse effects

As determined from physicians reports for the 34 patients who received the MVA E2 vaccine, only few mild adverse events were observed, considered to be related to the vaccine treatment. Side effects were headache in 12 patients, flu-like symptoms in nine patients, temperature above 39°C lasting for 1 day in six patients, chills in eight patients, mild abdominal pain in four patients, and joint pain lasting for 1 day in two patients. These results demonstrate that the MVA E2 vaccine is a safe and effective agent for treatment of HPV-induced cancerous lesions.

Discussion

In the present study, we found that the MVA E2 therapeutic vaccine is capable of eliminating high-grade *in situ* cancer cervical lesions (CIN 3), associated with infection by oncogenic HPV. The major findings of this report are: (1) the MVA E2 vaccine induced regression of lesions in all treated patients, (2) papilloma-virus DNA was almost completely eliminated in 50% of MVA E2-treated patients, and (3) very few mild side effects were observed in the MVA E2-treated patients. In fact, our MVA E2 recombinant virus, which expresses the E2 protein, is capable of stopping human tumor growth in immunodeficient animals,⁴ showing that the E2 protein promotes apoptosis in HPV-transformed cells and these cells can be recognized by the immune system to generate an immune response against them. In an organism with a functional immune system, the E2 protein still promotes apoptosis in HPV-transformed cells, but now these dying cells can be recognized by the immune system and induce an immune response against the cells. Therefore, the MVA E2-induced regression of lesions is due principally to the amount of cell destruction achieved by effector cells of the immune system.

In activating the immune system, we also have the effect of the MVA virus inside cells. Cells infected by vaccinia virus are efficiently recognized by the immune system. It is known that the vaccinia virus is a good vector for presenting antigens to the immune system.⁷ Consequently, there is an additive effect, from the E2 protein and the vaccinia virus, in stimulating the immune system when the recombinant MVA E2 is used. The immune response generated by the MVA E2 virus involves the production of specific antibodies and also of specific cytotoxic cells. These elements of the immune response contribute to the elimination of the cancer cells. Although the HPV E2 protein is probably the major target of the immune response induced by the MVA E2 vaccine, it is very likely that other HPV antigens, such as E6, E7, E5, and E4 proteins

present in tumor cells, are also involved. The lytic effect of vaccinia virus on tumor cells results in the release of these antigens, which can be picked up by various antigen-presenting cells (APCs). This effect will also contribute to expose other cell antigens and then generate a wider immune response against the tumor cells.^{3,4} The overall result of this immune response is the regression of papilloma-induced tumors. In the present work, we found that all MVA E2-treated patients developed an immune response against cancer cells. Patients had antibodies and cytotoxic T cells against MVA E2 and HPV-transformed cells, by the end of treatment. Antibodies generated against tumor cells contribute, at least to some extent, to neutralize the proliferation of transformed cells. The cellular immune response (cytotoxic lymphocytes) generated against the MVA E2 vaccine is probably responsible for the elimination and reduction of HPV-transformed cells in patients. Patients, in whom the lesion was not completely eliminated, underwent conization in order to remove the remaining lesion, and to secure their health. This procedure was performed because, according with Mexican Health Regulations, patients with high grade lesions must be treated with conization rather than going untreated. The MVA E2 treatment used in this study resulted in successful elimination of many CIN 3 lesions. However, in some cases, the lesions were only reduced in size. In these cases, the initial lesions were already very large covering almost the total area of the transformation zone. We used only six doses of MVA E2, but may be increasing the treatment to eight doses could improve the results, because after the six doses, lesions were reduced by more than 60% of its original size (Table 1) in most cases. On the other hand, it is also possible that our protocol is already enough and that larger lesions would be eliminated at later times, longer than 9–12 weeks. In any case, we have not yet observed any recurrences, or increases in the size of lesions, in any of the MVA E2-treated patients. This strongly suggests that the protection generated by the MVA E2 treatment is long lasting. Another important finding of this study is that the MVA E2 therapeutic vaccine significantly reduced the presence of papilloma-virus in the cervix. In contrast, conization, although is a good procedure for eliminating lesions, is not a procedure for eradicating HPV. All these results clearly indicate that therapeutic vaccination using recombinant vaccinia virus expressing papilloma antigens are excellent candidates to stimulate the immune system against papillomavirus-induced lesions. Moreover, the value of MVA recombinant viruses as potent therapeutic candidates has been demonstrated for several other diseases such as Influenza, Toxoplasmosis, HIV, SIV, Syncytial virus, and Measles.^{8–11} In conclusion, our study shows that the MVA E2 recombinant vaccinia virus vaccine is a very promising candidate for treating high-grade CIN 3 HPV-induced cervical lesions. Treatment leads to elimination of lesion and also elimination of viral DNA, leaving patients with better protection against future recurrences due to HPV re-infection.

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