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# Preclinical evaluation of the therapeutic effect of adenoviral vectors in Human papillomavirus-dependent neoplasias

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**ABSTRACT**

Gene therapy with adenoviral vectors can eliminate neoplasic cells through selective replication and/or through pro-apoptotic, immunogenic or suicide gene expression. However, an adenoviral vector may provide anti-cancerous effects even in the absence of replication or therapeutic gene expression. The present study evaluates the therapeutic effects caused by the administration of an adenoviral vector, alone, in HPV- dependent neoplasias (HPV-N). *In vivo* trials were carried out in two HPV-N mouse models. One model was immunocompetent and the other was immunodeficient. In both models, the effect of intratumoral administration of saline solution (PBS) was compared with administration of an adenoviral vector that had no replicative capacity or therapeutic gene (Ad-BGal). In the immunocompetent mice, Ad-BGal adenoviral vector administration significantly reduced tumor growth, compared with PBS. No differences were observed in the immunodeficient mice. In conclusion, the present study lends support to the use of adenoviral vectors in HPV-N treatment since they are capable of generating an antitumoral effect in immunocompetent individuals, even in the absence of a therapeutic gene or viral vector replication.

**Key words.** Neoplasms. Human papillomavirus. Human adenovirus. Gene therapy. Selective replication.

***Evaluación preclínica del efecto terapéutico de vectores adenovirales en neoplasias dependientes del papilomavirus humano***

***RESUMEN***

*Los adenovirus (vectores adenovirales) usados en la terapia génica pueden eliminar a las células neoplásicas mediante una replicación selectiva y/o a través de la expresión de genes proapoptóticos, inmunogénicos o tóxicos. Sin embargo, un vector adenoviral puede provocar efectos anticancerosos aun en ausencia de replicación o de la expresión de un gen terapéutico. El presente estudio evalúa la eficacia terapéutica de los vectores adenovirales, por si solos (sin efecto por replicación o por un gen terapéutico), en neoplasias dependientes del papilomavirus humano (N-PVH). Los ensayos in vivo fueron realizados en dos modelos murinos (ratones) de N-PVH, un modelo fue inmunocompetente y otro inmunodeficiente. En ellos se comparó el efecto de la administración intratumoral de solución salina (PBS) con la administración de un vector adenoviral sin capacidad replicativa ni gen terapéutico (Ad-BGal). En ratones inmunocompetentes, la administración del vector adenoviral Ad-BGal redujo significativamente el crecimiento tumoral en comparación con PBS, en tanto que en ratones inmunodeficientes no se observaron diferencias. En conclusión, el presente trabajo apoya el uso de vectores adenovirales en el tratamiento de N-PVH, pues son capaces de generar un efecto antitumoral en individuos inmunocompetentes, aun en ausencia de gen terapéutico o replicación del vector viral.*

***Palabrasclave.*** *Neoplasias. Papilomavirus humano. Adenovirus. Terapia génica. Replicación selectiva.*

## INTRODUCTION

Human papillomavirus-dependent neoplasias (HPV-N) are a public health problem in Latin American countries, the Carribbean and other developing regions.[1] HPV is the causal agent of a variety of malignant tumors, and cervicouterine cancer (CCA) stands out among them. Some prophylactic HPV vaccines are currently available,[2] but there is still a need for the development of new therapeutic tools to treat HPV-related precursor lesions and neoplasms. Gene therapy with viral vectors is one such alternative presently being researched.

A gene therapy viral vector is a genetically modified virus which serves as a "vehicle" for transporting a gene or therapeutic nucleotide sequence to the interior of a cell.[3] An objective of vector genetic modification can also be to cause tumorous cell lysis through viral vector replication, as though an infection limited to the tumor were being treated.[4] The therapeutic efficacy of these viral vectors largely depends on their genetic modifications, although the characteristics of the virus type used as a vector or "vehicle" (adenovirus, herpes virus, vaccinia virus, etc.) are also a factor. In gene therapy for cancer, adenoviral vectors are among the most commonly used since they can be introduced into a wide variety of cell types, there are few adverse side effects at therapeutic doses and their activity is transitory (weeks).[3-5] It is worth noting that among the diverse types of adenoviruses, type 5 is the one used in gene therapy because, up to now, it has been shown to be the safest.[5] It is not capable of integrating the viral genome into cellular chromosomes or of acquiring foreign genes in natural processes.[5]

Adenoviral vectors are more than simple vehicles for genes. They can increase therapeutic efficacy by setting off an immunological response to neoplasia as a secondary effect of an immune system reaction to the adenoviruses found inside the neoplasic cells. However, this adenoviral vector immunological effect has only been clearly evaluated in rectal cancer models.[6]

At the present time, diverse adenoviral vectors carrying antisense sequences or expressing pro-apoptotic, suicide or immunogenic genes have been designed to fight CCA.[7-20] Other adenoviruses take advantage of tumor-specific changes to increase their selective replication in HPV-N (oncolytic vectors).[21] Many of these vectors have successfully proven their antineoplasic effect in cell lines and in mouse models. However, when the antitumoral effects of adenoviral vectors are studied *in vivo*, it is generally done in immunodeficient mouse models, eliminating a possible immunological response. Therefore, it is possible that the true therapeutic capacity of many of these vectors is being underestimated.

The objective of the present study is to evaluate the therapeutic efficacy of adenoviral vectors alone for HPV-N in immunocompetent individuals, without taking the effect of therapeutic genes or vector replication into account. The purpose of this study is not to validate an adenoviral vector on its own as an HPV-N treatment option, but rather to demonstrate that adenoviral vector gene therapy can provide an additional effect to that of replication or therapeutic genes in HPV-N. Thus the study was carried out in *in vivo* trials to compare the antitumoral effect of the administration of a vector with no replicative capacity or therapeutic gene versus the administration of saline solution in an HPV-N immunocompetent mouse model.

## MATERIALS AND METHODS

### Cells and viruses

Human embryonic kidney (HEK) -293, human cervical cancer SiHa cells (HPV-16 carriers) and mouse TC-1 cells (syngeneic to C57Bl/6 mice) were used in these studies. Cells were maintained in DMEM 10% fetal bovine serum (FBS) and supplemented with L-glutamine, penicillin and streptomycin at 37 °C and 5% $CO_2$ in a humidified environment. Ad-wt (virus widely used as a wild-type adenovirus) and Ad-BGal, which is an E1A deleted adenovirus (vector with no replicative capacity or therapeutic gene; B-galactosidase gene carrier), have been described.[21,22] Viruses were grown on HEK-293 and purified with ViraKit AdenoMini-24 (Virapur LLC, San Diego CA, USA) according to the manufacturer's instructions. The viruses were titered on HEK-293 cells, as previously described.[23]

### Viral replication assay

The majority of mouse cells do not allow adenoviral replication.[24] However, there are exceptions,[6] and the adenoviral replicative capacity in TC-1 cell line had to be demonstrated. Cells were plated on 24-well plates at 100,000 cells per well, with a 2% FBS medium. Six hours later, the cells were infected with viruses at $1X10^7$ pfu/ml for 1 h. The medium containing the virus was removed and the cells were washed twice with 1ml of the culture medium. The cells were incubated in 1ml of 2% FBS medium and on

post-infection days 0 (2 hours after exposure to vectors), 2, 4, 6 and 14, both cells and supernatants were collected and subjected to titer determination on HEK-293 cells. The culture medium was renovated every four days. All experiments were performed in triplicate.

### Animal experiments

C57Bl/6 mice (4 to 6 week old females from Harlan Mexico, Mexico City) were used for the immunocompetent mouse model and Foxn1nu mice (4- to 6-week-old females from Harlan Mexico, Mexico City) were used for the immunodeficient nude mouse model. Both models were generated with a dorsal subcutaneous injection of 400,000 TC-1 mouse tumor line cells (syngeneic to C57Bl/6 mice). They contained a cDNA which encoded the HPV-16 E7 protein to generate a subcutaneous tumor that served as a surrogate for an HPV E7-expressing human tumor, such as a human cervical carcinoma. When tumors reached a diameter of approximately 4mm, they were injected with $1X10^9$pfu of Ad-wt, Ad-BGal or PBS. The antitumor efficacy of the treatments was evaluated by tumor growth and survival curves. Tumor size was measured every third day in two perpendicular directions with a caliper, and tumor volume was estimated by the formula LS2/2; S being the shorter tumor diameter and L the longer diameter. All animal manipulations were performed following institutional guidelines and the Mexican Official Norm regulating laboratory animal use.

### Statistical methods

The MedCalc (version 8.1.0.0 for Windows; Mariakerke, Belgium) software package was used to carry out the Kaplan Meier survival analysis. Kolmogorov-Smirnov test was used to check normality of data distribution and the Student's t-test was used for tumor volume comparison (version 11.0; SPSS, Chicago, IL).

## RESULTS

All trials were performed using the Ad-BGal and the Ad-wt adenoviral vectors. Ad-BGal is a vector lacking both replicative potential and a therapeutic gene. Ad-wt is an unmodified wild adenovirus with a large replicative capacity in diverse human cell types. As shown in figure 1, adenoviral vectors (Ad-BGal and Ad-wt) were not capable of producing viral particles in the TC-1 mouse cell line. This is in contrast to the large adenoviral production observed in the SiHa human cell line (Figure 1).

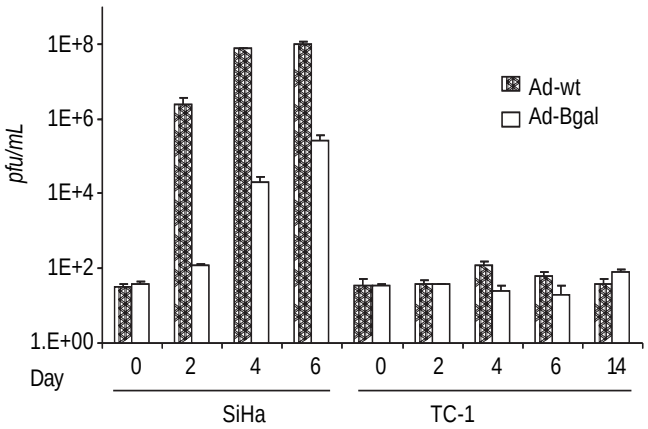


*Figure 1. Adenoviral vector production. SiHa and TC-1 cell lines were infected with vectors at 1 x $10^7$ pfu (vp) per milliliter for 1 h. At 2 hours after exposure (day 0) and on post-infection days 2, 4, 6 and 14, both cells and supernatants were collected and subjected to titer determination on HEK-293 cells. From post-infection day 4, the Ad-wt vector in the SiHa cell line reached the maximum viral production permitted by the number of cells in the trial. Adenoviral vectors were not capable of producing viral particles in the TC-1 murine cell line. Results are represented as mean ± standard deviation.*

The *in vivo* trials were carried out in two mouse models (with and without an immune system), to determine if the observed effects depended on an immune response or not. Another important aspect of the animal models is the fact that the tumors TC-1 cells. Since adenoviruses do not replicate in these cells (Figure 1), any antitumoral effect generated in this experiment would not be attributable to viral vector replication.

The trials in the mouse immunocompetent model showed that there was significantly less tumor growth in the adenoviral vector groups, compared with the PBS group (Figure 2). Tumor growth percentages were compared (Table 1) on post-injection day 21, which was the last register with 100% of the

Table 1. Percentage comparison of tumor growth on post-injection days 21 and 33 in the immunocompetent mouse model.

| Group/Day | Mean | Standard error | *P** |
|---|---|---|---|
| PBS/21 | 712.0 | 73.3 | Reference |
| Ad-wt/21 | 411.0 | 80.0 | 0.01 |
| Ad-Bgal/21 | 543.6 | 47.3 | 0.07 |
| PBS/33 | 1616.2 | 103.8 | Reference |
| Ad-wt/33 | 1021.4 | 231.4 | 0.04 |
| Ad-Bgal/33 | 1134.8 | 169.8 | 0.03 |

* Student's t-test.

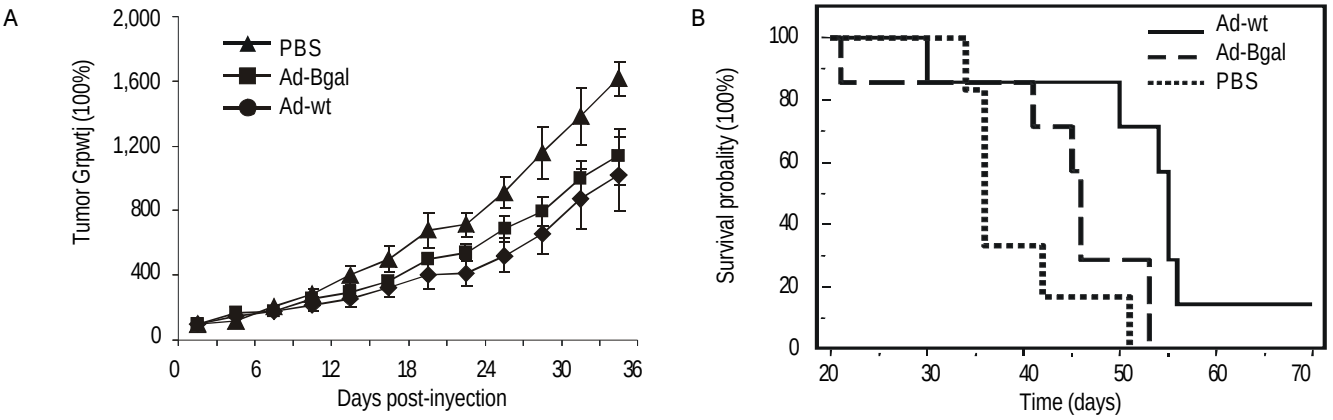


*Figure 2. Graphs showing tumor growth (A) and survival (B) of immunocompetent mice injected with 1x10$^9$ pfu of viral vectors or PBS. The growth of tumors injected with adenoviral vectors was significantly less than those injected with PBS. The survival rate of only the mice injected with Ad-wt was significantly greater than that of the mice injected with PBS (p = 0.02). Results of tumor growth are represented as mean ± standard error. Ad-wt (n = 7), Ad-BGal (n = 7), PBS (n = 6).*

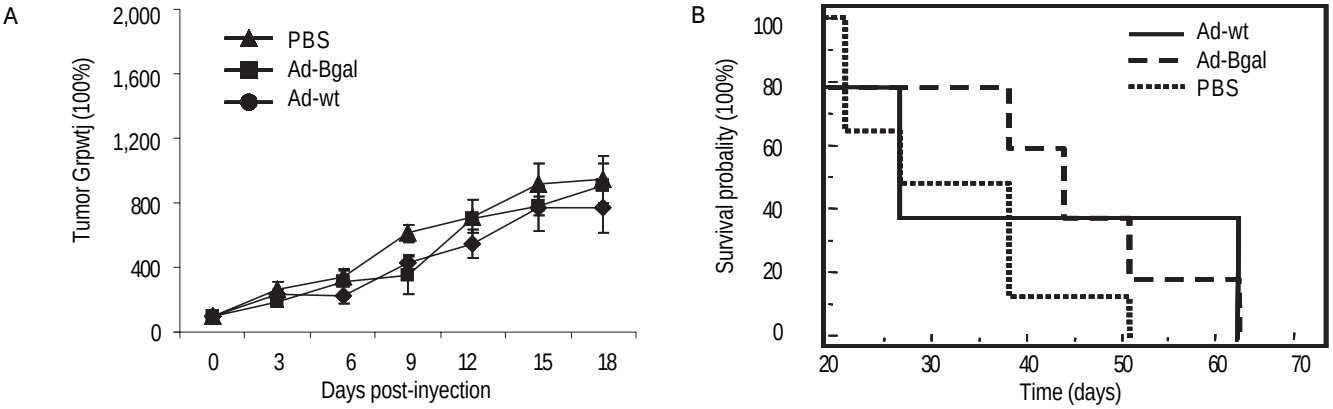


*Figure 3. Graphs showing tumor growth (A) and survival (B) of immunodeficient mice injected with 1x10$^9$ pfu of viral vectors or PBS. No significant changes in tumor growth or survival were found between groups. Results of tumor growth are represented as mean ± standard error. Ad-wt (n = 5), Ad-BGal (n = 5), PBS (n = 6).*

mice (no mouse had died) and on day 33, which was the last day with more than 80% of the mice alive in all groups (only one dead mouse per group). One of the most relevant results was that one-in-seven mice injected with Ad-wt had total tumor regression. This was not observed in any of the mice injected with Ad-BGal. In relation to survival, the mean was 55, 46 and 36 days for the Ad-wt, Ad-BGal and PBS groups, respectively (Figure 2). The mice injected with Ad-wt lived significantly longer than those of the PBS group (p = 0.02) while the survival rate of the mice injected with Ad-BGal was not significantly different from that of the other groups.

No significant changes in tumor growth or survival between groups in the immunodeficient nude mouse model were observed (Figure 3).

## DISCUSSION

The results of the present study lend support to the use of adenoviral vectors in the fight against HPV-dependent neoplasias, since they are capable of generating a significant antitumoral response in immunocompetent individuals, even in the absence of replication or a therapeutic gene.

In the immunocompetent mouse model, TC-1 cell tumors reduced their growth by an average of 30-37% (post-injection day 33) with the intratumoral administration of a dose (1x10$^9$ pfu) of adenoviral vectors. Contrastingly, no significant changes between groups in the immunodeficient model were observed, lending support to the idea that the antitumoral effect was mediated by the immune sys-

tem. Di Paolo, *et al.*, along with several other studies, have suggested that the interaction of adenovirus capsids with cellular receptors induces the expression of pro-inflammatory cytokines/chemokines, such as tumor necrosis factor-α (TNF-α), IFN-γ, interleukin (IL)-1, IL-6, IL-12, and monocyte chemoattractant protein-1 and 2. This results in the recruitment of innate and adaptive immune system effector cells to the site of infection[25]. Furthermore, presentation of adenovirus proteins of the incoming adenovirus particle and/or de novo expression of adenovirus proteins in tumor cells could provide an adjuvant effect on the activation of tumor-specific T cells. This idea is also supported by a recent study showing that HPV E6/E7-expressing TC-1 mouse tumor cells that underwent apoptosis after viral infection (with herpes simplex) increased the efficacy of dendritic cell vaccines more than TC-1 cells that died upon receiving UV-B radiation.[26]

No significant differences regarding tumor growth were found among the groups of mice injected with Ad-wt and Ad-BGal. However, it should be emphasized that one of the mice injected with Ad-wt had total tumor regression and that in general, the Ad-wt-injected mice lived significantly longer than the mice injected with PBS, an event not observed in the mice injected with Ad-BGal. However, it is striking that Ad-wt was more therapeutic than Ad-BGal in the TC-1 mouse model, and this cannot be attributed to the replicative capacity of Ad-wt, because the vectors were not capable of producing viral particles in the TC-1 mouse cell line (Figure 1). The principal difference between vectors is the presence of the E1A gene in Ad-wt. Its expression is the probable cause of the differences in mouse survival, given that this adenoviral protein has been shown to have pro-apoptotic effects[27] and induces susceptibility of cells to lysis by natural killer cells, activated macrophages and a variety of other immunologic and non-immune cellular injuries.[28]

The mouse models used in this work are for the study of HPV-N. However, it is very probable that the therapeutic effects reported here can also be seen in animal models of other neoplasia. In other words, this therapeutic effect is most likely not exclusive of HPV-N. Results of trials carried out in a rectal cancer mouse model concur with the findings of the present study[6]. Nevertheless, it is also probable that this effect which is secondary to the immune system, may vary according to the type of neoplasia treated with adenoviral vectors.

Another important aspect favoring the use of adenoviruses in HPV-N treatment is that HPV oncoprotein expression promotes adenoviral replication. This is because both viruses (HPV and adenovirus) have structurally and functionally similar proteins.[29] So then, adenoviral vectors designed to be replication-deficient were capable of generating large quantities of infectious viral particles in human tumoral cells with HPV (replication of Ad-BGal in SiHa human cells, Figure 1). However, it was evident that their production was much slower than that of a wild-type adenovirus.

The results of the present study will provide a better evaluation of the therapeutic effect of adenoviral vectors on HPV-N. The trials in immunodeficient mouse models underestimate the efficacy of these vectors on HPV-N. In can be inferred that their efficacy would be greater in immunocompetent individuals. Moreover, therapeutic gene effect or lysis by adenoviral replication can strengthen the immune system benefit since it is feasible to expect that the greater the cell dell death caused by the vector, the greater the secondary immunological response. Likewise, if an adenoviral vector with a therapeutic gene were tested in HPV-N immunocompetent models, the expected result would be that it had a greater efficacy than that found in the present study, demonstrating that the antitumoral effects were caused by the therapeutic gene and not only by the adenoviral vector.

Finally, it should be taken into account that an adenoviral vector utilized to fight against cancer is designed to kill cells and preferably, neoplasic cells. If an adenoviral vector, in a parallel manner, importantly affeects other organs, it is probable that the immune response may also increase the adverse effects. Therefore, the main challenge of each vector that is designed to work against a neoplasm is that its effect be limited to cancerous cells.

In conclusion, the present study results support the use of adenoviral vectors in the fight against HPV-N. These vectors are a good therapeutic "vehicle" since they are capable of generating a significant antitumoral response in immunocompetent individuals, even in the absence of replication or a therapeutic gene. Its purpose is not to validate an adenoviral vector lacking a therapeutic gene or replication as an HPV-N treatment option. Even though its effects are significant they should be added to the benefit obtained from the expression of vector-transported genes (pro-apoptotic, suicide or immunogenic) or by cellular lysis brought about by controlled, selective adenoviral replication.

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