Effect of Adeno-associated Virus on Transformation of NIH 3T3 Cells by ras Gene and on Tumorigenicity of an NIH 3T3 Transformed Cell Line

Ehud Katz and Barrie J. Carter

ABSTRACT

Transfection of NIH-3T3 cells with the plasmid pJ234, containing DNA from the human bladder carcinoma T24 cell line (ras gene), results in their transformation. Adeno-associated virus did not affect significantly the number of the transformed foci when different multiplicities of infection were used and when the virus was added to the cultures at different time intervals before or after transfection. A transformed cell line was derived following transfection of NIH 3T3 cells by the ras gene. Infection of these cells with adeno-associated virus resulted in a decrease in their growth rate and cloning efficiency. These infected cells showed a dose-dependent reduction in the frequency and an increase in the latent period for tumor appearance in nude mice.

INTRODUCTION

AAV1 particles significantly reduced tumor formation by human AD types 12 or 31, when both AD and AAV were injected into newborn Syrian hamsters (1, 2). The inhibition of tumors induced in Syrian hamsters by AD-12 did not depend on the infectivity of AAV, since defective interfering particles or DNA from these particles or from infectious AAV were found to be equally efficient (3). Furthermore, infection of adenovirus-transformed hamster cells with AAV, prior to inoculation into newborn hamsters, reduced the tumorigenic potential of these cells (3, 4). AAV infection also decreased the tumorigenicity of herpes simplex virus-transformed hamster cells (5). On the other hand, infection with AAV did not affect the tumorigenicity of a chemically transformed hamster cell line (4).

The aim of the present study was to examine the effect of AAV on the transformation of NIH 3T3 induced by transfection with the activated ras oncogene from human bladder cell carcinoma T24 and to follow the effect of the virus on the tumorigenicity of a ras-transformed cell line.

MATERIALS AND METHODS

Cells and Virus. NIH 3T3 cells are an established line of mouse fibroblasts (6). These cells were obtained at passage 7 beyond the original clonal isolation.EK-4 is an established line of NIH 3T3 cells transformed by pJ234.4 All cells were grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum and antibiotics. AAV2 was grown in KB-3 cells and purified in CsCl, as described previously (8).

Infection. Confluent cell cultures were infected with AAV at a multiplicity of 1000 TCID$_{50}$/cell. After 1 h, the cells were transfected with pJ234 and 2 weeks after they were fixed and stained. The results, presented in Table 1, show that AAV did not affect the formation of foci of transformed NIH 3T3 cells.

When NIH 3T3 cells were infected with different multiplicities of infection of AAV (1000, 200, 100, or 20 TCID$_{50}$/cell) and transfected 1 h later with pJ234, no significant differences were observed in the number of transformation foci which appeared in uninfected cultures, as compared with those in the AAV-infected cells. When AAV infected NIH 3T3 cells at a multiplicity of infection of 1000 TCID$_{50}$/cell, at 4 or 2 h before or 4 h or 1, 2, 5, or 9 days after transfection with pJ234, no significant differences were observed in the number of foci formed. The results show that AAV did not affect the number of transformation foci which appeared following transfection with pJ234 under the different infection conditions used. A significant delay in the time of appearance or a decrease in the size of the transformation foci were not observed either.

Tumorigenicity of AAV-infected EK-4 Cells. The EK-4 cell line was established by use following transfection of NIH 3T3 cells with pJ234, the plasmid containing DNA from the human bladder carcinoma T24 cell line (9). EK-4 shows typical morphology of transformed cells; i.e., the cells are piling up and have apparently lost contact inhibition. They are also able to induce tumors in nude mice. EK-4 cells have additional human ras genes to the resident mouse ras gene which cross-hybridized...
Table 1 Effect of AAV on formation of foci of NIH 3T3 cells by pJ234

<table>
<thead>
<tr>
<th>pJ234 (μg)</th>
<th>-AAV</th>
<th>+AAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>1.25</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>2.50</td>
<td>29</td>
<td>29</td>
</tr>
</tbody>
</table>

NIH 3T3 cells were infected with AAV at a multiplicity of 1000 TCID₅₀/cell and 1 h later were transfected with different amounts of pJ234 and with 1.25 μg of carrier NIH 3T3 DNA, as described in “Materials and Methods.” After 2 weeks the cells were fixed and stained and foci were counted. No. of foci

We wished to determine whether AAV is able to decrease the tumorigenicity of these cells in nude mice by infection of the cells with AAV in vitro, prior to inoculation into the mice. A culture of EK-4 cells was infected with AAV, as described in “Materials and Methods.” One day following infection, the cells were dispersed with trypsin, washed, and resuspended in fresh growth medium. Groups of NIH Swiss nude mice were given s.c. injections of 10², 10³, or 10⁴ cells and the appearance of tumors was followed (Fig. 2). Inoculation of 10² AAV-infected cells caused appearance of tumor in a single mouse of the ten that were injected; this tumor was observed 42 days following inoculation. On the other hand, three tumor-bearing mice were seen when 10³ uninfected EK-4 cells were injected; one of these mice developed a visible tumor on the 26th day and the other two on the 35th day following inoculation. A highly significant inhibition (P < 0.01) in the number of tumor-bearing mice and in the time of appearance of tumors was observed when 10⁴ AAV-infected cells were injected (Fig. 2). However, AAV infection did not exhibit any inhibitory effect on the tumorigenicity of EK-4 when the mice were inoculated with 10⁴ cells (Fig. 2). In another experiment (results not shown), a delay in tumor appearance was caused by AAV also when 10⁴ cells were injected and, to a limited extent, at a dose of 10³ cells. These findings indicate that the inhibitory effect of AAV on the tumorigenicity is no longer exhibited.

Effect of AAV on the Growth of EK-4 Cells in Vitro. In order to determine whether the effect of AAV on the tumorigenicity of EK-4 cells resulted from a direct interaction of the virus and the cells, the growth of the infected cells in vitro was followed. EK-4 cells were infected with AAV, as described in “Materials and Methods,” and dispersed with trypsin on the next day. Flasks (25 cm²) were seeded with 5 x 10⁵ cells and the number of cells was noted daily. The results are shown in Table 2. The most significant effect (P < 0.01) of AAV on the growth of AAV-infected EK-4 cells in vitro is the reduction of 33.3% observed in the saturation density level of the cells in the flasks.

Fig. 1. Detection of pJ234 sequences. KpnI-digested DNA of EK-4 and NIH 3T3 cells was electrophoresed in agarose gel, transferred to nitrocellulose paper, and hybridized to a 3²P-pJ234 probe, as described in “Materials and Methods.” pJ234 = 10 μg of pJ234 containing 10 μg of NIH 3T3 DNA carrier, cleaved with KpnI.

to the human ras probe (Fig. 1). A dot blot examination revealed that EK-4 cells have approximately five additional copies of the human c-Ha-ras-1 gene.³

We wished to determine whether AAV is able to decrease the tumorigenicity of these cells in nude mice by infection of the cells with AAV in vitro, prior to inoculation into the mice. A culture of EK-4 cells was infected with AAV, as described in “Materials and Methods.” One day following infection, the cells were dispersed with trypsin, washed, and resuspended in fresh growth medium. Groups of NIH Swiss nude mice were given s.c. injections of 10², 10³, or 10⁴ cells and the appearance of tumors was followed (Fig. 2). Inoculation of 10² AAV-infected cells caused appearance of tumor in a single mouse of the ten that were injected; this tumor was observed 42 days following inoculation. On the other hand, three tumor-bearing mice were seen when 10³ uninfected EK-4 cells were injected; one of these mice developed a visible tumor on the 26th day and the other two on the 35th day following inoculation. A highly significant inhibition (P < 0.01) in the number of tumor-bearing mice and in the time of appearance of tumors was observed when 10⁴ AAV-infected cells were injected (Fig. 2). However, AAV infection did not exhibit any inhibitory effect on the tumorigenicity of EK-4 when the mice were inoculated with 10⁴ cells (Fig. 2). In another experiment (results not shown), a delay in tumor appearance was caused by AAV also when 10⁴ cells were injected and, to a limited extent, at a dose of 10³ cells. These findings indicate that the inhibitory effect of AAV on the tumorigenicity is no longer exhibited.

Effect of AAV on the Growth of EK-4 Cells in Vitro. In order to determine whether the effect of AAV on the tumorigenicity of EK-4 cells resulted from a direct interaction of the virus and the cells, the growth of the infected cells in vitro was followed. EK-4 cells were infected with AAV, as described in “Materials and Methods,” and dispersed with trypsin on the next day. Flasks (25 cm²) were seeded with 5 x 10⁵ cells and the number of cells was noted daily. The results are shown in Table 2. The most significant effect (P < 0.01) of AAV on the growth of AAV-infected EK-4 cells in vitro is the reduction of 33.3% observed in the saturation density level of the cells in the flasks.

³ E. Katz, unpublished observation.
and the DNA was selectively extracted and analyzed following agarose gel electrophoresis and hybridization, as described in "Materials and Methods." The results (Fig. 3) show that while it is possible to detect single stranded AAV DNA in EK-4-infected cells, there is no evidence for the presence of a double stranded replicating form of AAV DNA in these cells. The single stranded AAV DNA is unreplicated infecting viral genomes. The results suggest that AAV penetrated into the cells but that its DNA failed to replicate in them. A similar fate of AAV DNA occurred in NIH 3T3 cells (Fig. 3).

### DISCUSSION

Both AD and herpes simplex virus are helpers for AAV replication (reviewed in Ref. 16). The tumorigenicity of transformed cells induced by these two viruses was reduced following AAV infection (1—5). On the other hand, AAV did not affect the tumorigenicity of a chemically induced transformed hamster cell line (4). The present study shows that AAV did not affect the in vitro transformation of NIH 3T3 cells by human activated ras oncogene but did decrease the saturation density level, the cloning efficiency in vitro, and the tumorigenicity in vivo of EK-4, a ras-transformed NIH 3T3 cell line.

The results showed that the effect of AAV in reducing the tumorigenicity of EK-4 cells was quantitative and inversely proportional to the cell inoculum. There was also a threshold in the number of cells above which AAV had no effect. These

### Table 2 Growth of AAV-infected EK-4 cells in vitro

Cultures of EK-4 cells were infected with AAV at a multiplicity of 1000 TCID<sub>50</sub>/cell. One day later the cells were dispersed and 5 x 10<sup>4</sup> cells were seeded in each 25-cm<sup>2</sup> flask. The number of cells was noted daily. Cell viability, as determined by trypan blue exclusion, was greater than 95% in all countings.

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of cells in culture</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK-4</td>
<td>5.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>EK-4</td>
<td>1.2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>EK-4</td>
<td>1.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>8.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**Materials and Methods.** The results (Fig. 3) show that while it is possible to detect single stranded AAV DNA in EK-4-infected cells, there is no evidence for the presence of a double stranded replicating form of AAV DNA in these cells. The single stranded AAV DNA is unreplicated infecting viral genomes. The results suggest that AAV penetrated into the cells but that its DNA failed to replicate in them. A similar fate of AAV DNA occurred in NIH 3T3 cells (Fig. 3).

### Table 3 Effect of AAV on the efficiency of cloning

NIH 3T3 and EK-4 cells were infected with AAV at a multiplicity of infection of 1000 TCID<sub>50</sub>/cell and 1 h later growth medium was added. On the following day the cultures were dispersed with trypsin and 2 x 10<sup>7</sup> cells were seeded in each 75-cm<sup>2</sup> flask. After 9 days the colonies were fixed with methanol and stained with 0.1% crystal violet in 0.1 M citric acid.

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of colonies</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK-4</td>
<td>456 ± 10</td>
<td>55.7</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>372 ± 8</td>
<td>52.7</td>
</tr>
</tbody>
</table>

While the average number obtained, for the period of time between the second and the sixth day following seeding, for the uninfected cells was 1.1 ± 0.1 (SD) x 10<sup>9</sup>, that of the AAV-infected cells was 7.6 ± 1.0 x 10<sup>7</sup> cells per culture (Table 2). In another experiment (results not shown), seeding of a smaller number of cells per flask (2 x 10<sup>3</sup>) did not reveal a significant difference in the exponential growth rate of the cells between AAV-infected and uninfected EK-4 cells, during the first 3 days following seeding, before reaching saturation density. Thereafter, a lower saturation density level was observed here too with the AAV-infected cells, as compared to uninfected cells. These results indicate that the most significant difference seen in the growth characteristics of EK-4 cells in vitro, between uninfected and AAV-infected EK-4 cells, involved the saturation density level obtained and not the exponential growth of the cells, until they reach this state. These results are consistent with those of Ostrove et al. (4), who observed that AAV-infected H14b cells stop dividing at a lower density.

The effect of AAV infection on the efficiency of cloning of EK-4 and NIH 3T3 cells was studied. Cultures of EK-4 and NIH 3T3 cells were infected with AAV as described in "Materials and Methods."—, uninfected cells; +, AAV-infected cells. The results (Fig. 3) show that while it is possible to detect single stranded AAV DNA in EK-4-infected cells, there is no evidence for the presence of a double stranded replicating form of AAV DNA in these cells. The single stranded AAV DNA is unreplicated infecting viral genomes. The results suggest that AAV penetrated into the cells but that its DNA failed to replicate in them. A similar fate of AAV DNA occurred in NIH 3T3 cells (Fig. 3).

### DISCUSSION

Both AD and herpes simplex virus are helpers for AAV replication (reviewed in Ref. 16). The tumorigenicity of transformed cells induced by these two viruses was reduced following AAV infection (1—5). On the other hand, AAV did not affect the tumorigenicity of a chemically induced transformed hamster cell line (4). The present study shows that AAV did not affect the in vitro transformation of NIH 3T3 cells by human activated ras oncogene but did decrease the saturation density level, the cloning efficiency in vitro, and the tumorigenicity in vivo of EK-4, a ras-transformed NIH 3T3 cell line.

The results showed that the effect of AAV in reducing the tumorigenicity of EK-4 cells was quantitative and inversely proportional to the cell inoculum. There was also a threshold in the number of cells above which AAV had no effect. These

### Table 3 Effect of AAV on the efficiency of cloning

NIH 3T3 and EK-4 cells were infected with AAV at a multiplicity of infection of 1000 TCID<sub>50</sub>/cell and 1 h later growth medium was added. On the following day the cultures were dispersed with trypsin and 2 x 10<sup>7</sup> cells were seeded in each 75-cm<sup>2</sup> flask. After 9 days the colonies were fixed with methanol and stained with 0.1% crystal violet in 0.1 M citric acid.

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of colonies</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK-4</td>
<td>456 ± 10</td>
<td>55.7</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>372 ± 8</td>
<td>52.7</td>
</tr>
</tbody>
</table>
observations are in agreement with those reported by Ostrove et al. (4) for AAV inhibition of AD-12 tumorigenesis in newborn hamsters. They observed that AAV infection was effective only when the inoculum was smaller than 2 x 10⁶ cells while it was completely abolished when 10⁶ cells were injected into the hamster (4). The reduction in the tumorigenicity of EK-4 cells caused by AAV infection was expressed both as a delay in the appearance of the tumors and a decrease in the number of mice bearing tumors. We followed the fate of the virus in these cells in vitro. The virus did penetrate into the cells; the AAV-DNA remained intact within these cells for at least 3 days but failed to replicate. The infected EK-4 cells showed slower growth rate than the uninfected cultures; the saturation density level of AAV-infected EK-4 cells was reduced by 33.3% as compared to uninfected cells and their cloning efficiency was decreased by more than 50%. It is possible that the direct effect of AAV on the transformed cells shown for the growth in vitro is the cause for the reduction in the tumorigenicity of these cells in vivo. Thus, a slowed cell growth rate may be sufficient to delay or inhibit tumor formation.

These results show that inhibition of tumor formation by transformed cells following infection with AAV is not limited to cells transformed by AAV helper viruses (3–5). Furthermore, the mechanism of tumor inhibition may be similar in each case, in that infectivity or replication of the AAV genome is not required. It is possible that some structural feature of the AAV genome, perhaps in the termini, mediates the tumor inhibition by binding a critical factor involved in growth or replication, as suggested before (3).

It was reported that the minute virus of mice, an autonomous parvovirus, inhibited in vitro transformation of mouse 3T3 cells by SV40, as measured by a decrease in transformed cell foci (7). However, our results indicate that AAV did not show a similar decrease in the in vitro transformation of NIH 3T3 cells by the ras oncogene. This suggests that the mechanisms of miniretrovirus tumour inhibition of in vitro transformation and AAV inhibition of in vivo tumorigenesis may be different.

ACKNOWLEDGMENTS

We wish to thank J. Austin and R. Pozzatti from the NIH, Bethesda, MD, for providing us with NIH 3T3 cells and the plasmid pJ234, respectively.

REFERENCES

Effect of Adeno-associated Virus on Transformation of NIH 3T3 Cells by ras Gene and on Tumorigenicity of an NIH 3T3 Transformed Cell Line

Ehud Katz and Barrie J. Carter


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/6/3023

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/46/6/3023.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.