



# ADENOVIRAL MEDIATED GENE TRANSFER CAN BE ACCOMPLISHED IN HUMAN MYELOID CELL LINES AND IS INHIBITED BY ALL-TRANS RETINOIC ACID-INDUCED DIFFERENTIATION

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## ABSTRACT

**Background and Objective.** Gene transfection could potentially represent a useful therapeutic tool for genetic and neoplastic hematological diseases. After having long been considered poorly able to transfect myeloid cells, adenoviral vectors have recently been demonstrated to be capable of introducing *foreign* DNA into purified CD34<sup>+</sup> cells from human bone marrow or cord blood. In the present study we evaluated the feasibility of adenoviral-mediated gene transfer in two human leukemic cell lines, both at baseline and after differentiation induction by all-trans retinoic acid (ATRA).

**Methods.** We used a recombinant adenovirus expressing  $\beta$ -galactosidase (Ad-RSV- $\beta$ -gal) to transfect K562 and HL-60 cell lines. The effects of 10<sup>-6</sup>M ATRA were evaluated after 8 days of expo-

sure. The efficacy of transfection was verified by X-gal staining.

**Results.** Ad-RSV- $\beta$ -gal was able to transfect both the HL-60 and, to a minor extent, the K562 cell lines. The addition of ATRA had no effect on transfection of K562 cells, while a lower percentage of  $\beta$ -gal-positive cells was detected in HL-60, which underwent differentiation upon ATRA treatment.

**Interpretation and Conclusions.** These data suggest that adenoviral-mediated gene transfer could be feasible in myeloid leukemia cell lines and that it is inhibited by ATRA in differentiation-sensitive cells. The latter effect merits further investigation in order to verify whether this represents an ATRA-related or a differentiation-related phenomenon.

*Key words: adenoviral vectors, cell lines, ATRA*

The availability of gene transfer technologies could contribute to understanding the mechanisms underlying genetic and neoplastic diseases.<sup>1,2</sup> The development of vector systems that can efficiently deliver therapeutic genes to appropriate target cells *in vivo* or *ex vivo* could permit clinical use of gene therapy. Of the various gene delivery systems currently in use, viral vectors appear to be the most efficient, since they allow transfection of genetic material in a large percentage of exposed cells.<sup>3</sup> In hematology, retroviral vectors have been the most widely employed so far because they have demonstrated a capacity to transfect a high percentage of cells, both normal<sup>4</sup> and neoplastic.<sup>5</sup> Conversely, a few studies have been published concerning the interaction of adenoviral vectors and myeloid cells<sup>6,7</sup> and discordant results have been reported concerning transfection of lymphoid cells.<sup>8-10</sup> Adenoviral vectors, however, could be potentially interesting for the transfection of hematopoietic cells, since they are also able to interact with non-cycling cells.<sup>3</sup> In addition, the

slight possibility of incorporation into the host genome reduces the chances of recombination. The short-term transgene expression caused by adenoviral vectors could render them attractive for short-term delivery of cytokines, such as thrombopoietin,<sup>11</sup> or anti-oncogene expression for bone marrow purging.<sup>12</sup> Recently, several authors have reported that adenoviral-mediated gene transfer can easily be achieved in CD34<sup>+</sup> cells from human bone marrow or cord blood.<sup>13,14</sup> In the present study we evaluated the feasibility of adenoviral-mediated gene transfer in two human leukemic cell lines, HL-60 and K562. For this purpose, we used a vector carrying a reporter ( $\beta$ -gal) gene; this could represent the first step towards the infection of cells with adenoviral vectors carrying a functional gene, such as p53 or herpes virus thymidine kinase (TK). Furthermore, we tested the effects of pre-treatment with all-trans retinoic acid (ATRA) on viral transfection. ATRA currently represents the first-line treatment for acute promyelocytic leukemia (APL).<sup>15</sup> This compound exerts its action by inducing differ-

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Acknowledgments: work supported in part by MURST 40%. The authors are grateful to Dr. Michel Perricaudet and Dr. Isabella Saggio (Institute Gustave Roussy, Villejuif, France) for kindly providing the recombinant adenovirus.

Received January 17, 1997; accepted May 20, 1997.

entiation of leukemic promyelocytes into mature granulocytes.<sup>16</sup> HL-60 cells have been observed to undergo differentiation upon treatment with ATRA,<sup>17</sup> while K562 cells are relatively insensitive.<sup>18</sup> The investigation of adenoviral mediated gene transfer in these two models could contribute to our understanding of whether granulocytic differentiation has any effect on adenovirus entry into cells.

## Materials and Methods

### Cell lines

Exponentially growing HL-60<sup>19</sup> and K562<sup>20</sup> cell lines were used throughout the study. The cells were cultured in 25mm sterile plastic flasks containing RPMI 1640 medium (Gibco Europe, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco). The flasks were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub> and the cultures were renewed three times a week. Under these conditions, the doubling time was approximately 24 hours for both cell lines.

### Recombinant adenovirus

Recombinant adenovirus expressing  $\beta$ -galactosidase (Ad-RSV- $\beta$ -gal, kindly provided by Dr Michel Perricaudet, Institut Gustave Roussy, Villejuif, France) was derived from an E1-E3 deleted type 5 adenovirus.<sup>21</sup> Defective virus contains the *Escherichia coli* lac-Z gene, driven by the Rous sarcoma virus (RSV) promoter as a reporter. The virus was produced in the human embryonic kidney cell line 293, and was purified and titred using standard techniques.<sup>21</sup>

### ATRA treatment

ATRA, kindly provided by Hoffman-La Roche (Basel, Switzerland), was dissolved in absolute ethanol to a concentration of 10<sup>-2</sup>M and further dilutions were performed in RPMI 1640 medium. Next, 1 × 10<sup>6</sup> HL-60 or K562 cells/mL were seeded in 10 mL RPMI + 10% FCS; ATRA was added at 10<sup>-6</sup>M and control cultures received the same amount of media without the drug. Every other day cells were pelleted and resuspended at 1 × 10<sup>6</sup>/mL in fresh medium  $\pm$  ATRA. After 8 days cells were collected and treated as described below. This ATRA dose and incubation time were chosen on the basis of previously reported data<sup>22</sup> which showed that maximal differentiation of HL-60 cells was thus obtained.

### Transfection of leukemic cell lines

One  $\times$  10<sup>6</sup> HL-60 or K562 cells, either untreated or treated with ATRA, were resuspended in 1 mL RPMI 1640 + 2% FCS. The cells were incubated in triplicate in 24-well plates with Ad-RSV- $\beta$ -gal at different multiplicities of infection (MOI: 0, 5, 10, 25 and 100). After 24 hours cells were washed three times, and following viability assessment by trypan blue dye exclusion, they were either immediately assayed for transfection or resuspended in fresh RPMI + 10% FCS for an additional 24 and 48 hours, after which transduction efficiency was evaluated. This was accomplished by washing HL-60 or K562 cells in PBS and fixing them with 0.05% glutaraldehyde in PBS for 10<sup>1</sup> at room temperature. After two washings, cells were stained with X-gal solution containing 10 mM K<sub>3</sub>Fe(CN)<sub>6</sub> + 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O + 1 mM MgSO<sub>4</sub> + 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galacto-pyranoside, Boehringer Mannheim, Germany). Cells were incubated overnight at 37°C and cytopspins prepared; dark blue cells were counted as  $\beta$ -gal positive. At least 500 cells were scored. All experiments were performed in triplicate and repeated a minimum of three times.

### Immunophenotyping of HL-60 cells

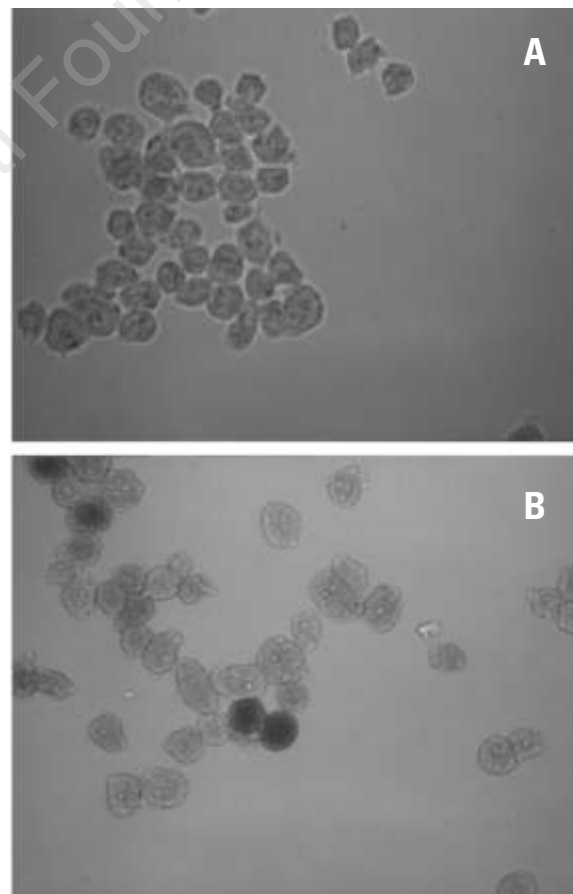
The membrane expression of CD13, CD33, CD15, CD11b (Becton Dickinson, San José, CA, USA) was evaluated in HL-60 cells both at baseline and after 8 days of incubation with ATRA. Data analysis, upon acquisition of 10,000 events, was performed using a FACScan flow cytometer (Becton Dickinson)

equipped with Lysis II software. The results were expressed as percentage of positive cells (compared to background fluorescence, for which mouse IgG was used in place of monoclonal antibodies). Experiments were repeated at least three times.

## Results

### Transfection of leukemic cell lines

The efficiency of adenoviral-mediated gene transfer to K562 and HL-60 cell lines is reported in Figure 1. A dose-response relationship between the MOI used and the percentage of  $\beta$ -gal-positive cells was observed in both cell lines. The highest percentage of cells expressing  $\beta$ -gal was detected 48 hours after infection; this is in line with what has been reported by other groups in different cellular models.<sup>6,23,24</sup> HL-60 cells were more efficiently transfected: 51.5%  $\beta$ -gal-positive cells were observed using Ad RSV- $\beta$ -gal at 100 MOI, compared to 39.2% in K562. It is interesting to notice that we exposed the cells to a lower viral load than that used in the majority of other studies.<sup>7,13,14</sup> At 72 hours  $\beta$ -gal expression reached a *plateau* or started



**Figure 1.** HL-60 cells untreated (A) or treated with Ad-RSV- $\beta$ -gal at 10 MOI for 24 hours (B). Both control and infected cells were incubated for 48 hours in RPMI medium and subsequently stained with X-gal solution as described in the Methods section. Dark blue cells were scored as positive.

a slight decline; this could be attributed to a *dilution* of the reporter gene by cell doubling or to degradation of viral DNA, or to a combination of both mechanisms as reported elsewhere.<sup>6</sup> No viral-related toxic effects were observed since cell growth and viability were comparable in infected and non-infected samples.

#### Effects of ATRA

ATRA is known to induce differentiation of HL-60 cells along the granulocytic pathway,<sup>17</sup> while it does not affect K562 cells (Table 1). Immunophenotypic evaluation of HL-60 cells after incubation with ATRA (Table 2) showed increased expression of CD11b and CD15, and a slight decrease of CD13, as previously reported.<sup>25</sup> CD33 expression remained unchanged.

After 8 days of exposure to ATRA,  $\beta$ -gal expression in K562 cells did not differ significantly with respect to untreated samples (Figure 2A); by contrast, ATRA-treated HL-60 showed a significantly lower percentage of  $\beta$ -gal positive cells compared to control, both at 24 ( $p = 0.02$ ) and 48 hours ( $p=0.04$ ) after infection (Figure 2B).

#### Discussion

Adenoviral vectors are currently considered to be potentially useful for human gene therapy. Deletion of the E1 and E3 regions renders them capable of harboring a large amount of *foreign* DNA and, unlike retroviruses, they have the advantage of infecting quiescent cells too.<sup>3,26</sup> Their poor ability to integrate into the DNA of host cells makes them safer than retroviruses for clinical use since the possibility of insertional mutagenesis is reduced. Several pre-clinical or clinical studies have been conducted using adenoviral vectors, the most remarkable of which are probably the ones concerning the treatment of cystic fibrosis by inserting the cystic fibrosis transmembrane conductance regulator gene into bronchial or nasal epithelium.<sup>27-29</sup> Other experimental applications of adenoviral vectors include the introduction into neoplastic cells of tumor suppressor genes like wild type p53,<sup>30</sup> the insertion of cytokine genes for cancer immunotherapy<sup>23,24</sup> and the transfection of suicide genes in order to eradicate selected cell populations.<sup>31</sup>

Contrasting results, however, have been reported concerning the transfection of bone marrow cells with adenoviral vectors; Mitani *et al.*<sup>6</sup> described productive infection of exposed cells, while Sanchez *et al.*<sup>32</sup> used an adenoviral vector harboring a suicide gene for bone marrow purging, exploiting its selectivity for epithelial tumors vs bone marrow cells. But recently several reports have pointed out that CD34<sup>+</sup> cells from bone marrow or cord blood can be efficiently transfected using adenoviral vectors.<sup>13,14</sup>

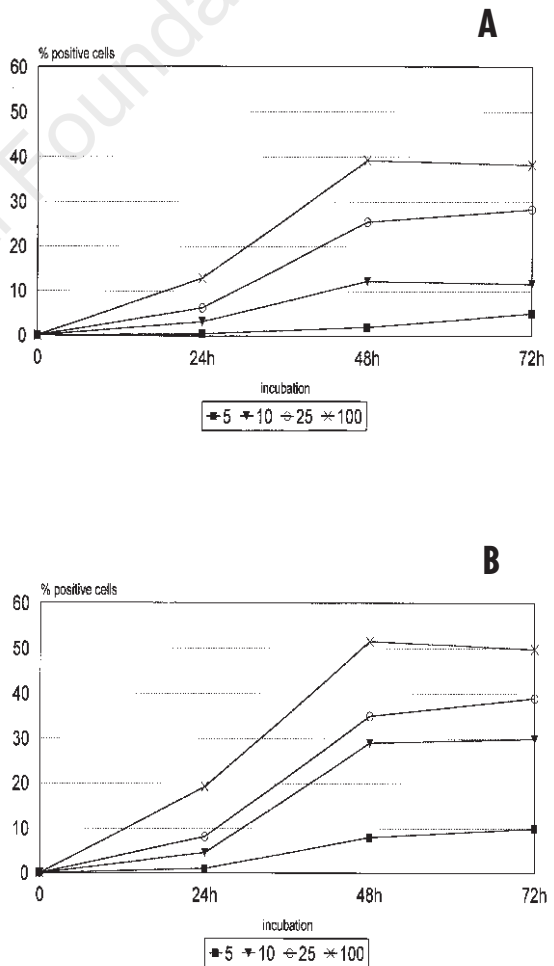
In the present study we transfected two human

**Table 1. Differentiation inducing effects of ATRA.**

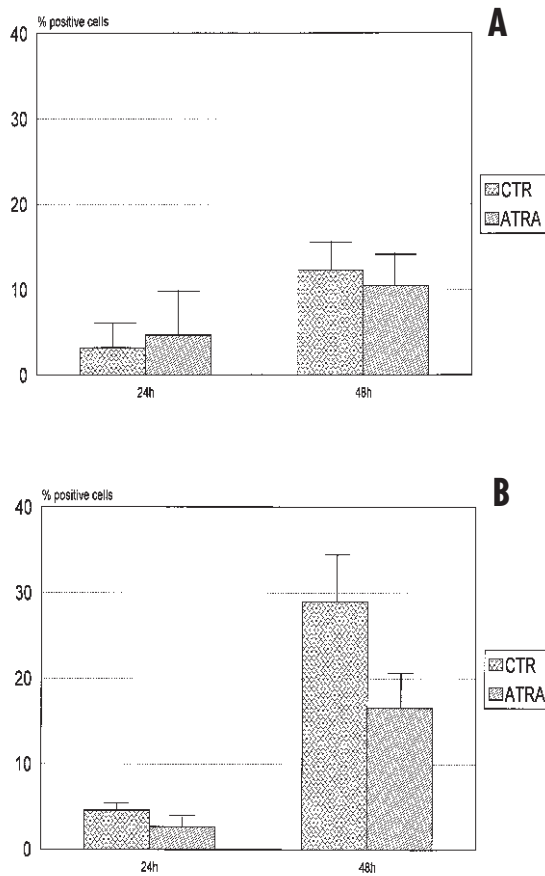
	control (%)				ATRA (%)			
	blasts	myelo	metamyelo	gran	blasts	myelo	metamyelo	gran
K562	100	0	0	0	100	0	0	0
HL-60	100	0	0	0	15	3	52	30

**Table 2. Immunophenotypic evaluation of HL-60 cells.**

	control (%)	ATRA (%)
CD13	100 $\pm$ 0.5	87 $\pm$ 1.5
CD33	100 $\pm$ 2.0	100 $\pm$ 1.5
CD15	8 $\pm$ 5.5	67 $\pm$ 7.5
CD11b	12 $\pm$ 3.5	100 $\pm$ 3.0



**Figure 2. Percentage of  $\beta$ -gal-positive K562 (A) or HL-60 (B) cells after incubation with Ad-RSV- $\beta$ -gal at different MOI (5, 19, 25 and 100).**



**Figure 3. Percentage of  $\beta$ -gal-positive K562 (A) or HL-60 (B) cells with or without ATRA pre-treatment. Cells were infected with Ad-RSV-8- $\beta$ -gal at 10 MOI and scored 24 hours and 48 hours after transfection.**

myeloid cell lines with an adenoviral vector, and we achieved reporter gene expression in a high percentage of cells. Our data are slightly dissimilar from those obtained by Wattel *et al.*;<sup>7</sup> this is probably due to a difference in mode and time of incubation. According to our findings adenoviral vectors can be considered suitable systems for gene delivery, even in neoplastic myeloid cells, and their potential should be further exploited in hematology. Short-term transgene expression can be useful when permanent correction of a defect is not required (e.g. cytokine secretion or immunotherapy),<sup>23, 24</sup> or when the side effects of continued production of the designed protein are too dangerous.<sup>11</sup>

In order to evaluate the effects of differentiation on adenoviral-mediated gene transfer, both cell lines were incubated with ATRA prior to transfection and we observed a reduction in the percentage of cells expressing  $\beta$ -gal only in HL-60, which is

known to undergo granulocytic differentiation upon treatment with ATRA. We do not yet know whether this behavior was due to differentiation or to ATRA itself. This compound is able to modulate surface adhesion molecules in APL cells,<sup>33</sup> and this phenomenon was confirmed by our finding of a modified HL-60 phenotype after ATRA treatment. It has been demonstrated that adenovirus entry into cells is promoted by surface expression of  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrins,<sup>34</sup> even though this does not seem to influence attachment to cells.<sup>35</sup> It could be hypothesized that in HL-60 cells the modification of surface integrins induced by ATRA could inhibit adenoviral-mediated transfection. On the other hand, up to now no study had been performed to evaluate variations of adenoviral transfection when target cells were exposed during the various stages of differentiation. This phenomenon merits thus further investigation in view of a wider application of adenoviral vectors in hematology.<sup>36</sup>

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