CORRESPONDENCE

Adenoviral Vectors and Hematopoietic Cells

To the Editor:

Watanabe et al[1] concluded that the adenoviral vector could be used to deliver transcription units into early bone marrow precursor cells at the $CD34^+$ stage, and into precursors of colony-forming unit–granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and high proliferative potential–colony-forming cells (HPP-CFC). We request that Watanabe et al respond to questions about their data that may lead to other interpretations of their data. Is it possible that their data do not conclusively prove that the adenovirus is a useful vector for modification of early hematopoietic precursor cells?

Watanabe et al based their claims that they have succeeded in using the adenoviral vector to introduce β-galactosidase transcription unit into $CD34^+$ cells on two-color flow cytometry analysis (see Fig 2 of their report) of bone marrow cells exposed to a β-galactosidase adenoviral vector for 24 hours. In Fig 2 of their report, Watanabe et al attempted to measure the total number of cells that were simultaneously positive for a lineage- or stage-specific hematopoietic differentiation marker and a fluorescein-conjugated substrate (FDG) for the β-galactosidase reaction. (The adenoviral vector used for their infections contained a transcription unit for an enzyme, β-galactosidase, that would modify the FDG and thereby promote its retention within the cells infected by the adenoviral vector.) We were surprised by the fact that exposure of the hematopoietic cells for 24 hours to the adenoviral vector appears to have resulted in a threefold increase in the percentage of cells that are positive for the markers CD2, CD13, and CD56, whereas exposure to the adenoviral vector for 24 hours does not increase the percentage of cells that are $CD34^+$. Our analysis of their data, which led to this conclusion, is presented in Table 1. We have taken the data in Fig 2 of their report and added the percentages of cells that are positive for each marker in the uninfected cells and in cells exposed for 24 hours to a 5-, 50-, and 500-fold excess of plaque-forming units of the vector.

Two things are immediately apparent from this data. (1) Surprisingly, the total percentage of marker-positive cells (CD2, CD13, and CD56) in the uninfected or vector nonexposed cells is not the same as in the cells exposed to 24 hours of exposure to the vector. This exposure induces a threefold increase in the percentage of cells that are positive for the lineage-restricted antigens CD2, CD13, and CD56. In the uninfected cells, most of the cells are double-negative; in the infected cells, most are double-positive. Clearly, the percentage of cells positive for each of the differentiated markers is not stable in the population. In addition, the higher the dose of vector, the more there is a change from differentiated marker-negative to differentiated marker-positive cells. This finding suggests that the presence of the vector induces differentiation in the hematopoietic cells. (2) The virus does not induce an increase in the percentage of cells that are positive for the CD34 antigen. This finding suggests that, whatever the vector is doing to induce differentiation in the lineage-committed cells, this does not occur with the earlier $CD34^+$ cells. Their number appears to remain constant in the presence of the vector.

Table 1. Increase in Differentiated Cells After Adenovirus Exposure

	Uninfected		Infected	
	0 PFU/Cell	5 PFU/Cell	50 PFU/Cell	500 PFU/Cell
Total $CD2^+$ (%)	20.2	46.7	68.8	60.3
Total $CD13^+$ (%)	24.6	46.5	87.9	73.6
Total $CD56^+$ (%)	13.1	15.1	47.0	49.4
Total $CD34^+$ (%)	74.0	70.5	63.3	73.0

The fact that the percentage of marker-positive cells is changing in the uninfected and infected cells calls into question the validity of the method used by the investigators to calculate the percentage of cells that was infected by the vector. The formula they used is as follows:

$$\text{Frequency (\%) of Infected Cells (for } CD2^+\text{, as an example)} = \frac{\%\ CD2^+FDG^+\ \text{(Infected} - \text{Uninfected) Cells}}{\%\ CD2^+\ \text{of Infected Cells}} \times 100$$

Because the total percentage of differentiated marker-positive cells is not equal in the uninfected and vector exposed cells, because the number of differentiated marker-positive cells increases in the viral exposed cells, and because the investigators subtract the total number of double-positive cells (differentiated marker-positive and vector marker-positive cells) in the uninfected from the vector exposed cells to calculate the total number of infected cells, their calculated increase in the fluorescein-positive or infected cells before and after exposure to the vector is generated both by the increase in the number of differentiated marker cells and by any uptake of vector by these cells. This invalidates the method used to estimate infectivity. For their method to be valid, the percentage of the differentiated marker-positive cells should be the same in the unexposed and exposed cells.

An additional weakness of the design of the experiments in Fig 2 is that Watanabe et al chose not to restrict their measurements to live cells through the use of propidium iodide gating. In view of the dramatic direct effect on the cells exposed to the vector, as evidenced by the threefold increase in the level of differentiated cells, it is possible that the majority of the cells on which the investigators were making measurements, which formed the basis for their claims of gene delivery to hematopoietic cells by adenoviral vectors, were dying or dead, due to a direct differentiation induction effect of the vector. If Watanabe et al have data on propidium iodide-gated cells, they should share it.

Another weakness is the high endogenous β-galactosidease–like activity within the population of cells that the investigators were studying, as evidenced by the positivity in the uninfected cells in the FDG gate of up to 14%. Watanabe et al could have used chloroquin to inhibit this activity, but they chose not to do that, further complicating the interpretability of their experiments. If the cells were being differentiated by the exposure to the vector, it is possible that the level of endogenous β-galactosidase activity was increasing in the vector-exposed cells as well. If Watanabe et al had used chloroquin, the contribution of this type of potentially artifactual positivity to their measurements of infectivity would have been more limited.

A third surprising feature of their data in Fig 2 is the very marginal and low intensity of most of the fluorescein- or vector-positive $CD34^+$ cells. This finding suggests that the transcriptional promoter used in their vectors is not very active in $CD34^+$ cells. They used the cytomegalovirus (CMV) promoter to drive the β-galactosidase

transcription unit. We and others have found that this promoter is very active in $CD34^+$ cells and that flow cytometry of positive CD34 cells is usually in the range of 10^2 to 10^3, and not in the range of 10^1, as in the data of these investigators. This suggests that the adenoviral vector really did not infect the $CD34^+$ cells or that the uptake by the cells of the vector was not receptor mediated and therefore not released from the endosome, which is a necessary prerequisite for high levels of transgene expression in adenoviral vectors.

To strengthen their claim that the marginal increases in the percentages of $CD34^+$ cells that are positive for the vector reflect significant uptake of the vector and functional transcriptional activity of the vector transgenes within the $CD34^+$ cells, Watanabe et al should present data on the variation of the percentages of fluorescein-positives in uninfected $CD34^+$ cells to strengthen their argument that there is actually a significant increase in double-positives in the vector-exposed $CD34^+$ cells. In other words, what is the range of variation in the uninfected $CD34^+$ cells for fluorescein positivity. In Table 1 of their report, the range of experimental variation is strikingly different in individual experiments. The question we are asking is whether the small percentage increases of fluorescein-positive cells in the $CD34^+$ population could have arisen from the range of variation of endogenous positives in the uninfected cells? In addition, the investigators should comment on why the majority of their fluorescein-positive $CD34^+$ cells are positive for fluorescein at such a low level of fluorescence intensity and not two or three logs higher for fluorescein, as is observed in other laboratories for the CMV promoter-driven transcription units in $CD34^+$ cells?

The decrease in plating efficiency in Fig 4 of the CFU-GM and BFU-E that accompanied increasing ratios of the vector to the nucleated cell count could have been due to the differentiation induction effect of the vector, seen in Fig 2, rather than a direct toxic effect on the precursors of the CFU-GM and BFU-E.

The claim of the investigators that they have shown that the vector-modified cells were proliferating into CFU-GM may not be unambiguously substantiated by their data. As outlined by Watanabe et al, the starting population of $CD34^+$ cells was between 70% and 80% $CD34^+$. The FDG-sorted positive cells were only 88% to 96% positive. Depending on the plating efficiency of $CD34^+$ cells in methylcellulose, the investigators may or may not be able to conclude that the CFU-GM they saw from the FDG positively sorted population were from the FDG-negative or -positive population. To have had more decisive data on this point, Watanabe et al could have chosen a transcription unit that coded for a resistance phenotype so that they could have more formally tested if CFU-GM containing functional adenoviral vector transcription units were growing from their sorted population.

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REFERENCES

1. Watanabe T, Kuszynski C, Ino K, Heimann DG, Shephard HM, Yasui Y, Maneval DC, Talmadge JE: Gene transfer into human bone marrow hematopoietic cells mediated by adenovirus vectors. Blood 87:5032, 1996

Response

We thank Fu et al for their interest in our report.[1] They correctly point out the controversial nature of the observation that adenoviruses can infect human $CD34^+$ cells. Recently, the observation that adenoviruses are capable of infecting $CD34^+$ cells was confirmed by Neering et al.[2] In addition, at the recent International Society of Experimental Hematology meeting, this observation was also confirmed by Spence et al[3] and Abraham et al.[4,5] We note that studies by Seth et al[6] and Garcia-Sanchez et al[7] have not been able to confirm this observation.

We based our finding that adenovirus vectors containing the β-galactosidase gene can infect $CD34^+$ cells on studies using the flow cytometric detection and isolation of infected cells followed by the analysis of functionality as compared with similarly sorted uninfected cells. In these studies, $CD34^+$ cells were isolated using magnetic beads to a purity of 70.2% to 81.7% that were then infected with Ad/β-gal at a multiplicity of infection (MOI) of 50:1. The infected cells were sorted for double-positive cells ($CD34^+FDG^+$ cells) by flow cytometry, achieving a double-positive cell purity of 88% to 96%. The assay used to show functionality was a CFU-GM assay of 1,000 $CD34^+$ cells/plate. Assuming a cellular purity of 96% $CD34^+$ cells, this would result in the plating of 40 FDG^- cells. In these studies, we obtained 7.3 to 40.7 colonies, suggesting a nearly 100% plating efficiency. In the studies of $CD34^+FDG^-$ cells, which were 95% to 98% pure, 10.6 to 44.4 colonies were obtained per 1,000 cells plated. Thus, the hypothesis proposed in Fu et al's letter that FDG^- cells form the colonies would require a 100% plating efficiency, an unlikely occurrence and one not supported by the observed 4% plating efficiency of the FDG^- cells. Furthermore, the principle concern raised in the letter is based on the information shown in Fig 2, which is not directly relevant to the observation that adenovirus infects $CD34^+$ cells. Rather, this figure suggests that adenoviruses are not only able to infect $CD34^+$ cells but also other hematopoietic and lymphopoietic origin cells.

We do not believe that the hematopoietic cells infected by adenovirus vectors were dying due to a direct differentiation/induction effect of the vector. The isolated cells that were FDG^+CD34^+ were fully functional after isolation by flow cytometric sorting. Furthermore, dead cells would not be found in the gate used in the analysis of these cells, because they have a different forward by side scatter profile.

The suggestion of using chloroquin to inhibit any putative endogenous β-galactosidase activity, although a good suggestion, is difficult to apply because chloroquin is toxic for hematopoietic cells at β-galactosidase–inhibiting levels. The marginal and low intensity of the fluorescein vector-positive $CD34^+$ cells is actually more than a log higher than uninfected cells. However, this is less than other investigators have observed using retroviral vectors for the infection of $CD34^+$ cells. However, this is a similar increase in fluorescence intensity as reported by other investigators using adenovirus vectors.[2,8] Furthermore, we suggest that a comparison of protein levels by retrovirus verses adenovirus vectors, even with the same promoter, may not be valid, because the presence of the Itr and chromosomal integretion can influence gene expression. Furthermore, the range of variation in the uninfected $CD34^+$ cells for fluorescein

positivity was subtracted in all studies; thus, any increase is not seen in control stained cells. Furthermore, the functional analysis of greater than 90% pure $CD34^+$ sorted cells would suggest that it is unlikely that uninfected FDG^- cells contaminating the sorted cells would be responsible for the colony outgrowth.

In other studies (not shown in this letter), we have infected bone marrow cell populations with adenovirus vectors expressing p53 and have shown by reverse transcription-polymerase chain reaction that specific message occurs in the infected but not in control adenovirus infected cells. This provides additional proof that the marrow populations can be infected by adenovirus vectors.

The principle question of this letter is directed to the observation ''that exposure of the hematopoietic cells for 24 hours to the adenoviral vector appears to have resulted in a threefold increase in the percentage of cells that are positive for one of the markers CD2, CD13, or CD56, whereas exposure to the adenovirus vector for 24 hours does not increase the percentage of cells that are $CD34^+$.'' As discussed in the original report, the studies that examined CD2, CD13, and CD56 expression were undertaken on a total bone marrow cell population. In contrast, the studies on $CD34^+$ cells were undertaken on isolated $CD34^+$ cell populations that were approximately 80% pure at the initiation of infection. Thus, there are significant differences in the composition of these two populations, and it is unlikely that such a purified population would vary in their phenotype frequency. Furthermore, because there is no decrease in the frequency of $CD34^+$ cells, this would speak against differentiation as a mechanism, because we would expect CD34 positivity to be lost as progenitor cells differentiate. However, as Fu et al indicate, their appears to be an induction of differentiation of lymphoid cells or some other mechanism that results in an increase in positive cells. We had not stressed this in the original manuscript because it was not central to the original hypothesis and would like to thank Fu et al for identify this intriguing hypothesis. We agree that it warrants further study.

As Fu et al are aware, it is very difficult to do four- or five-color analysis in FACS. Studies using propidium iodide results in fluorescence that can be observed in both the phycoerythrin and the fluorescein channel due to the wide emission spectra of propidium iodide. This would preclude the use of FDG fluorescein as an analysis parameter. Although compensation protocols are available, compensation may cause loss of data. Thus, we have not undertaken propidium iodide studies; furthermore, we suggest that gating parameters would remove the majority of dead cells and believe that the functional data shown in Table 2 successfully speak to this, where, in seven studies, no decrease in functionality was seen in the sorted cells.

Clearly, all studies can be improved upon and the use of a marker construct such as p53 infection of enriched $CD34^+$ cells and the demonstration of message and/or protein would be an excellent improvement. Similarly, membrane expression of adenovirus proteins on $CD34^+$ cells would be another way to further substantiate our conclusion. Both of the studies mentioned above have been initiated and will be published in due course. However, we believe that the studies shown in Table 2 constitute a solid demonstration of adenovirus infectivity of $CD34^+$ cells with the retention of hematopoietic function. Furthermore, the studies of Neering et al,[2] Spence et al,[2] and Abraham et al[4,5] provide confirmation of these observations.

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REFERENCES

1. Watanabe T, Kuszynski C, Ino K, Heimann DG, Shephard HM, Yasui Y, Maneval DC, Talmadge JE: Gene transfer into human bone marrow hematopoietic cells mediated by adenovirus vectors. Blood 87:5032, 1996

2. Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT: Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood 88:1147, 1996

3. Spence S, Schwarzenberger P, McCauslin C, Gooya J, Ruscetti F, Keller J: Stable, targeted gene transfer to human hematopoietic progenitor cell lines by molecular conjugate vectors containing recombinant adenovirus, in Quesenberry PJ (ed): International Society of Hematology, 1996. Charlottesville, VA, Carden Jennings, 1996, p 1059 (abstr 197)

4. Abraham NG, Cook P, Feldman E, Ahmed T: Gene transfer of alpha interferon (IFN-α) into hematopoietic stem cells in chronic myelogenous leukemia (CML), in Quesenberry PJ (ed): International Society of Hematology, 1996. Charlottesville, VA, Carden Jennings, 1996, p 1053 (abstr 157)

5. Abraham NG: Adenovirus mediated gene transfer into human hematopoietic $CD34^+$ stem cells, in Quesenberry PJ (ed): International Society of Hematology, 1996. Charlottesville, VA, Carden Jennings, 1996, p 1053 (abstr 158)

6. Seth P, Brinkmann U, Schwartz GN, Katayose D, Gress R, Pastan I, Cowan K: Adenovirus-mediated gene transfer to human breast tumor cells: An approach for cancer gene therapy and bone marrow purging. Cancer Res 56:1346, 1996

7. Garcia-Sanchez F, Pizzorno G, Adams E, Hanania E, Giles R, Crystal R, Deisseroth AB: Use of a safety recombinant adenovirus vector for selective bone marrow purging carrying the chemosensitization gene cytosine deaminase, in Quesenberry PJ (ed): International Society of Hematology, 1996. Charlottesville, VA, Carden Jennings, 1996, p 1138 (abstr 619)

8. Berger CN, Tan S, Sturm KS: Simultaneous detection of β-galactosidas activity and surface antigen expression in viable hematopoietic cells. Cytometry 17:216, 1994

2-Chlorodeoxyadenosine Treatment in the Sezary Syndrome

To the Editor:

We read with interest the report by Kuzel et al[1] describing the results of 2-chlorodeoxyadenosine (2-CdA) therapy in patients with mycosis fungoides (MF) and the Sezary syndrome (SS). We have also treated four patients for SS. Three of them had advanced disease and were resistant to prior treatments (PUVA, chlorambucil, antilymphocyte globulin, and cyclosporin). One patient (no. 3) was enrolled to the study shortly after diagnosis. Two patients (nos. 2 and 3) had a large-cell variant of SS. The patient characteristics, stage of disease at treatment, and time from diagnosis to treatment are detailed in Table 1. 2-CdA (cladribine; Biodribin; Foundation for Development of Diagnostics and Therapy, Warsaw, Poland) was administered by 2-hour intravenous infusion at a dose of 0.14 mg/kg for 5 days. Courses were repeated at 28-day intervals. 2-CdA treatment was terminated either if there was no response after 5 courses or once prohibitive toxicity occurred. Two patients (nos. 1 and 3) received 5 cycles of therapy and one (no. 2) received 4 cycles,