A new delivery system for 5-fluorouracil using prodrug and converting enzyme

M Akimoto, T Miyahara, J Arai, A Akimoto, H Hamada, Y Yoshida, N Yoshimura

Aims: To evaluate a new delivery system of 5-fluorouracil (5-FU) using 5-fluorocytosine (5-FC) as a prodrug and cytosine deaminase induced in vitro and in vivo.

Methods: Fibroblastic cells from rabbit Tenon’s capsule were cultured. The cells were exposed to 5-FU and 5-FC with or without cytosine deaminase induced by recombinant adenovirus. In the in vitro study, cell proliferation and DNA synthesis were assessed by MTS, BrdU assay. The effect of 5-FC removal after the treatment of 5-FC and cytosine deaminase induction was also assayed. In the in vivo study cells with or without cytosine deaminase induction were transplanted into the subconjunctival space of mice, followed by eye drops of 1000 µg/ml of 5-FC three times a day. The mice were sacrificed at days 1, 5, and 10, and then the cells transplanted were evaluated.

Results: Cell proliferation was inhibited by exposure to 5-FU in a dose dependent manner; however, up to 1000 µg/ml of 5-FC did not affect cell proliferation. Cell proliferation was inhibited by exposure to 5-FC in a time dependent manner with induction of cytosine deaminase following infection of recombinant adenovirus. When 5-FC was removed 3 or 6 days after the treatment, the cells grew again. The effect was reproduced in the in vivo model of subconjunctival cellular proliferation although 5-FC was administrated as eye drops. There were no cases with corneal erosion.

Conclusion: Cell proliferation was inhibited by co-exposure of 5-FC and cytosine deaminase. This new delivery system may merit controlled delivery of 5-FU after filtering surgery.

Materials and methods

Cell culture
Albino rabbit Tenon’s capsule fibroblasts were established in culture as described previously. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, MA Bioproducts, Walkersville, MD, USA), penicillin G (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 mg/ml), then cultured in an incubator at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cells from the 4th to the 10th passage were used.

Construction of adenovirus vector
The replication deficient recombinant adenovirus vector containing cDNA of cytosine deaminase was constructed according to the COS/TF2 method, as previously described. Briefly, a 3664 bp fragment containing the coding region of Escherichia coli cytosine deaminase was obtained from E coli genomic DNA and blunt ended. After purification by gel electrophoresis, this fragment was subcloned into the vector.
expression unit of pAxCAt: a cassette cosmid bearing an expression unit and full length adenovirus type 5 genome with deletions of the E1 and E3 region (a generous gift from Drs I Saito and Y Kanegae). The constructed cosmid was then co-transfected to 293 cells together with the EcoT22I digested adenovirus DNA terminal linking dilution technique, and the virus with the desired gene through homologous recombination were purified by the lim- phosphate precipitation method. The viruses generated protein complex (from Dr I Saito) using the calcium expression unit and full length adenovirus type 5 genome — 80°C (AxCACD).

Cell proliferation

After growing to confluence, the cells were enzymatically detached with 0.05% trypsin (Sigma, St Louis, MO, USA) at 37°C for 3 minutes, and trypsinisation was stopped by the addition of growth medium with 10% FBS (GM). The suspended cells were centrifuged at 1000 rpm for 3 minutes, and after which the pellets were resuspended, diluted to 10^6 cells/ml with GM, and seeded into 96 well plates as 10^3 cells in 100 µl GM. The virus and drug solutions were diluted with GM to reach the final concentrations desired. Of the diluted virus solution or GM, 50 µl were added to each well. The plate was then incubated at 37°C for 30 minutes.

Finally, 50 µl of GM with or without 5-FC (Wako, Osaka, Japan) or 5-FU (Wako, Osaka, Japan) was added to each well. GM, with or without 5-FU or 5-FC, was exchanged every 3 days. The number of cells was evaluated with colorimetric assay using the CellTiter 96 AQ One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) at days 0, 1, 3, 6, 9, 12, and 15, according to the manufacturer’s instructions. Briefly, cells were cultured on 96 well plates. The cell counting solution was made by mixing 100 µl of GM with 20 µl of the reagent for each well. The GM was removed using multichannel micropipettes, after which 120 µl of cell counting solution was added. The plate was placed at 37°C for 45 minutes, and the absorbance at 490 nm was then measured.

DNA synthesis was also evaluated with a 5-bromo-2’-deoxyuridine (BrDU) Labelling and Detection kit III (Roche, Mannheim, Germany) at day 1. Cells were cultured on 96 well plate. Serum free medium with BrdU (50 µM) was added into each well 4 hours before assay. The cells were fixed in 70% ethanol with 0.5 M HCl at –20°C for 30 minutes, then the DNA was fragmented with nuclease at 37°C for 30 minutes. Monoclonal antibody for BrdU conjugated with peroxidase was applied at 37°C for 30 minutes. The cells were then incubated with substrate solution at room temperature for 10 minutes, and absorbance was measured at 405 nm.

**In vivo study**

To evaluate the effect of cytosine deaminase induction in situ followed by eye drops of 5-FC, the method of Khaw et al.19,25 was modified. Fibroblastic cells were infected at multiplicity of infection (MOI) of 10 with AxCAlacZ, or with AxCACD and AxCAlacZ. AxCAlacZ carrying β galactosidase was used as a marker of the cells. Cells were washed with PBS, then harvested, and diluted at a concentration of 10^6 cells/ml GM.

Eight week old mice were anaesthetised by an intraperito- neal injection of diluted sodium pentobarbitone. Subconjunc- tival injections were performed under an ophthalmic surgical microscope. A Hamilton syringe with a 30 gauge needle was inserted into the superior lid until the needle could be observed through the tarsal conjunctiva. A pinhole at the thin bulbar conjunctiva may alter the efficiency of drug delivery, so we used the tarsal conjunctiva although the ultimate target is the bulbar conjunctiva. A total of 10^6 cells in 10 µl of GM were injected. Eye drops of 10 mg/ml of 5-FC were administered three times a day beginning immediately after the treatment. The mice were sacrificed at days 1, 5, or 10, and the lids harvested, and diluted at a concentration of 10^6 cells/ml GM.

The mouse corneas were fixed in 4% paraformaldehyde/ PBS for 2 hours. The lids were then washed in cold PBS overnight and incubated in PBS containing 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 0.01% sodium deoxycholate, 0.02% NP40, 2 mM MgCl_2, 5 mM K,Fe(CN)_6, and 5 mM K,Fe(CN)_4, overnight at room temperature. Each sample was observed by microscopy. All animals were cared for in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and with federal and local regulations.

**Statistical analysis**

All values are reported as mean (SD). Statistical significance was determined with an analysis of variance followed by Dunnet’s procedure in multiple comparisons. The group without treatment was used as the control. The group without
removal of 5-FC, however, was used as the toxic control when the effect of removal of 5-FC was assessed. All experiments in vitro were repeated twice with quadruplicates. Non-parametric analysis was performed using the Kruskal-Wallis test followed by the Mann-Whitney U test. A difference at a level of \( p < 0.05 \) was considered to be statistically significant.

RESULTS

In vitro study

Cytotoxicity of 5-FU, 5-FC, and adenovirus infection

Firstly, the cytotoxicity of each drug or virus was assessed independently. Various final concentrations from 1 \( \mu \text{g/ml} \) to 1000 \( \mu \text{g/ml} \) of 5-FU and 5-FC were tried. As shown in Figure 2A, up to 1000 \( \mu \text{g/ml} \) of 5-FC does not affect cell growth, although 5-FU inhibits cell growth in a time dependent and dose dependent manner (Fig 2B). The toxic effect of 5-FU was not observed on day 1, but was observed on days 3, 6, and 9.

Next, the cytotoxicity of adenovirus infection was assessed. Infection of AxCACD at various concentrations, up to an MOI of 100, did not produce a cytotoxic effect (Fig 2C).

Cytotoxicity of 5-FC after adenovirus infection

The cytotoxic effect of a combination of 5-FC and AxCACD was assessed. The concentration of 5-FC was 1000 \( \mu \text{g/ml} \) because this concentration did not exert a cytotoxic effect. Virus concentration was varied from MOI 1 to 100. A cytotoxic effect was not observed with either 5-FC or infection of AxCACD, although the combination treatment resulted in a cytotoxic effect in a time dependent and dose dependent manner (Fig 3A). The effect on DNA synthesis was also evaluated. The DNA synthetic activity was reduced by 1000 \( \mu \text{g/ml} \) with 5-FC (\( p < 0.001 \)) or high titres of AxCACD (\( p < 0.001 \)), whereas moderate titres of AxCACD had no effect. DNA synthetic activity was markedly suppressed by the combination treatment (\( p < 0.001 \)) (Fig 3B).
Reversal of cell viability with treatment of 5-FC and adenovirus infection followed by removal of 5-FC
A cytotoxic effect was observed following treatment with 5-FC and adenovirus infection. However, it is not known whether cell viability is reversible after removal of 5-FC. Accordingly, 5-FC was removed on days 3, 6, or 9, after which the cells were washed three times with GM. Cell viability was then determined after every 3 days. Cell growth was observed at day 9, 6 days after 5-FC removal on day 3, and at day 15, 9 days after 5-FC removal on day 6. Cellular viability did not reverse after 5-FC removal on day 9 in this observation (Fig 4).

In vivo study
Each sample was observed by microscopy and the tissue staining graded as follows: grade 1, no staining; grade 2, patchy staining; grade 3, marked staining. Blue staining means the cells injected remain. Representative photographs of each grade are shown in Figure 5. In vivo cytotoxic effects of gene induction followed by instillation of 5-FC eye drops were observed with a mouse model (Fig 6). Most treated animals showed marked staining of X-gal on day 1, and there were no significant differences among the groups, with almost all of the control cases showing marked staining on days 1 and 5. Many cases that underwent only cytosine deaminase induction or 5-FC application showed staining up to day 10, whereas no cases showed staining of tarsal conjunctiva at day 10 after cytosine deaminase induction followed by 5-FC application. No animals exhibited corneal erosion.

DISCUSSION
Both 5-FU and MMC have been used as an adjunctive treatment in glaucoma filtering surgery, although MMC is preferred because of its more convenient application. It is easy to apply and is not unpleasant for patients, although application must be limited to a few minutes during surgery and it may be difficult to select the appropriate dosage for each individual eye. In contrast, 5-FU can be administered over a course of several days after surgery. Thus, with the use of 5-FU it is easier to avoid the problems of overdosage, although its administration is less inconvenient for both patients and surgeons. Based on results of previous studies, various factors that are released gradually after administration were studied, which may reduce the inconvenience of 5-FU administration. However, the material should be removed surgically when the drug effect exceeds optimal conditions. Recent studies revealed a possible beneficial effect of a single application of 5-FU during surgery. However, the appropriate dosage for each case appears to be different. Intraoperative single application is presently effective only for low to moderate risk cases and a more controllable drug delivery system is needed.

In this study, we showed that cytosine deaminase induction and 5-FC administration leads to production of 5-FU in vitro and in vivo. Vector administration and 5-FC eye drops induce
Figure 6  In vivo effect of cytosine deaminase induction followed by of 5-FU eye drops. Staining of the cells was evaluated on days 1, 5, and 10. Each number above the bar is the number included. There were significant differences among the groups on day 5 (p < 0.05) and day 10 (p < 0.01). In each subgroup comparison, significant differences are indicated by asterisks. [*p < 0.05].

Kruskal-Wallis test was used for statistics.

Further investigation is needed in rabbit and monkey models mimicking the clinical situation, although we have shown the feasibility of a new drug delivery system.

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Authors’ affiliations

M Akimoto, T Miyahara, J Araji, A Akimoto, N Yoshimura, Department of Ophthalmology, Shinshu University, Matsumoto, Nagano, Japan

H Hamada, Department of Molecular Medicine, Sapporo Medical University, Sapporo, Hokkaido, Japan

H Hamada, Y Yoshida, Department of Molecular Biotherapy Research, Cancer Chemotherapy Center, Cancer Institute, Tokyo, Japan

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