The Use of *E. coli* Cytosine Deaminase Gene as an Example of Suicide Gene Therapy of Cancer

Ewa Missol
Aleksander Sochanik
Stanisław Szala
Department of Tumor Biology
Institute of Oncology
Gliwice, Poland

1. Introduction

Gene therapy of cancer diseases relies upon transferring certain exogenes into affected somatic cells in order to obtain the expected therapeutic effect (1). Two strategies are currently being explored for tumor gene therapy: indirect and direct killing of cancer cells (2-4).

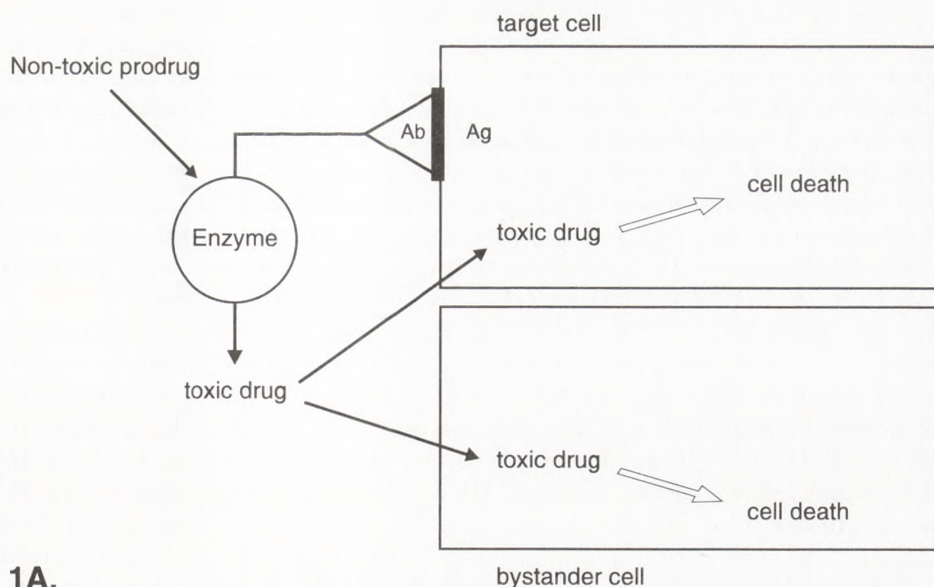
In the indirect approach immunostimulatory genes (such as genes for Il-2, Il-4, Il-6, TNF-α, GM-CSF) are transferred to enhance the local or systemic antitumor response (5,6).

The direct approach to the cancer gene therapy is based on the transfer of the so-called suicide genes (7,8). These bacterial, fungal or viral genes are not normally found in eucaryotic cells. They encode enzymes which are capable of activating a nontoxic prodrug into a cellular toxin. Thus genetically modified cells can be killed after systemic administration of a prodrug.

In the first part of this publication we would like to describe the properties of suicide genes used in gene therapy. In the other part, we will discuss the *E. coli* cytosine deaminase (CD) suicide gene transfer into the growing melanoma tumor *via* cationic liposomes.

2. Suicide Genes in Tumor Gene Therapy

The concept of using suicide genes in cancer gene therapy (genes that induce toxic phenotype in tumor cells only) is explored by the strategy used in conventional chemotherapy (which enhances selective destruction of tumor cells while simultaneously preserving and protecting normal cells) (9-11). The concept of suicide genes is a modification of the so called ADEPT



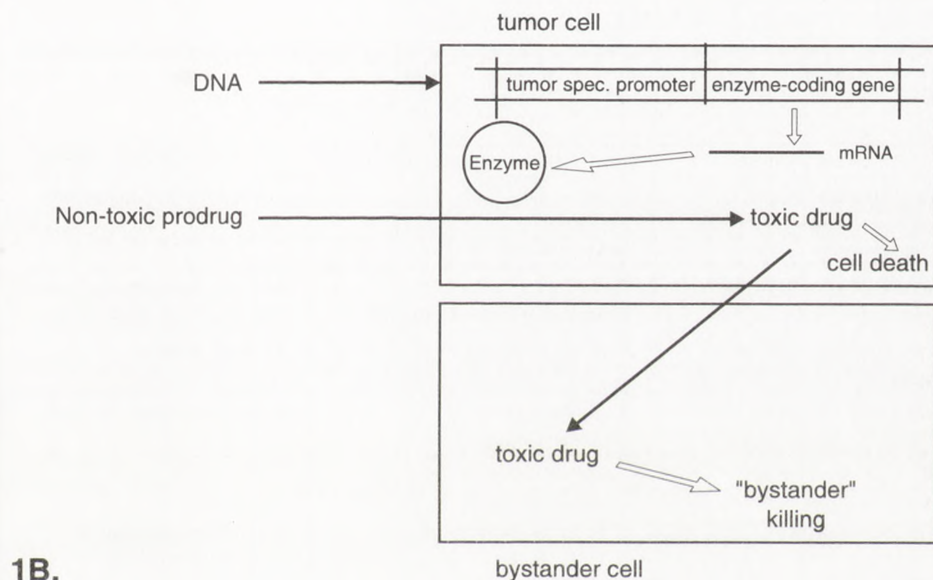


Fig. 1. Two approaches to prodrug activation.

1A. Antibody-directed enzyme prodrug activation (ADEPT). Antibody-connected enzyme is located on the surface of target tumor cell. After systemic administration of non-toxic prodrug toxin is generated by the enzyme. The toxin can penetrate into nearby cells.

1B. Transfer of the so-called suicide genes into tumor cells using carriers. These genes, under the control of tumor-specific promoters are expressed in tumor cells only. Toxin generated within target cells can penetrate into adjacent cells and thus kill them. The killing of unmodified cells is called "bystander" effect.

(antibody-directed enzyme prodrug therapy) concept, and its main idea is to convert nontoxic prodrugs into toxic compounds (drugs) by enzymes linked to antibodies to tumor antigens (12,13) (as shown in Fig. 1A). Due to specific binding of antibodies by tumor cells, high specificity of cell destruction has been achieved by generated toxin.

This method has, however, some disadvantages. The heterogeneity of antigen expression in tumor cell population reduces cells' recognizability by the antibody (a part of tumor cells may loose the antigen or may not contain it at all). Additionally, the introduced enzymes manifest antigenic properties and are recognized as foreign proteins by the immune system.

The modification of the ADEPT method that eliminates immunogenic properties of introduced proteins is based on the transfer of genes encoding toxins or chemosensitizing enzymes to the target cells (for examples see 2). These genes, called suicide genes, do not normally occur in host cells. They are most often of viral, bacterial or fungal origin and upon transfer they can be expressed in tumor tissues, thus inducing a toxic phenotype (14). Enzymes encoded by suicide genes are able to convert nontoxic prodrugs to toxic metabolites which are often active antitumor drugs (e.g. 5-fluorouracil, generated from 5-fluorocytosine via deamination by bacterial cytosine deaminase) (see: Tab. 1).

TABLE 1
SUICIDE GENES USED IN CANCER THERAPY

Encoded protein	Non-toxic substrate (prodrug)	Toxic product (drug)
herpes simplex virus thymidine kinase (HSV-tk)	gancyclovir acyclovir valacyclovir	nucleotide triphosphate
Varicella zoster thymidine kinase (VZV-tk)	6-methoxypurine arabinonucleoside (araM)	adenine arabinonucleoside triphosphate (araATP)
Escherichia coli cytosine deaminase	5-fluorocytosine	5-fluorouracil
Escherichia coli purine nucleoside phosphorylase (PNP)	6-methylpurine-2-deoxyriboside	6-methylpurine (6-MeP)
Escherichia coli nitroreductase	CB1954 (5-aziridin 2,4-dinitrobenzamidine)	5-aziridin 2,4-hydroxyamino 2-nitrobenzamidine
cytochrome P450 2B1	cyclophosphamide (CPA)	acrolein, phosphoramide mustard

Cell sensitivity to given prodrugs, obtained by introducing a suicide gene, depends on the activity of the encoded enzyme, the number of gene copies in the cell and on the strategy of prodrug administration (doses and number of injections).

Transcriptional targeting (*i.e.* use of tissue-specific tumor promoters which prevent exogene expression in incidentally transfected normal cells (Fig. 1B) has been proposed for suicide gene cancer therapy in the absence of carriers (viral or nonviral) able to specifically recognize tumor cells (15) (Tab. 2). Suicide genes are introduced using plasmids which do not integrate with the host genome and, after a transient period of activity, are degradated and removed from the cells.

Table 2
Selective gene expression of potential use in cancer gene therapy

Tumor specific protein	Tissue/Tumor
Carcinoembryonic antigen	Gastrointestinal tract, lung
α-fetoprotein	Hepatoma, teratoma
Dopa decarboxylase	Small-cell lung cancer, neuroectoderm
Neuron-specific enolase	Small-cell lung cancer
Prostate-specific antigen	Prostate
Calcitonin	Thyroid (medullary)
Thyreoglobulin	Thyroid
Polymorphic epithelial mucin	Breast, pancreas
Villin	Gastrointestinal tract, pancreas
erb-B2	Breast, gastrointestinal tract
erb-B3	Breast, gastrointestinal tract
Tyrosinase	Melanoma
Tyrosinase related protein	Melanoma

The suicide genes most often used in *in vitro* and *in vivo* experiments include: *herpes simplex virus* thymidine kinase gene (HSV-tk) (9-11), *Varicella zoster* thymidine kinase gene (VZV-tk) (16), cytosine deaminase gene from *Escherichia coli* (CD) (17-19), purine nucleoside phosphorylase gene from *E. coli* (PNP or DeoD) (20), nitroreductase gene from *E. coli* (21) and cytochrome P450 2B1 gene (22).

Induction of toxic phenotype in the treated tumor does not require suicide gene transfer into all tumor cells. In *in vivo* experiments, the introduction of suicide *herpes simplex virus* thymidine kinase gene into glial cells using vector-producer cells (VPC) resulted in a complete tumor regression even though retroviral particles did not penetrate all cells (23,24). The phenomenon based on penetration of the generated toxin into unmodified adjacent cells and their subsequent destruction is called "bystander" effect (Fig. 1B). Its magnitude depends on the type of toxin generated: for example *E. coli* PNP enzyme converts nucleosides into toxic nucleotides which easily diffuse across cellular membranes (20,25). Sorscher et al. (20) observed that 1-2%

of DeoD gene-transfected tumor cells were sufficient to destroy remaining 98-99% of unmodified tumor cells. Similar "bystander" effect was observed in the case of CD gene from *E. coli* (26). In the case of HSV-tk gene this effect was less significant (10-50% HSV-tk gene transfected cells were needed) (23,24,27,28). Phosphorylated derivatives of gancyclovir, acyclovir etc. are transported inside the cells by active transport or penetrate into other cells via gap junctions (27,28). These two processes are less effective than simple diffussion.

The "bystander" effect in the case of HSV-tk gene appears to be related to the process of apoptotic cell death when HSV-tk positive cells are exposed to gancyclovir. Detailed studies by Freeman et al. (27), confirmed by microscopic observations, show that HSV-tk gene transfected cells exhibit characteristics of apoptotic cell death: degeneration of nuclear membrane, chromatin condensation, cell detachment and vesicle formation. Apoptotic vesicles, probably containing toxic nucleotide triphosphates, are phagocytized by adjacent cells. Prevention of apoptotic vesicles' formation also prevented the "bystander" effect.

In accordance with general strategy of cancer gene therapy it appears that suicide genes should be introduced directly either into tumor cells or systemically into the bloodstream (for more about nonviral and viral carriers see 44).

One way of introducing suicide genes intratumorally *in vivo* is to use viral vectors. But viruses are strongly immunogenic. They cannot be used for multiple injections. In addition, they cannot be used for systemic administration into the bloodstream.

A much safer method of gene delivery in cancer therapy consists of introducing genes via nonviral systems like liposomes and "synthetic" viruses (carriers).

In the next part of this article we shall demonstrate the results of introducing *E. coli* CD gene into B16(F10) melanoma tumor cells *via* cationic liposomes.

3. The Use of Cytosine Deaminase Suicide Gene from *Escherichia coli* in *In Vivo* Cancer Gene Therapy

Cytosine deaminase gene from *E. coli* encodes an enzyme which converts nontoxic 5-fluorocytosine to highly toxic 5-fluorouracil. 5-fluorouracil is a chemotherapeutic agent, generally applied to gastrointestinal tumors. Its metabolites (phosphorylated nucleotides) disrupt DNA and RNA synthesis (17-19). Some of the first trials using CD gene for producing toxic 5-fluorouracil in tumor gene therapy were those of Nishiyama et al. (29). These researchers were introducing semipermeable capsules filled with CD enzyme into solid glial tumors. These capsules released a sufficient quantity of 5-fluorouracil to obtain growth reduction of experimental tumors. Cytosine deaminase in

combination with monoclonal antibody (MAb-enzyme/prodrug combination) was used in a few experiments (30-32) analogous to the ADEPT approach.

Independently, Mullen et al. (17,33), Huber et al. (18,26) and Sikora et. al (19) initiated experiments testing therapeutic usefulness of *in vitro* CD gene-transfected cell lines (colorectal carcinoma, fibrosarcoma, adenocarcinoma and breast cancer).

Our own model of murine B16(F10) melanoma therapy that makes use of CD suicide gene strategy is based on cationic liposomes acting as *in vivo* carriers of the exogene (34). *E. coli* CD gene (35) was cloned into the plasmid pBCMGSNeo (36). The plasmid pBCMGSNeo is a bovine papilloma virus DNA expression vector non-integrating with the host genome. The 14,5 kb plasmid contains the neomycin resistance gene(neo) whose presence makes the selection of transfected cells in *in vitro* culture possible. For transcriptional targeting the CMV promoter was deleted and instead the tyrosinase promoter specific for melanoma was cloned upstream of the CD gene (37). Under the control of this promoter, the CD gene is expressed only in melanoma cells (38,39). Cloning and the procedures of plasmid preparation (pBCMGSNeo/CD/Tyr with CD gene and tyrosinase promoter) are described in detail (see 45). For C57Bl/6 mice implanted subcutaneously with B16(F10) melanoma cells previously transfected *in vitro* with the CD gene, tumor growth inhibition was observed after intraperitoneal injections of 5-fluorocytosine (750 mg/kg body weight/one injection). This provides evidence that the suicide system with the CD gene inserted into pBCMGSNeo plasmid does indeed work according to our expectations.

We then tried to apply an effective method for selective killing of tumor cells. For this purpose we introduced the suicide CD gene *in vivo* using cationic liposomes DDAB/DOPE (DDAB is dimethyldioctadecylammonium bromide, DOPE-dioleoyl-L-α-phosphatidylethanolamine) (40). Nonviral carriers such as liposomes are safe but not very efficient (41). Effectiveness of *in vivo* transfection after single injection of the gene amounts to 0,1-1% of all tumor cells and can be increased by multiple injections of the transfection complexes (DNA and liposomes) (42). Even though the percentage of transfected cells is not very high (only 1-2%) we think that a suitable strategy of gene and prodrug administration can result in a sufficient therapeutic effect.

In a typical experiment 2,5 x 10^5 B16(F10) melanoma cells in 0,1 ml PBS were injected subcutaneously in C57Bl/6 mice. Intratumoral injections of transfection complexes were done as follows: 10 μg of DNA (plasmid pBCMGSNeo/CD/Tyr) and 20 μg of DDAB/DOPE (1:1 w/w) liposomes in 200 μl lactated Ringer solution was injected every other day in the morning for two weeks beginning on the sixth day of the experiment after cell injection. 5-fluorocytosine (5-FC) prodrug solution in PBS (15 mg/2mlPBS/one injection) was injected peritoneally twice a day (or once on the day when the mice received the transfection complex). After the treatment with liposome-DNA complexes and simultaneous administration of 5-fluorocytosine we observed a strong therapeutic effect: tumor growth inhibition and prolonged

survival of treated mice compared to control mice, receiving only B16(F10) cells or B16(F10) cells and transfection complexes but no 5-fluorocytosine (Fig. 2 and 3).

We think that the therapeutic effect mainly depends on two elements: high "bystander" effect (CD enzyme generates membrane-permeant toxin 5-fluorouracil) and multiple-injection strategy. The best results of suicide gene therapy were observed in the case of small tumors (3-5 mm of diameter). In the case of large tumors the growth was too fast in comparison with the rate of tumor cells' destruction and only tumor growth stabilization was observed but not complete tumor regression.

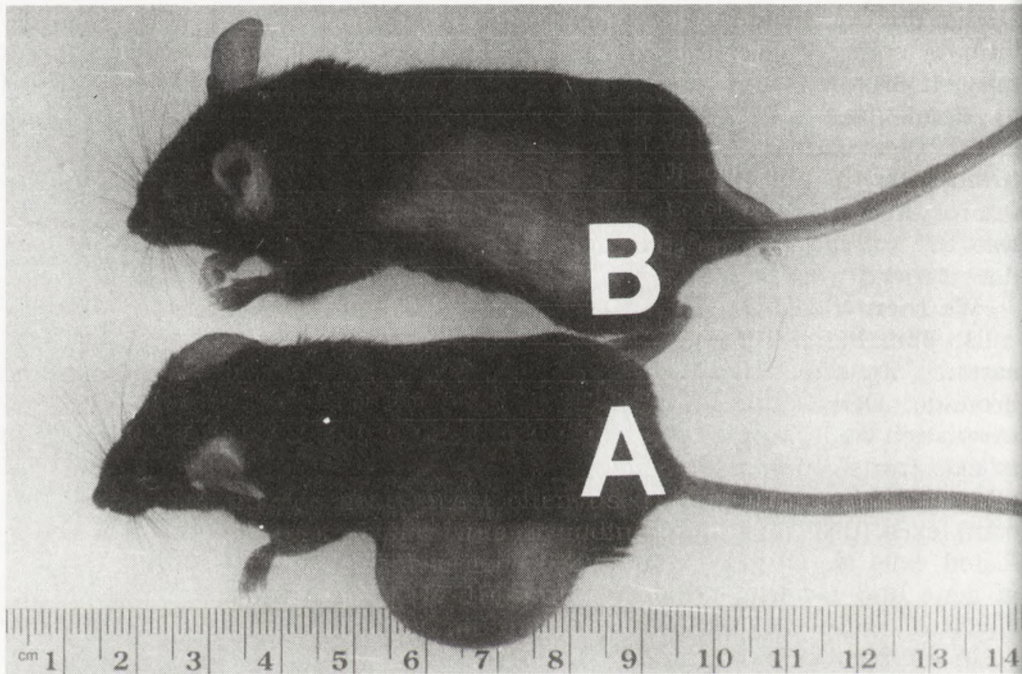


Fig. 2. Tumor growth inhibition of established B16(F10) melanoma tumors following injections of DNA-liposome complexes and concominant administration of 5-fluorocytosine.

Control mouse (A) received subcutaneously 2,5 x 10^5 B16(F10) cells in 0,1 ml PBS.

Treated mouse (B) after initial injection of 2,5 x 10^5 B16(F10) cells received intratumoral injections of DNA-liposome complexes as follows: 10 μg of DNA and 20 μg of DDAB/DOPE (1:1 w/w) in 0,2 ml lactated Ringer solution every other day in the morning for two weeks begining on the sixth day of the experiment. 5-fluorocytosine solution in PBS (15 mg 5-FC in 2 ml of PBS) was injected intraperitoneally twice a day (or once on the day when the mouse received the transfection complexes).

Animals are pictured on the 26th day of the experiment.

Additionally, the therapeutic effect *in vivo* was limited by high intratumoral pressure and by ineffective penetration of the prodrug as well as DNA into solid tumors.

Similar experiments were carried out with DC-Chol/DOPE liposomes (DC-Chol is 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol) (43). The results were not as good as those obtained after treatment with DDAB/DOPE. DDAB/DOPE liposomes are probably more toxic to cells than DC-Chol/DOPE and due to that higher toxicity they destroy greater numbers of tumor cells.

Both types of liposomes were also used to cause another genetic modification of the tumor. Injecting liposomes complexed with plasmid pBCMGSNeo carrying Interleukin-4 gene into the growing tumor resulted in a sufficient local antitumor immunological response (42). The influx of eosinophils and macrophages to the tumor site caused tumor cell killing.

In conclusion, cationic liposomes can be used as an efficient *in vivo* delivery system of suicide genes and their application for DNA introduction could be an attractive method for local controlling of the growth of tumor cells sensitive to 5-fluorouracil.

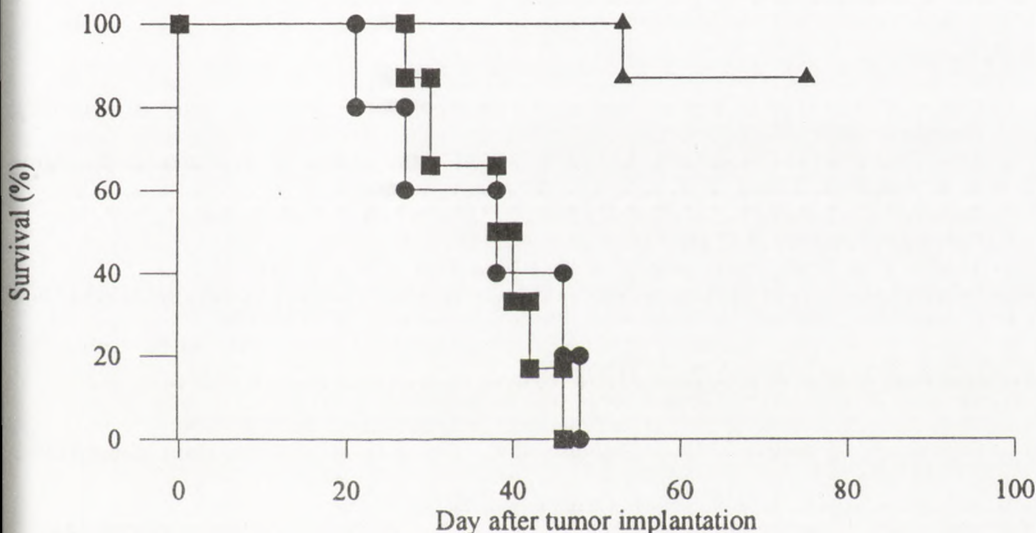


Fig. 3. Survival of C57B1/6-treated mice vs. control mice.
—●— B16(F10) control
—■— liposomes + pBCMGSNeo/Tyr/CD-5FC
—▲— liposomes + pBCMGSNeo/Tyr/CD+5FC

4. Conclusion

Our goal was to introduce *E. coli* cytosine deaminase gene directly into the growing murine melanoma tumor via cationic liposomes.

This was the first attempt to directly transfer *E. coli* cytosine deaminase suicide gene into the tumor via liposomes in order to obtain selective tumor cell killing following systemic administration of 5-fluorocytosine prodrug. Transcriptional targeting was achieved using tyrosinase promoter specific for melanoma and controlling expression of the therapeutic gene.

In spite of a relatively low percentage of transfected cells, multiple injections of therapeutic complexes into an established tumor can inhibit its growth or, sometimes, cause a total tumor regression.

Synthesis of cationic liposomes is simple. They are suitable for direct transfer of therapeutic genes into solid tumor cells. These liposomes do not cause genetic complications and can safely be used as carriers of DNA in gene therapy of established tumors.

Acknowledgement

This work was supported by a grant from the State Committee for Scientific Research No.4PO5A.028.08.

References

1. Culver K. W., (1994), *Gene Therapy. A Handbook for Physicians*, Mary Ann Liebert Inc.; New York.
2. *Gene Therapy for Neoplastic Diseases*, (1994), Eds. Huber B. E., Lazo J. S., Ann. N. Y. Acad. Sci., 176, 1-351.
3. Gore M. E., Collins M. K., (1994), Eur. J. Cancer, 30A, 1047-1049.
4. Herrman F., (1995), J. Mol. Med., 73, 157-162.
5. Tepper R. I., Mule J. J., (1994), Hum. Gene Ther., 5, 153-164.
6. Schmidt-Wolf G. D., Schmidt-Wolf I. G. H., (1995), Immunol. Today, 16, 173-175.
7. Culver K. J., Blease M. R., (1994), Trends in Genet., 10, 174-178.
8. Blaese R. M., Ishii-Morita H., Mullen C., Ramsey J., Ram Z., Oldfield E., Culver K., (1994), Eur. J. Cancer, 30A, 1190-1193.
9. Moolten F. L., (1968), Cancer Res., 46, 5276-5281.
10. Moolten F. L., Wells J. M., (1990), J. Natl. Cancer Inst., 82, 297-300.
11. Moolten F. L., Wells J. M. J., Heyman R. A., Evans R. M., (1990), Hum. Gene Ther., 1, 125-134.
12. Bagshawe K. D., (1989), Br. J. Cancer, 60, 275-281.
13. Deonarain M. P., Spooner R. A., Epenetos A. A., (1995), Gene Ther., 2, 235-244.
14. Borelli E., Heyman R., Hsi M., Evans R. M., (1988), Proc. Natl. Acad. Sci. USA, 85, 7572-7576.
15. Gutierrez A. A., Lemoine N. R., Sikora K., (1992), Lancet, 339, 715-721.
16. Huber B. E., Richards C. A., Krenitsky T. A., (1991), Proc. Natl. Acad. Sci. USA, 88, 8039-8043.
17. Mullen C. A., Kilstrup M., Blaese R. M., (1992), Proc. Natl. Acad. Sci. USA, 89, 33-37.
18. Huber B. E., Austin A. E., Good S. S., Knick C. V., Tibbels S., Richards C. A., (1993), Cancer Res., 53, 4619-4626.

19. Sikora K., Harris J., Hurst H., Lemoine N., (1994), Ann. N. Y. Acad. Sci., 716, 115-125.
20. Sorscher E. J., Peng S., Bebok Z., Allan P. W., Bennet L. L., Parker W. B., (1994), Gene Ther., 1, 233-238.
21. Bridgewater J. A., Springer C. J., Knox R. J., Collins M. K. L., (1995), Third Symposium on gene therapy in Berlin-Buch P9.
22. Wei M. X., Tamiya T., Chase M., Boviatsis E. J., Chang T. K. H., Kowall N. W., Hochberg F. H., Waxman D. J., Breakefield X. O., Chiocca E. A., (1994), Hum. Gene Ther., 5, 969-978.
23. Culver K. W., Ram Z., Wallbridge S., Ishii H., Oldfield E. H., Blaese M. R., (1992), Science, 256, 1550-1552.
24. Ram Z., Culver K. W., Wallbridge S., Blaese M. R., Oldfield E. H., (1993), Cancer Res., 53, 83-88.
25. Hughes B. W., Wells A. H., Bebok Z., Gadi V. K., Garver R. I., Parker W. B., Sorscher E. J., (1995), Cancer Res., 55, 3339-3345.
26. Huber B. E., Austin E. A., Richards C. A., Davis S. T., Good S. S., (1994), Proc. Natl. Acad. Sci. USA, 91, 8302-8306.
27. Freeman S. M., Abboud C. N., Whartenby K. A., Packman H. C., Koeplin D. S., Moolten F. L., Abraham G. N., (1993), Cancer Res., 53, 5274-5283.
28. Bi W. L., Parysek L. M., Warnick R., Stambrook P. J., (1993), Hum. Gene Ther., 4, 725-731.
29. Nishiyama T., Kawamura Y., Kawamoto K., Matsumura H., Yamamoto N., Ito T., Ohyama A., Katsuragi T., Sakai T., (1985), Cancer Res., 45, 1753-1761.
30. Senter P. D., (1990), FASEB J., 188-193.
31. Senter P. D., Su P. C. D., Katsuragi T., Sakai T., Cosand W. L., Hellstrom I., Hellstrom K. E., (1991), Bioconjugate Chem., 2, 447-451.
32. Wallace P. N., MacMaster J. F., Smith V. F., Kerr D. E., Senter P. D., Cosand W. L., (1994), Cancer Res., 54, 2719-2723.
33. Mullen C. A., Coale M. M., Lowe R., Blaese R. M., (1994), Cancer Res., 54, 1503-1506.
34. *Gene Therapeutics. Methods and Applications of Direct Gene Transfer*, (1994), Ed. Wolff J. A., Birkhauser Boston.
35. Danielsen S., Kilstrup M., Barilla K., Jochimsen B., Neuhard J., (1992), Mol. Microbiol., 6, 1335-1344.
36. Karasuyama H., Melchers F., (1988), Eur. J. Immunol., 18, 97-104.
37. Yamamoto H., Takeuchi S., Kudo T., Sato C., Takeuchi T., (1989), Jpn. J. Genet., 64, 121-135.
38. Vile R. G., Hart I. R., (1993), Cancer Res., 53, 962-967.
39. Vile R. G., Hart I. R., (1993), Cancer Res., 53, 3860-3864.
40. Rose J. K., Buonocore L., Whitt M. A., (1991), BioTechniques, 10, 520-525.
41. Nabel G. J., Nabel E. G., Yang Z.-Y., Fox B. A., Plantz G. B., Gao X., Huang L., Shu S., Gordon D., Chang A. E., (1993), Proc. Natl. Acad. Sci. USA, 90, 11307-11311.
42. Missol E., Szala S., Sochanik A., (1995), Cancer Lett., 97, 189-193.
43. Gao X., Huang L., (1991), Bioch. Biophys. Res. Commun., 179, 280-285.
44. Sochanik A., Szala S., (1996), Acta Biochim. Polon., 43, 293-300.
45. Szala S., Missol E., Sochanik A., Gene Therapy, (in press).

The Use of *E. coli* Cytosine Deaminase Gene as an Example of Suicide Gene Therapy of Cancer

Summary

Suicide genes encode enzymes, which convert nontoxic substrates to toxic products. These genes are proposed for selective killing of tumor cells in cancer gene therapy. In this review we

demonstrate preliminary results of direct *in vivo* transfer of cytosine deaminase (CD) gene from *E. coli* into murine B16(F10) melanoma cells *via* cationic liposomes.

Key words:
gene therapy, cytosine deaminase gene, melanoma cells, liposomes.

Address for correspondence:
Stanisław Szala, Department of Tumor Biology, Institute of Oncology, Wybrzeże Armii Krajowej St. 15, 44-100 Gliwice, Poland, fax: (0-32) 313-512.