EFFECT OF IRRADIATION ON INTERLEUKIN 6 AND SOLUBLE INTERLEUKIN 6 RECEPTOR MODIFIED MELANOMA GENETIC VACCINE

J. MALICKI[1], M. ŁACIAK[2], G. KOSICKA[1], J. KIERZKOWSKI[1], M. WIZNEROWICZ[2], A. MACKIEWICZ[2]

[1]Medical Physics Department, GreatPoland Cancer Center, Garbary 15, Poznań
[2]Dept. of Cancer Immunology Chair of Oncology University School of Medical Sciences at GreatPoland Cancer Center

ABSTRACT

We have designed phase I/II human melanoma gene therapy clinical protocol. The aim of the study was to actively immunize HLA-A1 and/or HLA-A2-positive patients with melanoma with an admixture of irradiated autologous tumor cells and allogeneic melanoma cells genetically engineered to secrete IL-6 and sIL-6R in order to elicit or enhance specific and nonspecific anti-melanoma immune responses to autologous tumor cells to eradicate distant melanoma lesions. Irradiation of autologous and allogeneic tumor cells is a key step in preparation of cellular vaccine because of two major reasons, (i) it inhibits cell proliferation which is crucial in the case of autologous cells which may form a tumor; (ii) it increases melanoma vaccine immunogenicity. The aim of the study was to estimate the optimal dose of ionizing radiation which will provide sterilization of both autologous and allogeneic melanoma cells and will ensure cytokine secretion.

Human melanoma cells (Mich-1) were transduced with IL-6 and sIL-6R cDNA using double copy bicistronic retroviral vector. Parental and transduced cells were seeded at in six-well tissue culture plates and were irradiated with 10, 50, 100 and 200 Gy. Secretion of both recombinant proteins into culture was analyzed before and 24, 48, 72, 96 h and 6, 7, 10 and 12 days following irradiation. At the same time adherent cells were enumerated, evaluated for viability and proliferation. At 24, 48, 72 and 96 h postirradiation specific IL-6 and sIL-6R mRNA levels were analyzed.

Irradiation of gene modified cells inhibited their proliferation in the dose dependant manner. Dose of 50 Gy sufficiently affected cell proliferation, however, for safety reasons we decided to use the dose of 100 Gy for vaccine preparation. Irradiation did not inhibit secretion of IL-6 and sIL-6R. In contrary, on a per cell basis it significantly increased their secretion which lasted 12 days postirradiation. Interestingly, we did not observe dose or time dependent differences in specific mRNA cellular levels suggesting that increased secretion of both proteins is regulated not on the transcriptional but rather on the posttranscriptional level. Taking all these facts into account we concluded that irradiation of tumor cells may provide an effective and safe approach for gene-modified vaccine preparation.

INTRODUCTION

Recent developments of gene transfer technology and better understanding of immunological mechanisms related to melanoma growth resulted in an renewed interest in the use of melanoma cellular vaccines - the next generation - genetically modified vaccines (Dalgleish, 1995; Bystryn et al, 1993). The general principle of this approach is to locally enhance melanoma antigens presentation or/and to provide costimulatory signal to the immune system for the activation of specific anti-tumor immune response (Blankenstein, 1994; Tepper and Mulle, 1994). One version of this strategy is to insert cytokine genes into autologous or allogeneic tumor cells, or other carrier cells such as fibroblasts in order to deliver cytokines in high concentration locally to the tumor while keeping systemic concentrations low (Blankenstein, 1994; Tepper and Mulle, 1994)..

IL-6, multifunctional cytokine, exerts its activity via a receptor complex composed of two subunits, a (gp80) IL-6 binding glycoprotein, and ß (130gp) signal transducing protein (Mackiewicz et al, 1995). Soluble form of a subunit of the IL-6 receptor (sIL-6R) acts agonistically with IL-6 by enhancing its activity both *in vitro* and *in vivo* (Mackiewicz et al, 1992; Mackiewicz et al, 1995). Moreover IL-6/sIL-6R complex displays broader range of biological activities than IL-6 alone by activating cells which possess gp130 but lack gp80 (Mackiewicz et al, 1992). In our pre-clinical studies introduction of IL-6 and sIL-6R genes into low immunogenic murine melanoma cells (line B-78-H1) caused inhibition of tumor growth, ability to metastasize, and stimulated potent, specific and long lasting anti-melanoma immunity (Mackiewicz et al, 1995; Mackiewicz et al, 1995). Anti-

melanoma activity of IL-6/sIL-6 complex was significantly higher than IL-6 alone. Analysis of tumor infiltrating cells have demonstrated that IL-6/sIL-6R secreting melanoma cells attracted CD8+ T cells, NK cells but not CD4+ T cells or dendritic cells (unpublished results).

On the basis of obtained results we have designed phase I/II human melanoma gene therapy clinical protocol (Mackiewicz et al, 1995). The aim of the study was to actively immunize HLA-A1 and/or HLA-A2-positive patients with melanoma with an admixture of irradiated autologous tumor cells and allogeneic melanoma cells genetically engineered to secrete IL-6 and sIL-6R in order to elicit or enhance specific and nonspecific anti-melanoma immune responses to autologous tumor cells to eradicate distant melanoma lesions.

Irradiation of autologous and allogeneic tumor cells is a key step in preparation of cellular vaccine because of two major reasons, (i) it inhibits cell proliferation which is crucial in the case of autologous cells which may form a tumor; (ii) it increases melanoma vaccine immunogenicity. Melanoma is known to be radioresistant. In addition, significant differences in radiosensitivity of melanoma cells isolated from different patients were reported. Consequently the optimal dose of ionizing radiation providing sterilization of both autologous and allogeneic melanoma cells as well as ensuring cytokine secretion needs to be estimated. Moreover, the molecular mechanisms induced by irradiation and governing altered secretion of recombinant proteins need to be elucidated.

MATERIAL AND METHODS

Construction of retroviral vectors caring IL-6 and sIL-6R cDNA

Construction of double copy dicistronic (DCCMV) vectors was based on original MSCV vector as described in detail (Wiznerowicz et al, 1997). Briefly, into MSCV's U3 region of 3' LTR downstream of NheI site artificial polylinker containing ClaI, NruI, SacII and MluI unique restriction sites was cloned. Moreover pgk-Neo cassette was removed from the transcription region of the vector. Next, PstI fragment containing human CMV-IE promotor/enhancer was blunt ligated into NruI site and then SalI-BamHI fragment containing IRES-Neo cassette was blunt ligated into filled MluI site. Then human IL-6 or human sIL-6R cDNAs cut out from pEXIL-6 and pEXSIRI expression vectors (obtained from Dr. S. Rose-John, Mainz, Germany) were inserted into Sal I site of DCCMV.

Transduction of human melanoma cells

PA-317 amphotropic packaging cells were electroporated (300V/100ms) with 5 mg of ScaI linearized plasmid DNA (DCCMV-IL-6 and DCCMV-sIL-6R) and selected with geneticin (500 mg/ml). After 3 weeks of selection the infectious medium containing recombinant retroviruses was obtained by incubation for 16 h of subconfluent (70-80%) corresponding PA-317 cells. The media were collected, complemented with polybrene and added for 4 hrs to human melanoma cell line (Mich-1). Then the cells were selected in geneticine for 3 weeks.

Maintenance and irradiation of melanoma cells

Parental and transduced cells were seeded at identical concentrations (34 x 10^3/well) in six-well tissue culture plates (Corning). After 24 h wells were filled up with the medium and plates irradiated at room temperature with 10, 50, 100 and 200 Gy and energy 6MV using linear accelerator Saturn 43F (General Electric). Irradiated cells were cultured in DMEM supplemented with 5% FCS at 37°C in 5% CO_2/95% air incubator.

Determination of IL-6 and sIL-6 secretion by melanoma cells

Secretion of IL-6 and sIL-6R by melanoma cells into culture was analyzed before and 24, 48, 72, 96 h and 6, 7, 10 and 12 days following irradiation. Twenty four hours before each time point of analysis medium was changed. Then the medium was collected and IL-6 and sIL-6R concentration was determined using ELISA kits (Genzyme and R & D, respectively).

Analysis of cell viability and proliferation

At each time point adherent cells were washed, trypsinized, enumerated and evaluated for viability using trypan blue exclusion. In parallel wells proliferation of irradiated cells was analyzed using MTT assay (Waserman and Twentyman, 1988). Briefly, to each well 200 ml of MTT solution (4 mg/ml) was added and incubated for 4h at 37°C. Then to each well 1 ml of DMSO was added and incubated for 1h at room temperature. Following incubation absorbance was measured at 570/630 nm wave length. In three experiments parallel to MTT assay cell proliferation was assessed by ^{3}H-thymidine incorporation assay ().

Analysis of IL-6 and sIL-6 mRNA

RNA from melanoma cells transduced

with IL-6 and sIL-6R was isolated 24, 48, 72 and 96 h following irradiation using Chomczynski and Sacchi method (Chomczyński and Sacchi, 1987). Specific IL-6 and sIL-6R mRNA levels were analyzed by Northern blot using 440 bp TaqI-BanII fragment of IL-6 cDNA and PstI 600 bp fragment of sIL-6R cDNA, labeled by random priming. As a control ß-actin mRNA was analyzed.

RESULTS

Construction of DCCMV-IL-6 and DCCMV-sIL-6R vectors

Principles of construction of DCCMV-IL-6 and DCCMV-sIL-6R vectors are shown in Fig. 1. Three transcripts of both IL-6 and sIL-6R were found.

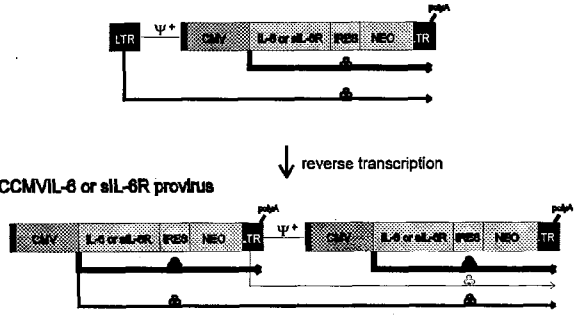

Fig. 1.
Structure and principle of double copy bicistronic vector (DCCMV-IL-6 or sIL-6R). Bicistronic gene driven by CMV-IE promoter was inserted into U3 region of 3'LTR of MSCV. Reverse transcription of DCCMV-IL-6 or sIL-6R RNA in target cells results in the CMV-IE-IL-6 or sIL-6R-IRES-Neo transcriptional unit in the U3 region of 3'LTR being transferred to the 5'LTR such that the DCCMV-IL-6 or sIL-6R provirus harbors two copies, one copy in each LTR. Arrows represent transcripts.

Effect of irradiation on cell proliferation and viability

Both cell proliferation assays, the MTT and ^{3}H-thymidine incorporation demonstrated similar results. Accordingly for further studies MTT assay was employed. Results of proliferation of irradiated cells were expressed as percentage of unirradiated corresponding cells. Irradiation caused dose dependent inhibition of melanoma cells proliferation. There were no differences between non transduced and IL-6 or sIL-6R gene transduced Mich-1 cells. Doses of 5, 10 and 20 Gy inhibited proliferation by 50% at 48h following irradiation, while dose of 1 Gy at about 90h (Fig. 2). Six days after irradiation there were no differences between doses of 5, 10 and 20 Gy.

Enumeration of adherent cells at different time points demonstrated progressive increase in cell number of unirradiated cells (Fig. 3A). Irradiation caused dose dependent reduction of adherent cells. Until 48h following irradiation an increase of cell number was still observed. However, after that time progressive decrease of adherent cells was seen (Fig. 3B-E). Trypan blue exclusion analysis demonstrated 93-98% viable cells.

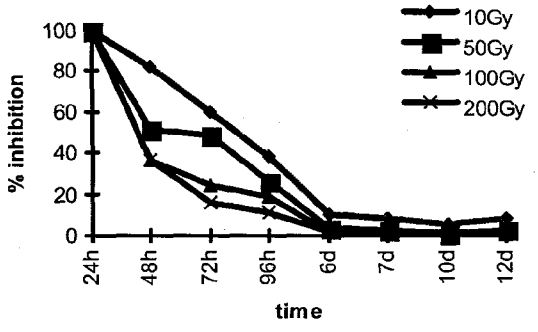

Fig. 2.
Inhibition of melanoma cells proliferation by irradiation with various doses as indicated.

Production of IL-6 and sIL-6R by melanoma cells following irradiation

Twenty four hours after seeding Mich-1 cells secreted approximately 300 and 100 ng/10^{6}cells/24h of IL-6 and sIL-6R respectively. During maintenance in culture Mich-1 cells demonstrated similar pattern of secretion of both proteins. Amount of IL-6 and sIL-6R accumulated in the medium of unirradiated cells kept increasing until the 7th day of culture and then despite the increase of cell number progressively declined (Fig. 3). Cells irradiated with various doses displayed similar pattern of IL-6 and sIL-6R secretion which reached maximum at 96h following irradiation. Amount of both proteins secreted was dependent on dose of irradiation. Calculation of secretion on a per cell basis demonstrated significant increase of secretion of both proteins by irradiated cultures.

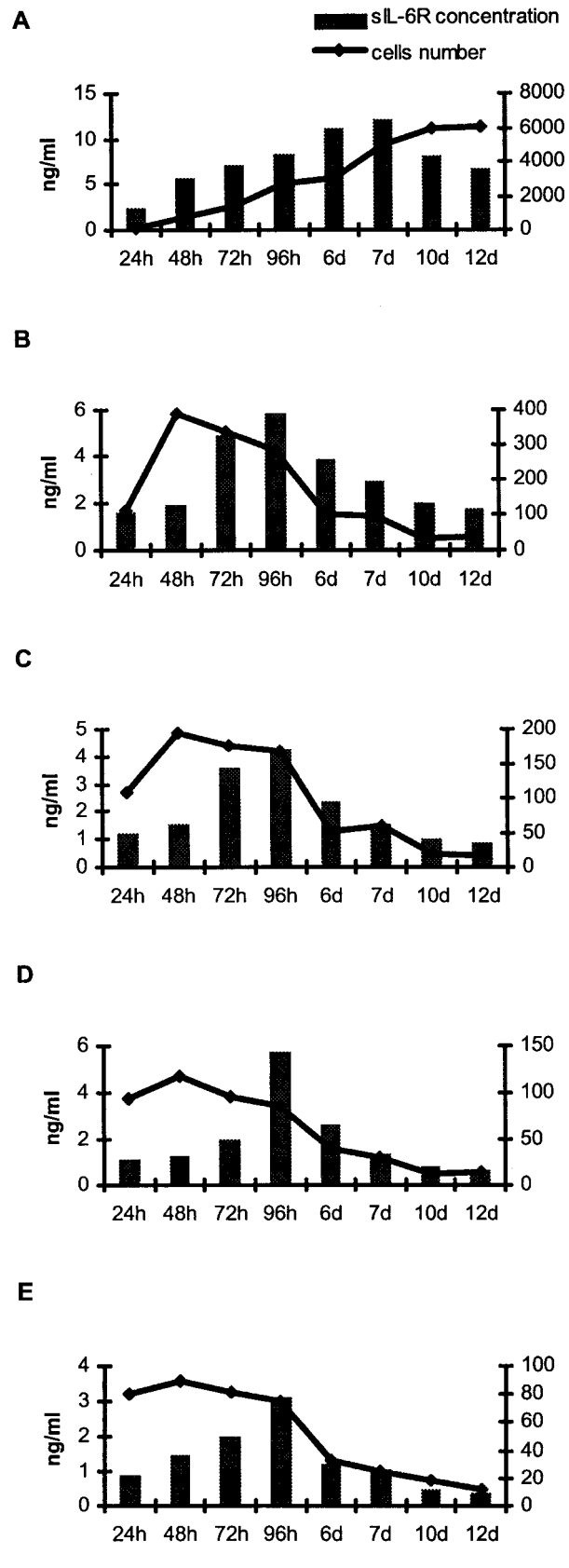

Fig. 3.

sIL-6R in the culture medium (bars) and adherent cells number at different time points postirradiation. A - unirradiated cells; B - cells irradiated with 10 Gy; C - with 50 Gy; D - with 100 Gy and E - with 200 Gy. Number of cells expressed in thousands. Note different scales on each sIL-6R concentration and cell number axis.

IL-6 and sIL-6R mRNA in irradiated melanoma cells

Northern blot analysis of IL-6 and sIL-6R specific mRNAs isolated from adherent cells demonstrated no significant differences between unirradiated and irradiated cells at 24, 48, 72 and 96 h postirradiation. Moreover, no differences between particular doses were seen (data not shown).

DISCUSSION

One of components of our genetic cellular vaccine is a human melanoma cell line modified to secrete recombinant IL-6 and sIL-6R. cDNAs encoding both factors were transduced into melanoma cells using recombinant retrovirus. Retroviral vector used in the system was especially designed for these studies. It combines two strategies double copy vector and dicistronic gene concept. Double copy strategy provides high number of copies of transduced gene while dicistronic gene provides simultaneous expression of two transduced genes within one cell ensuring expression of therapeutic gene in all selected cells. Moreover, such a construct allows application of various exogenous promoters depending on the target cell requirement. In our DCCMV vector we have employed very strong promoter with enhancer from CMV virus which provided high expression of both therapeutic genes. Secretion of IL-6 or sIL-6R did not affect melanoma cells growth in vitro (proliferation) even when both components were present.

Irradiation of gene modified cells inhibited their proliferation in the dose dependant manner. Dose of 50 Gy sufficiently affected cell proliferation and from theoretical point of view would be appropriate for further application. However, for safety reasons we decided to use the dose of 100 Gy in the vaccine preparation. Similar results were obtained by others who found the dose of 40 Gy was sufficient for irradiation of gene modified autologous melanoma cells derived from various patients (Abdel-Wahab et al, 1996). Similarly they choose to use the dose of 100 Gy for the same reason. Others have employed 100 Gy as a standard dose to irradiate interferon gamma (INF) and IL-2 transduced human melanoma cell lines (). They also found significant differences in sensitivity of particular cell line to irradiation, however, at day 14 postirradiation they saw between 10-30% of viable cells compared to day 3 postirradiation.

Irradiation did not inhibit secretion of both recombinant proteins. In contrary, on a per cell

basis it significantly increased their secretion which lasted 12 days postirradiation. Similar observations were made by others in relation to other recombinant proteins (Abdel-Wahab et al, 1996). Taking the reduction of cell number and the level of IL-6 and sIL-6R secretion into account we may conclude that irradiation of tumor cells may provide an effective and safe approach of gene-modified vaccine preparation. Interestingly, we did not observe dose or time dependent differences in specific mRNA cellular levels suggesting that increased secretion of both proteins is regulated not on the transcriptional but rather on the posttranscriptional level. Our results are in a disagreement with studies of Abdel-Wahab et al. (Abdel-Wahab et al, 1996) who demonstrated by RT-PCR analysis differences in IL-2 mRNA levels between 7 and 14 days postirradiation. In our studies we have analyzed mRNA levels until 96h postirradiation using Northern blot assay. This method is more reliable than RT-PCR, however, requires larger amounts of RNA for analysis. Accordingly, quantities of RNA obtained from cells on day 7 and 12 were not sufficient to perform the Northern blot. However, observed differences may be due to the various promoters used in the retroviral vectors. Abdel-Wahab et al. (Abdel-Wahab et al, 1996) used Moloney leukemia virus LTR while in our vector we employed CMV promoter. This is supported by their result showing no changes in ß-actin mRNA concentration what may indicate involvement of various nuclear transcription factors. In addition, Hauser et al. (Hauser et al, 1993) demonstrated increased expression of surface MHC class I (H-2D^b) antigens on B16 murine melanoma cells following irradiation what was accompanied by increased specific mRNA levels. Similarly, they did not observe changes in ß-actin mRNA and c-myc mRNA, a well known modulator of MHC class I transcription (Vesteeg et al, 1988).

In our studies we did not analyze expression of MHC surface molecules. As mentioned above (Hauser et al, 1993) and demonstrated by others (Abdel-Wahab et al, 1996) in human melanoma cell lines irradiation leads to increased expression of these surface antigens enhancing the immunogenicity of cancer cells. This phenomenon demonstrates another benefit of irradiation in the process of cellular vaccine preparation.

ACKNOWLEDGEMENTS

This work was supported by the State Committee for Scientific Research grant No 4S40209606.

REFERENCES

Abdel-Wahab Z, Dar MM, Hester D, Vervaert C, Gangavalli R, Barber J, Darrow TL, Segler HF. Effect of irradiation on cytokine production, MHC antigen expression, and vaccine potential of interleukin-2 and interferon-gamma gene modified melanoma cells. Cell Immunol, 1996; 171:246-254.

Blankenstein Th, Increasing tumor immunogenicity by genetic modification. Eur J Cancer 1994; 30:1182-1190. Bystryn JC, Ferrone S, Livingston F. eds. Specific Immunotherapy of cancer with vaccines. Ann NY Acad Sci, 1993; 690:1-401.

Chomczyński P, Sacchi N. Single-step method of RNA isolation by acud guanidinium thiocyanate-phenol chloroform extraction. Anal Biochem,1987;162:156-159. Dalgleish AG. Vaccine strategies for melanoma. Trends Exp Clin Med, 1995; 5:375-393.

Hauser SH, Calorini L, Wazer DE, Gattoni-Celli S. Radiation-enhanced expression of major histocompability complex class I antigen H-2D^b in B16 melanoma cells. Cancer Res, 1993; 53:1952-1955.

Mackiewicz A, Górny A, Laciak M, Malicki J, Murawa P, Nowak J, Wiznerowicz M, Hawley R.G, Heinrich P.C, Rose-John S. Gene therapy of human melanoma. Immunization of patients with autologous tumor cells admixed with allogeneic melanoma cells secreting interleukin 6 and soluble interleukin 6 receptor. Hum. Gene Ther, 1995; 6:805-811.

Mackiewicz A, Koj A, Sehgal P. (eds). Interleukin 6-type cytokines. Ann NY Acad Sci, 1995; 762:1-510.

Mackiewicz A, Schooltink H, Heinrich P, Rose-John S. Soluble human IL-6-receptor/IL-6 up-regulates expression of acute phase proteins. J Immunol, 1992; 149:2021-2027.

Mackiewicz A, Wiznerowicz M, Roeb E, Karczewska A, Nowak J, Heinrich P.C, Rose-John S.: Soluble Interleukin-6 Receptor is Biologically Active in vivo. Cytokine 1995; 7:142-149.

Mackiewicz A, Wiznerowicz M, Roeb E, Pawlowski T, Baumann H, Heinrich P, Rose-John S. Interleukin-6-type cytokines and their receptors for gene therapy of melanoma. Ann NY Acad Sci, 1995; 762:361-374.

Tepper RI, Mulle JJ. Experimental and clinical studies of cytokine gene-modified tumor cells. Hum Gene Ther, 1994; 5:153-164.

van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med, 1995; 182:689-98.

Vesteeg R, Noordermeer IA, Kruse-Wolters M, Ruiter DJ, Schrier PI. "c-myc" down-regulates class I HLA expression in human melanomas. EMBO J, 1988; 7:1023-1029.

Waserman TH, Twentyman P. Use of colorimetric microtiter (MTT) assay in determining the radiosensitivity of cells from murine tumours. Int J Radiat Oncol, 1998; 15:699-702.

Wiznerowicz M, Fong A, Mackiewicz A, Hawley R. Double-copy bicistronic retroviral platform for gene therapy and tissue engineering: application to melanoma vaccine development. Gene Ther. 1997 (submitted).