 ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(3):210-213

Transduction of human hepatocellular carcinoma cells with human γ-interferon gene via retroviral vector *

QIAN Shu-Bing and CHEN Shi-Shu

Subject headings Carcinoma,hepatocellular/therapy; interferon II/genetics;retroviridae;immunotherapy,adoptive

Abstract

AIM To investigate the therapeutic potential of gamma interferon (IFN-γ) genemodified human hepatocellular carcinoma (HCC) cells.

METHODS The IFN-γ gene was introduced retrovirally into four HCC cell lines. Secreted IFN-γ activity was assessed using bioassay. The expression of MHC molecules was detected by FACS. Tumorigenicity was analysed by tumor formation in nude mice.

RESULTS Four IFN-γ gene transduced HCC cell lines secreted different amounts of IFN-γ, as in the same case of five clones derived from one HCC cell line. Transduction with IFN-γ caused significant increase in the expression of major histocompatibility complex (MHC) antigens on HCC cells. The expression of HLA class I was increased by 2-3 times in terms of mean fluorescence intensities, while for class II expression, the percentage of positive cells augmented from <10% to >50%. When equal amount of tumor cells were injected into nude mice, the tumor igenicity some transduced cells decreased dramantically.

CONCLUSION IFN-γ gene transduction can convert weakly imunogenic HCC cells to activate antitumor immune response, and further pave the way for the future use of such gene modified tumor cells as a modality for the cancer immunotherapy.

Department of Biochemistry and Molecular Biology, Research Center for Human Gene Therapy, Shanghai Second Medical University, Shanghai 200025 China

Dr. QIAN Shu Bing, male, born on 1970-04-03 in Zhejiang province, Han nationality, graduated from Shanghai Second Medical University as a postgraduate in 1997, lecturer of biochemistry, majoring cancer molecular biology and gene therapy, having 6 papers published.

Presented at the First International Symposium on Gene Therapy, Beijing, July 7-10, 1997

***Supported by the National "863" High Tech Project Foundation No.863-102-16-05 and the National Natural Science Foundation of China, No.39470792.**

Correspondence to: Dr. CHEN Shi Shu, Research Center for Human Gene Chongqing Rd, Shanghai 200025, China

Tel. +86 • 21 • 63846590 ext 400 Fax. +86 • 21 • 6384961, E-mail. zpwu@fudan.ac.cn

Received 1997-10-28

INTRODUCTION

The past several years have seen an explosive growth in cancer immunotherapy using cytokine genetransduced tumor cell vaccines. This strategy seeks to locally alter the immunological environment of the tumor cell so as to enhance either antigen presentation of tumor-specific antigens to the immune system or both the activation of tumor-specific lymphocytes and nonspicific immunity. Many cytokine genes have been introduced into tumor cells with varying effects on both tumorigenicity and immunogenicity [1,2]. The success of cytokinesecreting tumor vaccines in murine models of cancer has led to the initiation of clinical trials in patients[3]. IFN-γ is a pleiotropic cytokine produced by activated T-lymphocytes, which can influence the outcome of an immune response in several distinct ways[4]. An important property of IFN-γ is the ability to up-regulate the expression of major histocompatibility complex (MHC) molecules, which play a central role in immune response. An increase in immunogenicity after MHC class I up-regulation by IFN-γ is thought to be due to improved presentation of tumor-specific antigens to CD8+ CTLs[5]. We successfully transduced IFN-γ into four HCC cell lines with retroviral vector results showed a significant up-regulation of surface MHC molecules. Moreover, transduced HCC cells decreased in tumor growth. The increased immunogenicity and decreased tumorigenicity might reflect the immunotherapeutic potential of such IFN-γ gene transduced HCC vaccines.

MATERIALS AND METHODS

Cell lines

The following human hepatocellular carcinoma cell line were used: QGY7701, SMMC7721, BEL7404 and HHCL. All the cell lines were purchased from Bank of Cell, Institute of Cell Biology, Chinese Academy of Sciences (Shanghai). HCC cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100U/mL), streptomycin (100 μg/mL) and 2mML-glutamine. The monolayer was propagated by trypsinization as required.

Construct of retroviruses and IFN-γ transduction

Human full-length IFN-γ cDNA encoding a leading peptide and IFN-γ was cloned into pLXSN retroviral vector (provided by Dr. AD Miller), generating recombinant construct pL (IFN-γ)SN. The inserted IFN-γ gene was driven by the long terminal repeat (LTR), of moloney murine leukemia virus (MLV) while the neomycin phosphotransferas gene (Neo-R) was driven by both the LTR and the simian virus early promoter (SV40). The vector was introduced into amphotropic packaging cell line PA317. Transfected cells were selected in 0.4mg/mL G418 (Gibco) and resistant colonies were isolated and amplified. Viral titers of the retroviral supernatant ranged from 5×104 to 2×106 colony forming units (CFU)/mL, when assayed for their ability to transfer neomycin-resistance to NIH3T3 cells.

HCC cells (0.5-1×106) were cultured overnight in T80 flasks (Nunc). Retroviral supernatants supplemented with 8mg/L polybrene (Sigma) were added to each flask to obtain a ratio of about 1MOI/cell. The following day, cells were fully selected by the neomycin analogue, geneticin G418. The concentration of G418 used for the selection ranged from 0.5 to 1g/L (active dose), depending on the sensitivity of each cell to the toxic effects of G418. Approximately, cells were maintained in selecting medium for 2 weeks. Colonies that survived the G418 selection were isolated and expanded.

IFN-γ assay

The amount of IFN-γ produced by transduced cells was determined by the standard cytopathic inhibition assay. Briefly, 5×105 cells were incubated for 24 hours, then supernatants were harvested, centrifuged and aliquoted as test asmples. Human fibroblasts were clutured with serial dilutions of test samples or standard IFN-γ (Boehringer Mannheim). The cells were challenged with vesicular stmatitis virus (VSV) and cultured overnight. The IFN-γ titer was calculated as the reciprocal of the dilution that protected 50% of the monalayer cells from the cytopathic effect of the virus.

Flow cytometric analysis

Flow cytometry was performed for quantitative analysis of surface MHC class I and II expression. One million cells were harvested by trypsinization, washed twice in phosphate balance solution (PBS) containing 2% FCS and 0.1% sodium azide (Sigma), and incubated for 30min at 4℃ with saturating amounts of monoclonal antibodies (anti-HLA class I and anti-HLA class II, Dept. Immunology, Beijing Medical University). After twice washing, cells were incubated with a 1×50 dilution of fluorescence isothiocyanatic-conjugated goat anti[a2]mouse IgG (Huamei Co., Shanghai) for 30min at 4℃ in the dark. The cells were washed twice, fixed with PBS containing 0.2% formaldehyde and examined by an FACScan flow cytometer (Becton Dickinson) for the percentage of positive cells and mean fluorescence intensities.

Tumor formation in nude mice

Female athymic nude BALB/c mice of 4-5 weeks (purchased from Animal Center, Chinese Academy of Sciences, Shanghai) were housed under pathogen free conditions. Six to eight animal in a group were used. Cells (3×106 in 200μL) were injected into the right flank of the mice. The tumors were measured by a caliper in two dimensions and the volume were calculated using the formula (width2×length/2). At the end of 45 days, the tumors were removed and weighed.

RESULTS

Production of IFN-γ from transduced clones

The tumor cell lines were successfully infected with recombinant retrovirus containing IFN-γ cDNA. G418-resistant colonies were isolated and expanded to cell lines for further analysis. According to the number of selected clone, each transduced cell line was designated as QGY7701. 3A, SMMC7721.2C, BEL7404.6A and HHCL.5D, respectively. Five different clones derived from the transduced HHCL were designated following the same rule. Southern blot analysis was made and showed that the intact gene of interest was present in the transduced cell lines in the form of provirus. Northern blot analysis further indicated that the correct mRNA species were transcribed (data not shown). Secretion of IFN-γ by HCC cells transduced with the retroviral vectors was determined by an appropriate bioassay. As shown in Table 1, transduced cell lines derived from different HCC cell lines produced different amounts of IFN-γ, as the same case of five clones derived from one parental HHCL.

Enhancement of the MHC molecule expression after IFN-γ gene transduction

All the four HCC cell lines used in this study were screened for cell surface expression of HLA molecules by flow cytometry as described above. As shown in Table 1, all the cell lines expressed HLA class I molecules, however the expression? of HLA. DR, a HLA class II molecules was very low. Transduction with the IFN-γ gene resulted in significant increases in the expression of HLA molecules. The expression of HLA class I molecules was increased 2-3 times in terms of

fluorescence intensities, while for class II molecules, the percentage of positive cells was augmented from <10% to >50%. There was nocorrelation between the degree of increase in HLA expression and the amounts of IFN-γ production, even when five transudced clones derived from one parental cell line HHCL were compared. But it is fairly clear that the cells with lower expression of HLA class I molecules, such as QGY7701 and BEL7404, have stronger response to IFN-γ transduction in inducing the expression of HLA molecules, and thus resulting in more increase in the intensities of such molecules (Table 1).

Tumor growth in nude mice

Live parental 3×106 or transduced HCC cells were injected s.c. into the thigh region of nude mice, and tumor growth was measured weekly. The tumorigenicity was different in each parental HCC cell. The tumor growth of QGY7701 was more rapid than HHCL. Injection of the former cells resulted in palpable tumor within 7 days, while the latter formed discernible tumor after 14 days. However, the tumor formation of respective IFN-γ transduced HCC cells was also different. Transduced QGY7701.3A showed no distinguishable decrease in its tumor growth as compared with that of parental cell line, whereas transduced HHCl.5D reduced its tumorigenicity dramatically (Table 2). In addition to the much lower tumor incidence (1/7), there was a significant decrease in both the size and the weight of the tumor (P<0.01). The other two transduced HCC cells also inhibited the tumor growth, although in different degrees. It is notable that the reduction in tumor growth following IFN-γ transduction was related to the original tumor formation potential of its parental HCC cell line.

Table 1 Secretion of IFN-γ and expression of HLA antigens by transduced hepatocellular carcinoma cells in culture

Cell lines	IFN-γ Production (U/5×105 cells/24h)	Control* MCN(% positive)△△	HLA class I * * MCN (% positive)	HLA class II△ MCN (% positive)
QGY7701	0	18(6)	159(99)	26(11)
QGY7701.3A	75	21(5)	306(100)	28(77)
SMMC7721	0	9(2)	200(100)	13(12)
SMMC7721.2C	150	21(3)	348(100)	14(58)
BEL7704	0	20(7)	34(93)	21(8)
BEL7404.6A	75	22(5)	117(100)	29(47)
HHCL	0	10(3)	232(100)	12(10)
HHCL.2C	100	22(6)	340(100)	37(56)
HHCL.2D	25	14(6)	440(100)	12(68)
HHCL.5C	100	11(4)	401(100)	19(83)
HHCL.6C	75	15(3)	550(100)	10(44)
HHCL.5D	200	10(3)	341(100)	10(40)

*Cells were stained with fluorescence isothiocyanatic-conjugated goat anti-mouse IgG (FITC-IgG) as a control.
**Cells were stained with anti-HLA class I McAb following with FITC-IgG.
△Cells were stained with anti-HLA class II McAb following with FITC-IgG.
△△Mean fluorescence channel number (MCN) and percentages of positive cells (in parenthese).

Table 2 Formation of tumors by parental and transduced hepatocellular carcinoma cells in nude mice

Cell	Incidence	Volume (cm3)	Weight (g)
QGY7701	8 / 8	5.58±0.90	1.62±0.36
QGY7701.3A	8 / 8	5.16±16	1.60±0.72
SMMC7721	7 / 7	3.87±0.24	0.55±0.12S
MMC7721.2C	5 / 6	1.26±0.32[a]	0.28±0.08[a]
BEL7404	8 / 8	4.32±0.28	0.78±0.14
BEL7404.6A	8 / 8	3.35±0.21	0.50±0.12
HHCL	8 / 8	2.04±0.32	0.35±0.18
HHCL.5D	1 / 7	0.15[b]	0.02[b]

All the values are measured at 6 weks after injection with equal amounts of cells (3×10^6 each), and presented as the average value ± the standard error of the mean
[a]P<0.05, compared with SMMC7721 value by Student's t test
[b]P<0.01, compared with HHCL value by Student's t test

DISCUSSION

In this study human hepatocellular carcinoma cell line were the genes for human IFN-γ were transduced successfully. Four different HCC cells transduced with the IFN-γ gene produced varying levels of IFN-γ, and such difference is also existed among the five clones derived from one parental HCC cell line (HHCL). Since the distinguished feature of retroviral vector is its integration into the host genomic DNA in the form of provirus[5], the expression difference might be due to the random integration resulting in varying efficiency of gene expression derived by interior promoter.

It has been repeatedly demonstrated that IFN-γ

can exert significant antitumor effects via either direct antiproliferative effects on the tumor or indirectly through the host immune system, including enhancement of MHC class I and II expression, activation of macrophage and natural killer cells, generation of cytotoxic T-lymphocytes and induction of tumor associated antigen[4]. MHC class I and II molecules play a central role in cellular immunity and tumor survillance. Recent studies have demonstrated that the loss of MHC class I expression was associated with tumor progression or metastasis[7]. Enhancing MHC class I expression by tumor cells may promote antitumor response against them. Our results in MHC as consistent with most others in melanoma and RCC [8-10]. The cell surface expression of both HLA class I and II molecules was increased in HCC cells transduced with the IFN-γ gene. Considering the original display of HLA class I, but not class II in parental HCC cells, it is not surprising that following IFN-γ transduction, HLA class I expression was significantly in creased in terms of mean fluorescence intensities, while for class II, in terms of percentage of positive cells. There was no definite correlation between the magnitude of the increase in the expression of HLA molecules and the amount of the IFN-γ secreted by tranduced HCC cells. However, the magnitude of the increase in the expression of HLA class I in mean fluorescence intensities appeared to be greater in transduced HCC cells with lower expression of such molecules in its parental cells. This suggests that the augmentation of MHC expression is associated with both the effects of IFN-γ and the potential of expression of such molecules.

Most animal studies have showed that IFN-γ secretion by tumor cells results in reduced tumorigenicity. However, when the tumorigenicity of human tumor cells was examined in nude mice, several element must be considered. Nude mice is a T cell deficient strain, but other components of the immune system may still exist. In addition, human IFN-γ secreted by transduced HCC cells have little effect on mouse immune system. Our results obtained from nude mice injected with parental or transduced HCC cells were different. That the tumor growth of the transduced QGY7701.3A in nude mice was not significantly different from that of the parental untransduced cell lines was surprising. But it should be noted that QGY7701 was of the highest tumorigenicity among the four HCC cell lines. In contrast to QGY7701, HHCL, which was of the lowest tumorigenicity, showed dramatic decrease in tumor growth in nude mice following transduction with IFN-γ gene. The observation of such difference might reflect either the heterogeneity of the cells with which we worked or the limited effects of IFN-γ for tumor cells of high tumorigenicity.

In summary, the data obtained in this study indicated that IFN-γ gene modified HCC cells might be useful in the treatment of human cancers, especially in inducing specific immune responses. However, the heterogeneity of tumor cells should be considered in establishing effective tumor vaccine, thus a more potential tumor vaccine can be selected and an increased antitumor immunity induced *in vivo* can be obtained. This study has laid ground for the future use of cytokine gene modified tumor cells as a modality for the cancer immunotherapy.

REFERENCES

1 Gansbacher B, Zier K, Daniels B, Cronin K, Mannerji R, Gilboa E. Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protevtive immunity. *J Exp Med,* 1990;172(5):1227-1224

2 Giboa E, Lyerly HK, Vieweg J, Satio S. Cytokine gene therapy in tumor. *Semin Cancer Biol,* 1994;5(3):409-417

3 Special report. Human gene marker / therapy clinical protocols. *Hum Gene Ther,* 1996;7(4):567-588

4 Sigel JP. Effects of interferon-gamma on the activation of human T-lymphocytes. *Cell Immunol,* 1988;111(3):461-472

5 Restifo NP, Spiess PJ, Karp SE, Mule JJ, Rosenberg SA. A nonimmunogenic sarcoma transduced with the cDNA for inteferon γ elicits CD8+T cells against the wild type tmor: correlation with antigen presentation capability. *J Exp Med,* 1992;175(6):1423-1431

6 Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. *Science,* 1995;270(4):404-414

7 Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical revelance. *Immunol Today,* 1995;16(7):487-497

8 Nayak SK, MeCallister T, Han LJ, Gangavalli R, Barber S, Dilloman RO. Transduction of human renal carcinoma cells with human γ-interferon gene via retroviral vector. *Cancer Gene Ther,* 1996;3(3):143-150

9 Gansbucher B, Bannerji R, Dannids B, Zier K, Cronin K, Gilboa E. Retroviral vector mediated γ interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res,* 1990;50(14):7820-7825

10 Ogasawara M, Rosenberg SA. Enhanced HLA molecules and stimulation of autologous human tumor infiltrating lymphocytes following transduction of melanoma cells with γ-interferon genes. *Cancer Res,*1993;53(7):3561-3568