添付資料

当該遺伝子治療に関する培養細胞，実験動物を用いた研究成果（論文添付）

Melanoma Research 2001, 11, pp. 337–342

Growth inhibition of human malignant melanoma transfected with the human interferon-β gene by means of cationic liposomes

T. Kageshita, M. Mizuno, T. Ono, K. Matsumoto, T. Saida and J. Yoshida*

Department of Dermatology, Kumamoto University School of Medicine, Japan (T. Kageshita, T. Ono). Departments of Molecular Neurosurgery (M. Mizuno) and Neurosurgery (J. Yoshida), Nagoya University, Postgraduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: (+81) 52 744 2355; Fax: (+81) 52 744 2360; Email: jyoshida@med.nagoya-u.ac.jp. Department of Dermatology, Shinshu University School of Medicine, Japan (K. Matsumoto, T. Saida).

Among the various types of human interferons, human interferon-β (HuIFNβ) has the strongest anti-proliferative activity against human melanoma cell lines. Therefore, we investigated the growth inhibitory effect of a cationic liposome containing the HuIFNβ gene on human melanoma cell lines *in vitro* and *in vivo*. After transfection with liposomes containing the HuIFN-β gene, human melanoma cell lines produced HuIFNβ in the culture medium at levels ranging from 67 to 3.8 IU/ml on day 6, and growth of the cells was inhibited by 71–92%. Moreover, six injections of liposomes containing the HuIFNβ gene completely eradicated human melanoma nodules transplanted onto the backs of nude mice 40 days after the first injection. Histological analysis of the injected nodules revealed that the HuIFNβ gene transfection induced apoptosis of the human melanoma cells. These data suggest that transfection of the HuIFNβ gene using cationic liposomes is a promising candidate for gene therapy of human melanoma.

Key words: cationic liposome, gene therapy, human melanoma, interferon-β

Introduction

The incidence of malignant melanoma has been increasing by 5% per year for the last 40 years in Caucasian as well as other populations. At present, there is no effective treatment for patients with recurrent and/or advanced melanoma. Gene therapy, including immunogene therapy, has received particular attention as a promising treatment modality for melanoma, since conventional chemotherapy and radiotherapy have limited efficacy. Thus, experimental studies and clinical trials have been undertaken using retrovirus and/or adenovirus vectors to deliver various kinds of genes.[1] However, clinically effective gene therapy has not yet been established. Liposomes have been used as a safer alternative to virus vectors in experimental and clinical trials in melanoma,[2,3] though the transfection rate is not so high.

Cationic liposomes are among the most attractive vectors for human gene therapy because they are not infectious and have little immunogenicity or toxicity. Morphologically, cationic liposomes are divided into three main types: small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). SUVs bind the genes of interest to their surfaces to produce DNA-lipid complexes. This type of liposome has already been used in human gene therapy for cancer. Nabel *et al.*[3] applied this type of liposome to gene therapy for patients with melanoma. They reported that direct injection of a DNA (a gene coding for HLA-B7)-liposome complex induced regression of some melanoma nodules in patients with metastatic melanoma.[3] In contrast, LUVs and MLVs generally entrap the genes within the liposomes, rather than at the surface. Recently, we found that cationic multilamellar liposomes containing *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) were a very useful vector for gene therapy against experimental brain tumours, especially malignant gliomas.[4–9] In April 2000, we started clinical trials of gene therapy for malignant glioma using MLV liposomes containing the HuIFNβ gene.

*To whom correspondence should be addressed

HuIFNβ is a multifunctional cytokine with antiviral, antiproliferative and antiangiogenic activity, and its protein has been already used as an antitumour agent against glioma and melanoma.[10,11] To our knowledge, no information is available about HuIFNβ gene therapy for melanoma using MLV liposomes. Therefore, in this study we assessed the antiproliferative activity of MLV liposomes containing HuIFNβ gene on cultured human melanoma cells and examined the *in vivo* effects on human melanoma nodules transplanted onto the backs of nude mice.

Materials and methods

Cell lines

The human melanoma cell lines RPM-EP, RPM-MC, G361, MM-AN and Colo38 were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 2 mM L-glutamine.[12] They were cultured at 37°C in a 5% CO_2 atmosphere.

Animals

Female nude mice, KSN, aged 6–8 weeks, were used. They were kept and bred under pathogen-free conditions in the animal facility of our university.

Monoclonal antibody (MAb) and conventional antisera (MAb MIB-1 to Ki 67 antigen) was purchased from Immunotech (Marseille, France). The Vectastain ABC kit and biotinylated anti-mouse Ig xenoantibodies were purchased from Vector Laboratories (Burlingame, California, USA). The apoptosis detection kit was purchased from Oncor (Gaithersburg, Maryland, USA).

Lipids

Positively charged lipid, TMAG, dilauroyl phosphatidylcholine (DLPC) and dioleoyl phosphatidylethanolamine (DOPE) were purchased from Sogo Pharmaceutical Co. Ltd, Tokyo, Japan, Sigma Chemical Co., St Louis, Missouri, USA, and Avanti Polar-Lipids, Inc., Pelham, Alabama, USA, respectively.[7]

Plasmids

A pSV2IFNβ was constructed by inserting the HindIII-BglII large fragment of pSVdhfr, HuIFNβ cDNA and deoxyoligonucleotides into an SV40-derived expression vector (Toray Industries, Inc., Tokyo).[4,6]

Preparation of liposomes containing pSV2IFNβ

For the preparation of the pSV2IFNβ-containing liposomes, TMAG, DLPC and DOPE in a molar ratio of 1:2:2 (total amount 1 μmol) were dissolved in 0.5 ml of chloroform. The solvent was then evaporated and the lipid film was wetted with 0.2 ml of phosphate-buffered saline (PBS) containing 20 μg of pSV2IFNβ and then suspended with a vortex agitator for 2 min. The volume of the suspension was adjusted to 0.5 ml with PBS.

Gene transfer and growth inhibition of cultured melanoma cells

For this, 1 ml of melanoma cell suspension in culture medium (5×10^4/ml) was placed in each well of a Falcon plate (047, Franklin Lakes, New Jersey, USA) and incubated at 37°C for 24 h in a humidified atmosphere of 5% CO_2. The culture medium was collected 3 and 6 days after addition of the liposomes (15 nmol/ml of lipid and 0.3 μg/ml of DNA) and the amount of HuIFNβ was measured.

The growth-inhibitory effect of HuIFNβ gene transfer to melanoma cells was evaluated by counting the number of viable cells as described previously.[4,5] In addition, blocking of the growth-inhibitory effect was evaluated using MCA (YSB-1), which reacts specifically with HuIFNβ. One unit of MCA (YSB-1) was able to neutralize HuIFNβ from 100 to 10 IU and was provided by Toray Industries, Inc., Tokyo, Japan.[13]

Gene transfer and *in vivo* growth inhibition of melanoma nodules in nude mice

Fragments of the tumours (3 mm in diameter) were transplanted subcutaneously onto the backs of mice by means of a trocar needle. When the transplanted tumours had grown to 7 mm in diameter, the mice were divided randomly into the following four treatment groups: group 1, one intratumoral injection of PBS (15 μl); group 2, one intratumoral injection of empty liposomes (150 nmol lipid in 15 μl); group 3, one intratumoral injection of liposomes containing pSV2IFNβ (3 μg DNA and 150 nmol lipid

in 15 μl); and group 4, six intratumoral injections of liposomes containing pSV2IFNβ (3 μg DNA and 150 nmol lipid in 15 μl). In this last group, the liposomes were injected every other day. Growth inhibition of transplanted tumours was evaluated by measuring the tumour size every 2 days with the aid of microcallipers. Tumour volume was calculated using the formula $ab^2/2$, where a is the width and b the length of the tumour. The relative tumour size (%) was calculated from the formula $T_n/T_0 \times 100$, where T_0 = tumour weight immediately before the intratumoral injections and T_n = tumour weight after the injections.[14]

Detection of HuIFNβ

The amount of HuIFNβ in the medium was determined by an enzyme-linked immunoassay modified by Yamazaki *et al.*[15] In this assay the accurate detection limit of HuIFNβ was 2.5 IU/ml.

Histopathological analysis

Tumours were removed from nude mice 30 days after administration of PBS, empty liposomes or liposomes containing pSV2IFNβ. Tumour tissue was fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded. Histopathological analysis was evaluated with conventional haematoxylin and eosin staining of tissue sections. The detection of apoptotic cells in tissue sections was performed using a TUNEL assay kit (ApopDETEC cell death assay system, Enzo Diagnostic, Inc, New York, USA) according to the manufacturer's instructions.

Results

Production of HuIFNβ by cultured melanoma cells transfected with liposomes containing pSV2IFNβ

HuIFNβ was detected in the culture medium of each of the five human melanoma cell lines at 3 and 6 days after the transfection of pSV2IFNβ (Table 1). The levels of HuIFNβ ranged from 67 IU/ml in RPM-EP cells to 3.8 IU/ml in Colo38 cells on day 6. Treatment with empty liposomes did not affect HuIFNβ production in any of the cell lines.

Table 1. Level of HuIFNβ in melanoma cells transfected with pSV2IFNβ. For this, 1 ml of melanoma cell suspension in culture medium (5×10^4/ml) was placed in each well. The culture medium was collected 3 and 6 days after addition of the liposomes containing pSV2IFNβ (15 nmol/ml lipid and 0.6 μg/ml DNA). The amount of HuIFNβ was measured by enzyme immunoassay ($n = 5$). Values given are means ± SD

Cells	Incubation time (days)		
	0	3	6
RPM-EP	ND	30.6 ± 4.3	67.3 ± 8.4
RPM-MC	ND	10.5 ± 2.4	12.2 ± 3.2
G361	ND	22.3 ± 5.2	28.5 ± 6.7
MM-AN	ND	13.5 ± 1.9	21.7 ± 4.2
Colo38	ND	4.0 ± 1.3	3.8 ± 0.9

ND, not detectable.

Growth inhibition of cultured melanoma cells transfected with liposomes containing pSV2IFNβ

Viable cultured melanoma cells were counted 3 and 6 days after transfection with pSV2IFNβ (Table 2). Growth inhibition was observed in all the melanoma cell lines; it ranged from 56% in G631 cells to 34% in RPM-MC and Colo38 cells on day 3, and from 92% in RPM-EP and G361 cells to 71% in Colo38 cells on day 6. On the other hand, adding exogenous 100 IU/ml HuIFNβ produced only 40% growth inhibition in non-transfected RPM-EP cells on day 6. The growth-inhibitory effects were correlated with the level of HuIFNβ in the culture medium. Adding 50 units of MCA (YSB-1) to the medium of transfected RPM-EP cells caused an undetectable level of HuIFNβ in the medium and the growth-inhibitory effects were reduced significantly but not completely. Treatment with empty liposomes did not affect cell growth in any of the cell lines.

In vivo growth inhibition of melanoma tumours transfected with liposomes containing pSV2IFNβ

Tumours injected with PBS (group 1) grew linearly from the time of injection to a volume three times the size by 60 days after the implantation (Figures 1 and 2). In contrast, a single injection of liposomes containing pSV2IFNβ (group 3) suppressed tumour size for 40 days and inhibited tumour size by 44% at 60 days after the implantation. No growth inhibition was observed in tumours receiving a single injection of empty liposomes (group 2). In mice receiving six intratumoral injections of liposomes with pSV2IFNβ

Table 2. Growth-inhibitory effects of endogenous HuIFNβ in cultured human melanoma cells transfected with pSV2IFNβ. For this, 1 ml of melanoma cell suspension in culture medium (5×10^4/ml) was placed in each well. Cell growth was evaluated on days 3 and 6 by counting the number of trypan blue-excluding cells in a haemocytometer ($n = 5$). Values given are means ± SD expressed as number of cells $\times 10^4$/ml

Cells	Control Incubation time (days)			Liposome Incubation time (days)		
	0	3	6	0	3	6
RPM-EP	4.8 ± 0.6	10.3 ± 1.1	18.7 ± 0.9	4.8 ± 0.6	5.0 ± 1.3	1.5 ± 0.6
RPM-MC	4.5 ± 0.7	8.4 ± 0.9	16.4 ± 2.1	4.5 ± 0.7	5.5 ± 1.1	2.8 ± 0.4
G361	5.1 ± 0.3	10.8 ± 1.9	18.4 ± 3.0	5.1 ± 0.3	4.8 ± 0.7	1.4 ± 0.2
MM-AN	4.5 ± 0.7	8.2 ± 1.6	14.6 ± 2.7	4.5 ± 0.7	4.0 ± 1.1	3.3 ± 1.2
Colo38	4.4 ± 0.7	6.4 ± 0.7	9.1 ± 2.0	4.4 ± 0.7	4.2 ± 0.8	2.6 ± 0.9

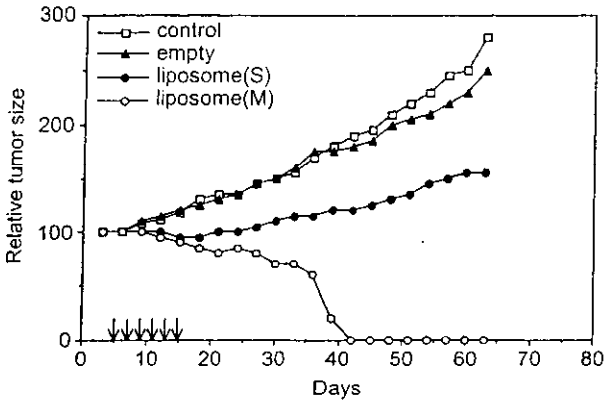

Figure 1. Effects of injection of cationic liposomes containing pSV2IFNβ on the growth of human melanoma transplanted to nude mice. □, six intratumoral injections of PBS (15 μl); ▲, six intratumoral injections of empty liposomes (150 nmol lipid/15 μl); ●, one intratumoral injection of liposomes containing pSV2IFNβ (3 μg DNA and 150 nmol lipid in 15 μl); ○, six intratumoral injections of liposomes containing pSV2IFNβ (3 μg DNA and 150 nmol lipid in 15 μl). Injection times are indicated by arrows on the *x* axis. Melanoma nodules were evaluated by measuring the size every 2 days with the aid of microcallipers, and tumour volume and relative tumour size were calculated (see Materials and methods).

(group 4) the size of the tumours was suppressed and the tumours disappeared completely within 40 days post-implantation. Repeated injection of HuIFNβ protein (5×10^4 IU six times) inhibited the size of melanoma nodules to some degree but they did not disappear completely (data not shown). Moreover, HuIFNβ protein was detected in the intratumoral region for at least 3 weeks in group 4, while it was detected for less than 10 days in the group receiving injections of HuIFNβ protein.

Histopathological analysis

Histopathological analysis of the haematoxylin and eosin stained sections of the tumour nodules obtained 30 days after the implantation revealed necrotic areas within the tumours in all four groups. Sections from tumours from group 4 mice displayed the highest degree of necrosis. Melanoma cells with eosinophilic cytoplasm and pyknotic nuclei were also seen in all tissue sections. The number of these cells was significantly higher in group 4 than in groups 1 and 2. Staining of serial sections revealed that these cells were identical with apoptotic kit-positive cells (Table 3). In addition, the number of Ki 67-positive cells in group 4 was significantly lower than that in groups 1 and 2.

Discussion

We have demonstrated for the first time that human cultured melanoma cells, transfected with pSV2IFNβ, produced HuIFNβ and were inhibited in their growth *in vitro*. The growth-inhibitory effect was HuIFNβ specific, since it was blocked by anti-HuIFNβ antibody. The growth of melanoma cells was much more strongly inhibited than that of glioma cells reported in our previous studies.[4,5] One possible reason for this difference is higher susceptibility of melanoma cells to HuIFNβ protein. Moreover, in this study, human melanoma nodules subcutaneously transplanted to nude mice completely disappeared after six injections of pSV2IFNβ-containing liposomes. In our previous study, growth of glioma nodules was significantly inhibited but did not disappear completely.[7] To the best of our knowledge, this is the first study showing complete disappearance of human melanoma nodules transplanted to nude mice with transfection of the HuIFNβ gene alone.

The mechanisms that may underlie the growth-inhibitory effect of HuIFNβ on human melanoma cells deserve some comments. Although addition of exogenous HuIFNβ has shown anti-proliferative but

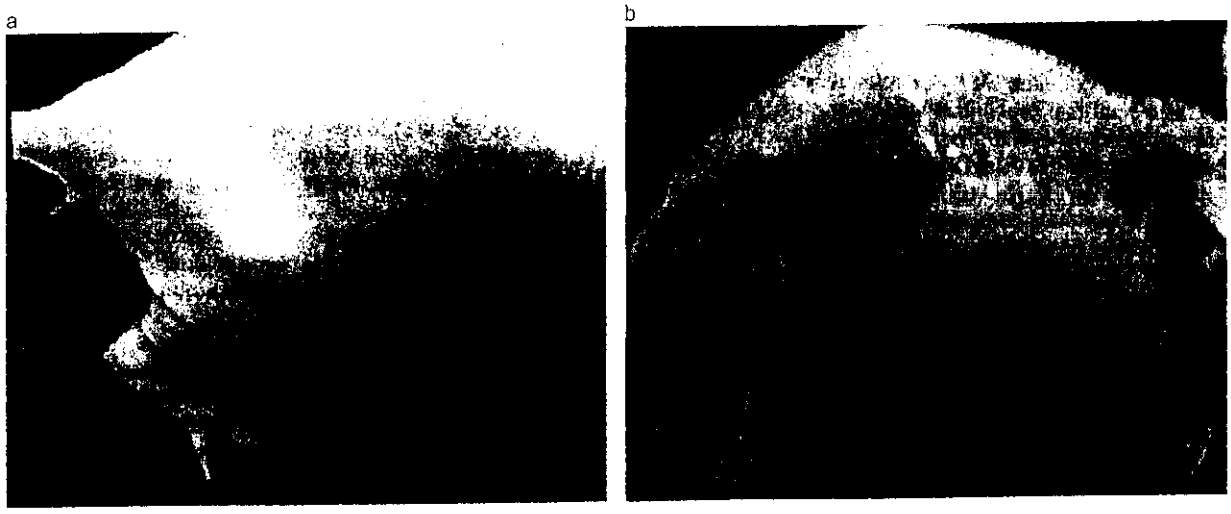

Figure 2. Macroscopic features of the transplanted human melanoma nodules 60 days after implantation. **a** The tumour nodule completely disappeared in mice after six intratumoral injections of liposomes containing pSV2IFNβ. **b** The tumour nodule in control mice increased rapidly in size.

Table 3. Number of Ki 67-positive and apoptotic cells in melanoma tissue sections. Apoptotic melanoma cells were detected immunohistochemically using a TUNEL assay kit. Values given are means ± SD

Group	Ki 67-positive cells (%)	Apoptotic cells (%)
1	48.9 ± 2.9	2.2 ± 1.4
2	45.1 ± 4.6	3.8 ± 2.2
3	ND	ND
4	39.6 ± 6.7	7.5 ± 3.9

ND, not detectable.

not cytotoxic activity against melanoma cells *in vitro*,[10,11] melanoma cells transfected with HuIFNβ gene were strongly inhibited in their growth in this study. Local higher concentrations of HuIFNβ continuously secreted from transfected melanoma cells may work more effectively on themselves *in vitro* and *in vivo*. Regarding this point, Hanson *et al.*[16] published recently an interesting paper. According to their study, the sensitivity of melanoma cells to interferons is dependent on the expression of the interferon gene, which is located at chromosome 9p21 and is often deleted from melanoma cells. Therefore, transduction of HuIFNβ gene might be able to make human melanoma cells much more susceptible to HuIFNβ. In addition, Qin *et al.*[17] demonstrated that *ex vivo* interferon-β gene transfection by replication-defective adenovirus, with a transfection rate less than 1%, blocked tumour formation in immune-deficient mice. Our present study, along with these reports, indicates that endogenously produced HuIFNβ caused by HuIFNβ gene transfection has a powerful cytocidal effect on human melanoma.

Immunohistochemical analysis revealed that HuIFNβ gene transfection reduced the number of cells in the cell cycle and induced a significantly higher degree of apoptosis, which was surely responsible for the growth inhibition. Melcher *et al.*[18] demonstrated that induction of necrosis is important in increasing tumour immunogenicity via heat-shock protein. Not only apoptosis but also necrosis of melanoma cells caused by HuIFNβ gene transfection could increase the efficacy of the therapy by activating the host immune system. Analysis of immune system activation by this gene therapy is underway in our laboratory using a syngeneic mouse melanoma system.

The present study suggests gene therapy using the HuIFNβ gene is promising in melanoma. We are currently investigating the systemic effects of immunoconjugated liposomes labelled with human single chain fragment of variable region of anti-high molecular weight-melanoma associated antigen antibody (HMW-MAA) in order to target the gene to metastatic lesions.[19,20]

Acknowledgements

This work was supported in part by the Grant-in-Aid for Cancer Research (11-7) from the Ministry of Health and Welfare of Japan and by the Grant-in-Aid for Scientific Research (B) from the Japanese Society for the Promotion of Science, Tokyo.

References

1. Parmiani G, Colombo MP. Somatic gene therapy of human melanoma: preclinical studies and early clinical trials. *Melanoma Res* 1995; **5**: 295–301.
2. Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, Huang L, Shu S, Gordon DD. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity. *Proc Natl Acad Sci USA* 1993; **90**: 11307–11311.
3. Nabel GJ, Gordon D, Bishop DK, Nickoloff BJ, Yang Z-Y, Aruga A, Cameron MJ, Nabel EG, Chang AE. Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. *Proc Natl Acad Sci USA* 1996; **93**: 15388–15393.
4. Mizuno M, Yoshida J, Sugita K, Inoue I, Seo H, Hayashi Y, Koshizaka T, Yagi K. Growth inhibition of glioma cells transfected with the β-interferon gene by liposomes coupled with a monoclonal antibody. *Cancer Res* 1990; **50**: 7826–7829.
5. Mizuno M, Yoshida J, Sugita K, Yagi K. Growth inhibition of glioma cells of different cell lines by human interferon-β produced in the cells transfected with its gene by means of liposomes. *J Clin Biochem Nutr* 1990; **9**: 73–77.
6. Yoshida J, Mizuno M, Yagi K. Secretion of human β-interferon into the cystic fluid of glioma transfected with the interferon gene. *J Clin Biochem Nutr* 1991; **11**: 123–128.
7. Yoshida J, Mizuno M, Yagi K. Antitumor effect of endogenous human β-interferon on malignant glioma and augmentation of the effect by tumor necrosis factor-α. *J Clin Biochem Nutr* 1992; **12**: 153–160.
8. Harada K, Yoshida J, Mizuno M, Kurisu K, Uozumi T. Growth inhibition of intracerebral rat glioma by transfection-induced human interferon-β. *J Surg Oncol* 1995; **59**: 105–109.
9. Mizuno M, Yoshida J. Effect of human interferon-β gene transfer upon human glioma transplanted into nude mouse brain involves induced natural killer cells. *Cancer Immunol Immunother* 1998; **47**: 227–232.
10. Garbe C, Krasagakis K, Zouboulis CC, Schroder K, Kruger S, Stadler R, Orfanos CE. Antitumor activity of interferon alpha, beta, and gamma and their combinations on human melanoma cells in vitro: changes of proliferation, melanin synthesis, and immunophenotype. *J Invest Dermatol* 1990; **95**: 231S–237S.
11. Horikoshi T, Fukuzawa K, Hanada N, Ezoe K, Eguchi H, Hamaoka S, Tsujiya H, Tsukamoto T. In vitro comparative study of the antitumor effects of human interferon-alpha, beta, and gamma on the growth and invasive potential of human melanoma cells. *J Dermatol* 1995; **22**: 631–636.
12. Byers HR, Etoh T, Doherty JR, Sober AJ, Mihm MC Jr. Cell migration and actin organization in cultured human primary, recurrent cutaneous and metastatic melanoma. *Am J Pathol* 1991; **139**: 423–435.
13. Sugi M, Kato H, Fujimoto M, Utsumi J, Hosoi K, Shimizu H, Kobayashi S. Monoclonal antibody to human beta interferon: characterization and application. *Hybridoma* 1987; **6**: 313–320.
14. Harada K, Yoshida J, Mizuno M, Sugita K, Kurisu K, Uozumi T. Growth inhibition of subcutaneously transplanted human glioma by transfection-induced tumor necrosis factor-alpha and augmentation of the effect by gamma-interferon. *J Neurooncol* 1994; **22**: 221–225.
15. Yamazaki S, Kuramoto K, Matsuo E, Hosoi K, Hara M, Shimizu H. Highly sensitive enzyme immunoassay of human interferon-beta1. *J Immunoassay* 1989; **10**: 57–73.
16. Hanson C, Kopf I, Weijdegard B, Weimarck A, Stierner U. Sensitivity to extrinsically supplied interferon and the endogenous expression of interferon in melanoma cell lines. *Melanoma Res* 1999; **9**: 451–456.
17. Qin X, Tao N, Dergay A, Moy P, Fawell S, Davis A, Wilson JM, Barsoum J. Interferon-β gene therapy inhibits tumor formation and causes regression of established tumor in immune-deficient mice. *Proc Natl Acad Sci USA* 1998; **95**: 14411–14416.
18. Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat Med* 1998; **4**: 581–587.
19. Desai SA, Wang X, Noronha EJ, Kageshita T, Ferrone S. Characterization of human anti-high molecular weight-melanoma associated antigen single chain Fv antibodies isolated from a phage display antibody library. *Cancer Res* 1998; **58**: 2417–2425.
20. Noronha EL, Wang X, Desai SA, Kageshita T, Ferrone S. Limited diversity of human scFv fragments isolated by panning a synthetic phage display scFv library with cultured human melanoma cells. *J Immunol* 1998; **161**: 2968–2976.

(Received 31 July 2000; accepted in revised form 22 January 2001)