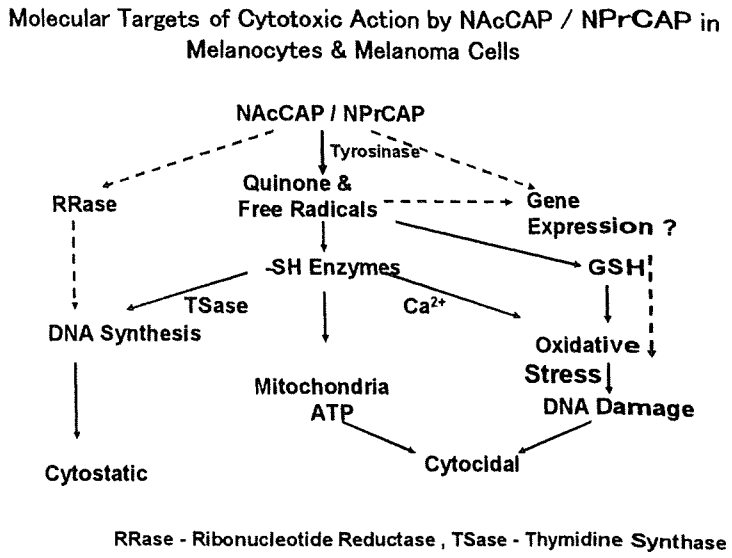

Fig. (3).

3 . Selective growth inhibition effect of cysteaminylphenols to melanoma cells

The selectivity and specificity of our synthetic compounds to melanoma cells were evaluated by the in vivo and in vitro studies. The selective uptake of our drug by melanoma cells and tissues was shown by employing ^{14}C-labelled cysteaminylphenol. A high, specific uptake of NAcCAP was seen by melanoma cell lines, such as SKmel 23. In addition, a melanoma-bearing mouse showed, on the whole body autoradiogram, the selective uptake and covalent binding of NAcCAP in melanoma tissues of lung and skin. In another experiment, we examined to what extent one can block the melanoma growth in both in vitro culture and in vivo lung metastasis assays by administration of NAcCAP combined with BSO, buthionine sulfoxide, which blocked the effect of anti-oxidants. There was a marked growth inhibition of cultured melanoma cells in the presence of BSO, indicating that the selective cytotoxicity by our CAP is related to the quinone and free radicals. The in vivo lung metastasis experiment also showed the decreased number of lung melanoma colonies [3]. The problem was, however, that a fairly large number of amelanotic melanoma lesions were seen to grow in the lung. NPrCAP has been developed with the hope of increasing the cytotoxicity and overcoming a part of the problem.

Ⅱ. STRATEGY FOR DEVELOPMENT OF CHEMO-THERMO-IMMUNOTHERAPY FOR MELANOMA BY MELANOGENESIS SUBSTRATES

1 . Synthesis for conjugate of N-propionyl cysteaminylphenol and magnetite nanoparticles

In order to further increase the cytotoxicity to both melanotic and amelanotic cells, we conjugated NPrCAP with magnetite nanoparticles, which generate heat upon exposure to an alternating magnetic field (AMF). We expected this combination of NPrCAP and magnetite nanoparticles to be a potential source for developing not only anti-melanoma pharmacologic but also immunogenic agent. It was expected that NPrCAP/magnetite nanoparticles complex could be selectively incorporated into melanoma cells. The degraded melanoma tissues from oxidative stress by NPrCAP and heat shock by AMF exposure would produce the synergistic effect for generating

tumor-infiltrating lymphocytes, TIL that will kill melanoma cells in distant metastases, Fig. (4). Four compounds were synthesized and two of them, i.e., NPrCAP/M and NPrCAP/PEG/M were used for animal and human studies respectively (Fig). 5.

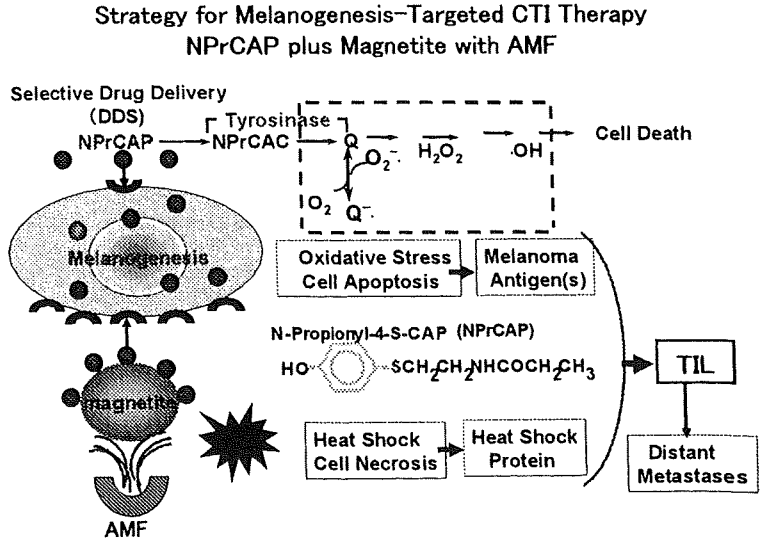

Fig. (4).

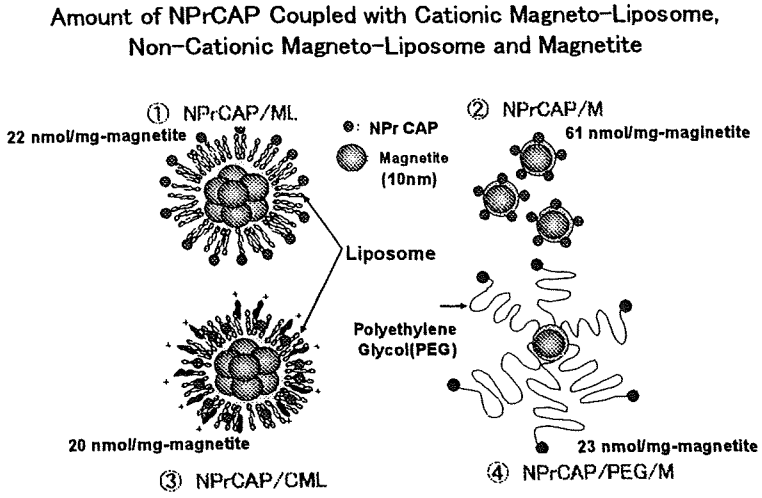

Fig. (5). ML : neutral magneto-liposome, CML : cationic magneto-liposome.

Magnetite nanoparticles have been employed for thermotherapy in a number of cancer treatments including human gliomas and prostate cancers [33-35]. They consist of 10-100nm-sized iron oxide (Fe_3O_4) with a surrounding polymer coating and become magnetized when placed in AMF [9]. We synthesized, in our initial study, the conjugate of NPrCAP with magnetite nanoparticles (NPrCAP/ML) and NPrCAP/CML in which NPrCAP were embedded in cationic liposomes, Fig. (5). There was, however, non-specific electrostatic interaction between cationic magneto-liposomes and various non-target cells [35] and non-specific aggregations in neutral magneto-liposomes. A promising technique is the use of tumor-targeted magnetite nanoparticles, and this approach is extended by synthesizing another type of magnetite nanoparticles, NPrCAP/M and NPrCAP/PEG/M, on which NPrCAP is superficially and directly bound on the surface of magnetite nanoparticles without using

liposomes. They are chemically stable, and can be produced in large quantities and employed to effect melanoma-targeted chemotherapy (by NPrCAP) and thermo-immunotherapy (by magnetite with HSP), hence providing a basis for a novel chemo-thermo-immunotherapy (CTI therapy). Most of the experiments described below were carried out by employing NPrCAP/M except in preliminary clinical trials to which NPrCAP/PEG/M was used.

2. Development of chemo-, thermo- and immunotherapy by exploiting melanogenesis substrates

Our basic strategy in designing chemo-thermo-immunotherapy (CTI therapy) drugs is that tyrosinase substrates, NPrCAP/M, will be selectively aggregated on the melanoma cell surface by active transport through a still unknown receptor system and that they will be incorporated into early and late endosomes to which tyrosinase will also be transported from TGN to form stage I melanosomes. Once NPrCAP/M is incorporated into melanosomes, it will be then retained and aggregated in the melanosomal compartments as there will be no melanosome transfer occurring in melanoma cells, Fig. (6). Thus we should be able to selectively destroy melanoma cells by heat generated by AMF exposure from magnetite nanoparticles which are accumulated only in melanosomal compartments. In fact, we could see NPrCAP/M nanoparticles which were selectively aggregated in melanoma cells compared to non-melanoma cells, Fig. (7). NPrCAP/M nanoparticles were found to be specifically incorporated and aggregated in melanosomal compartments at 2 weeks after ip administration by electron microscopy, Fig. (8). After AMF exposure, there will be selective disintegration of melanoma tissues as can be seen by Berlin Blue staining, Fig. (9). [36,37]

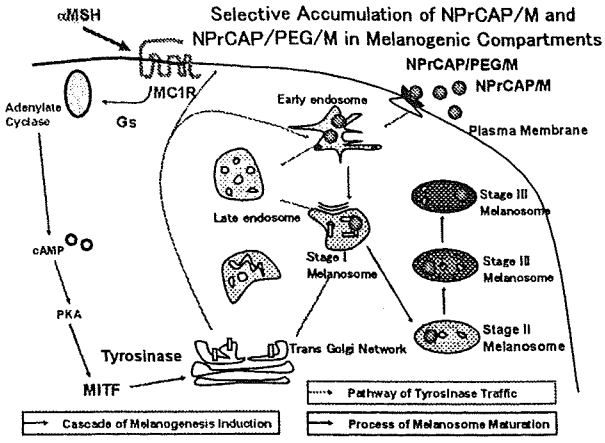

Fig. (6)

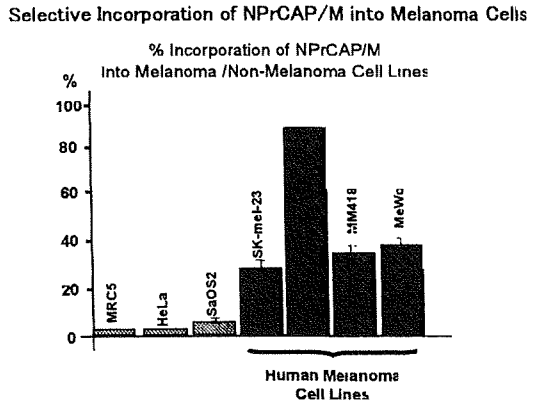

Fig. (7).

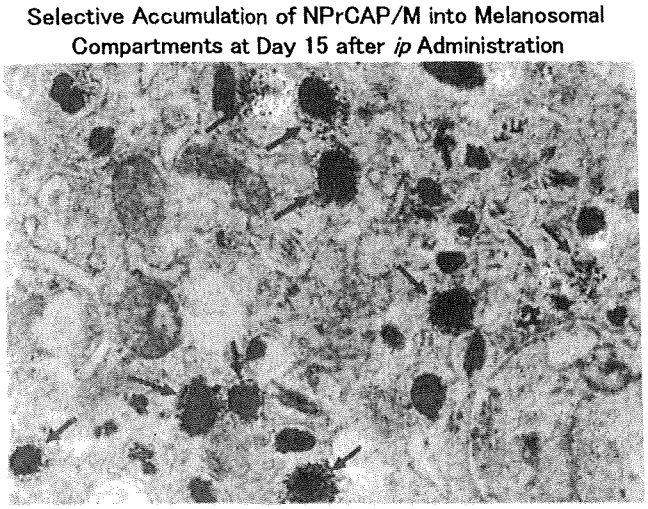

Fig. (8). Arrows indicate magnetite particles incorporated into melanosomes.

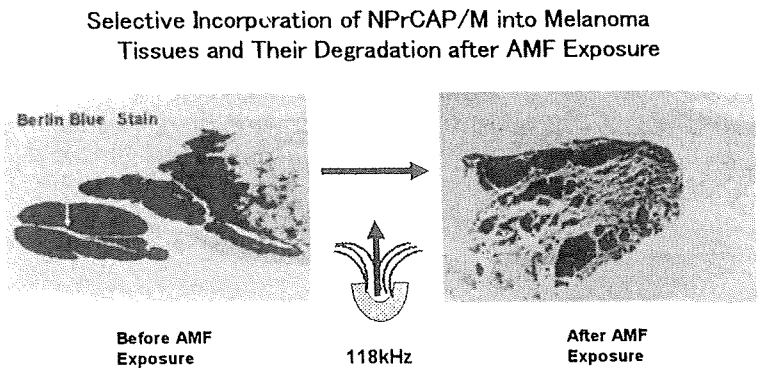

Fig. (9).

In hyperthermia treatment, the expression of heat shock proteins (HSPs) plays an important role in immune reactions [12-16, 38-39]. Accumulating evidence from our group [18-20] and from others [21] implicates HSP expression induced by hyperthermia in tumor immunity and opens the door to novel cancer therapy based on hyperthermia treatment (thermo-immunotherapy). In such a strategy, a tumor-specific hyperthermia system that can induce necrotic cell death via HSP expression without damaging non-cancerous tissues would be highly desirable. An intracellular hyperthermia system using tumor-targeted magnetite nanoparticles facilitates tumor-specific hyperthermia ; this can induce necrotic cell death via HSP expression, which in turn induces antitumor immunity.

2 . Protocols of experimental chemo-thermo-immunotherapy by employing melanogenesis substrates

In this study, we employed three cell lines of B16 melanoma, i.e., B16F1, B16F10 and B16OVA cells and compared the thermo-therapeutic protocols in detail by evaluating the growth of the re-challenge melanoma as well as the duration and rates of survival of melanoma-bearing mice, Fig. (10).

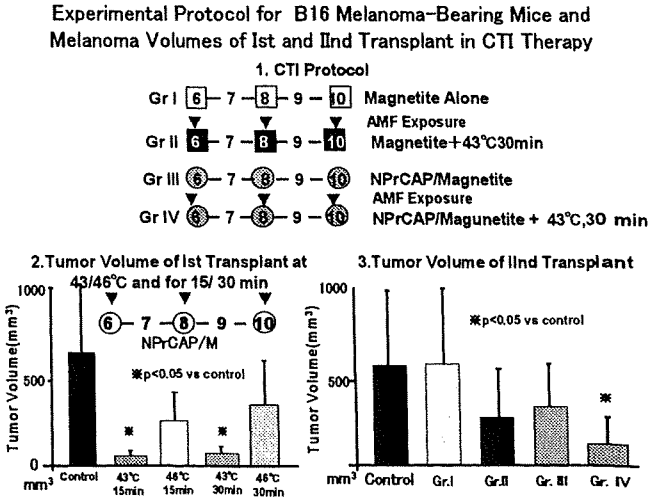

Fig.(10).

We first evaluated the chemotherapeutic effect of NPrCAP/M with or without heat. NPrCAP/M without heat inhibited growth of primary transplants to the same degree as did NPrCAP/M with heat, indicating that NPrCAP/M alone has a chemotherapeutic effect. However, there was a significant difference in the melanoma growth inhibition of re-challenge transplants between the groups of NPrCAP/M with and without heat. NPrCAP/M with AMF exposure showed the most significant growth inhibition in re-challenge melanoma and increased life span of the host animals, i.e., 30-50% complete rejection of re-challenge melanoma growth, indicating that NPrCAP/M with heat possesses a thermo-immunotherapeutic effect, Fig. (11). Specifically our study indicated that the most effective thermo-immunotherapy for re-challenge B16 melanoma can be obtained at a temperature of 43℃ for 30 min with the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma, Fig. (10). Our therapeutic conditions and their effects differ from those of magnetically mediated hyperthermia on the transplanted melanomas reported previously [40]. cationic magneto-liposomes-mediated hyperthermia for B16 melanoma showed that hyperthermia at 46℃ once or twice led to regression of 40-90% of primary tumors and to 30-60% survival of mice, whereas hyperthermia at 43℃ failed to induce regression of the secondary tumors with 0% survival of mice [40].

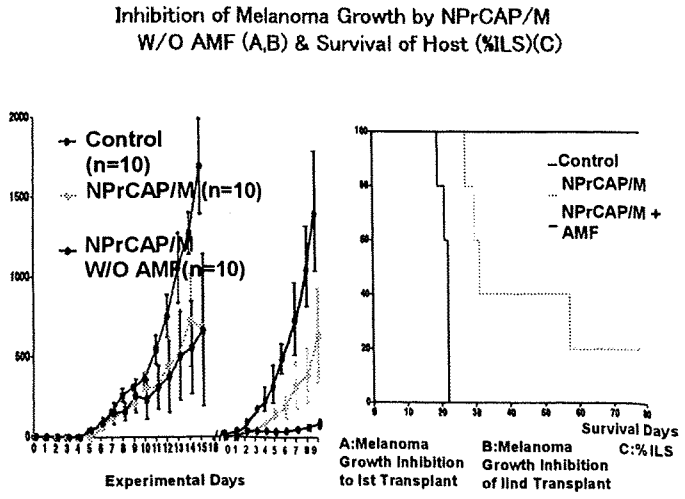

Fig. (11).

We analyzed HSP70 production in the primary tumor and $CD4^+$ and $CD8^+$ T cell infiltration into the re-challenge secondary tumor. Our study showed that NPrCAP/M-mediated hyperthermia at 43℃ for 15 to 30 min and 46℃ for 15 min produced a large amount of HSP70, Fig. (11). This stress protein forms a complex with intracellular peptides released from degrading tumor cells and presented by the MHC class I molecules of professional antigen-presenting cells [20]. Although thermotherapy at 46℃ for 15 min could induce HSP70 as abundantly as that at 43℃ for 30 min, this condition failed to suppress the re-challenge melanoma transplant as efficiently as 43℃ thermotherapy Fig. (12). This suggests that immunological factors other than HSPs are at least in part responsible for rejection of the second re-challenge melanoma. Hyperthermia at 43° for 1 hr revealed the expression of MHC class I molecules after 24 h in association with enhanced expression of HSP70 [41]. Heat treatment of tumor cells permits enhanced cross-priming, possibly via up-regulation of both HSPs and tumor antigen expression [21]. Thus, by inducing HSP70 and possibly MHC class I, our protocol of NPrCAP/M-mediated hyperthermia at 43℃ can be an effective therapy for the treatment of advanced metastatic melanoma.

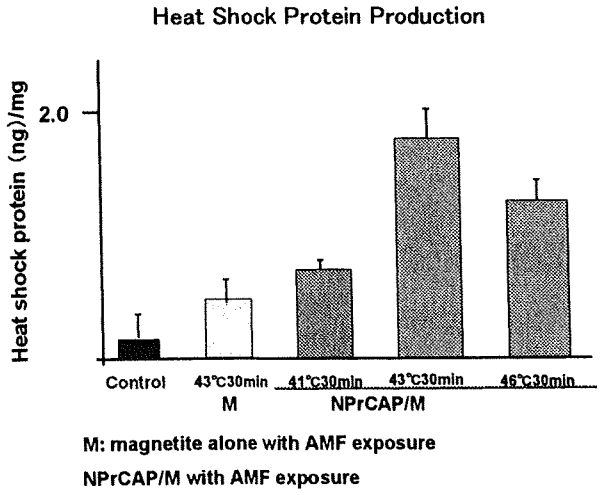

Fig. (12).

NPrCAP/M-mediated hyperthermia at a relatively low temperature (43℃) effectively inhibited the growth of second transplant, re-challenge melanoma. It may be possible that superficially bound NPrCAP possesses an important role not only in targeting nanoparticles to melanocytic cells and a chemotherapeutic effect on these cells but also in causing potentially an immunotherapeutic effect.

4. Melanocytotoxic and immunogenic properties of N-propionyl cysteaminylphenol (NPrCAP) and magnetite conjugates

Hyperthermia increases the expression of intracellular HSPs which is important in and necessary for the induction of antitumor immunity [42,43]. Over expression of HSPs, such as HSP 70, increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [44, 45] . In this process professional antigen presenting dendritic cells play unique and important roles in taking up, processing and presenting exogenous antigens in association with MHC class I molecules. Our working hypothesis for induction of in situ vaccination immunotherapy is that CTI therapy causes degradation of melanoma tissues which results in the release of HSP/melanoma antigen complex. This complex is taken up by professional antigen-presenting dendritic cells through HSP receptor. Subsequently after

internalization within the dendritic cells, MHC and antigen peptide complex is presented to CD8+ T cells with the induction of acquired immunity, Fig. (13).

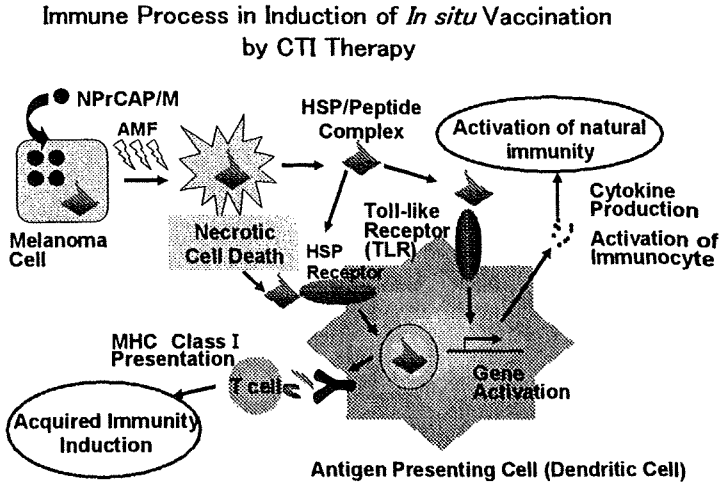

Fig. (13).

In our animal study it was indicated that NPrCAP/M by itself inhibits melanoma growth by not only chemotherapeutic effect but also a unique immunogenic property. Our current working hypothesis for this finding is that there is a difference in the cyototoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without AMF exposure. The animals with NPrCAP/M plus AMF exposure resulted in non-apoptotic necrotic cell death with immune complex production of melanoma peptide as well as HSP 70 and a small amount of HSP 90. The group with NPrCAP/M plus AMF exposure showed the most significant growth inhibition of the re-challenged melanoma growth which resulted in the almost complete survival of the host animals as long as for 3 months that we have conducted our experimental protocol.

It is, however, important to note that those animals bearing B16F1, B16F10 and B16OVA melanoma cells showed not only significant rejection of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure but also apoptotic or apoptotic cell death which was associated with immune complex production of HSP90 and melanoma peptide [44]. When NPrCAP was given systemically i.p. to black C57BL/6 mice, it caused depigmentation of black hair follicles which was found to be derived from selective apoptotic disintegration of follicular melanocytes [47]. Melanin intermediates produce reactive oxygen species such as superoxide and H2O2 [5, 47, 48]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. The molecular interaction between NPrCAP chemo-immunotherapeutic and magnetite/AMF thermo-immunotherapeutic properties needs to be further studied.

Ⅳ. SUMMARY AND PERSPECTIVES

In this communication, we are able to show that

(1) NPrCAP with conjugation of magnetite nanoparticles, NPrCAP/M, with/without AMF exposure can induce cytotoxic T cells that inhibit the growth of re-challenged melanoma transplanted at the opposite site of body ;

(2) NPrCAP alone appears to generate both chemotherapeutic and immunotherapeutic property to B 16melanoma cells through both apoptotic and non-apoptotic processes respectively ;
(3) Melanogenesis cascade can be utilized as the basis for developing melanoma-targeted DDS and chemo-thermo-immunotherapy agents.

Based upon these animal experiments, a preliminary human clinical trial has been carried out by employing NPrCAP/PEG/M plus AMF after we received the approval of our human clinical trials for a limited number of stage III and IV melanoma patients (Clinical Trial Research No. 18-67, Sapporo Medical University). The therapeutic protocol followed the basically identical experimental schedule as that of animal experiments. In this clinical trials, however, we utilized NPrCAP/PEG/M which was made by conjugating polyethylene glycol between NPrCAP and magnetite nanoparticles, Fig. (5). Among four patients two of them showed complete and partial responses to our treatment and have been able to carry out normal daily activities after CTI therapy. In one patient, for example, four distant cutaneous metastasis sites were evaluated and either significant regression or shrinkage of all of these four melanoma lesions was seen. The patient was able to survive 30 months after several trials of CTI therapy. The pathological and immunological specimens revealed dense aggregation of lymphocytes and macrophages at the site of CTI therapy. Importantly there was a trend to have an almost identical distribution of CD8+ T cells and MHC class 1 positive cells. Another patient had many lymph node metastases, but still has been surviving more than 32 months. In order to evaluate the overall therapeutic effect to advanced melanoma, it is important to have larger-scaled clinical trials and define concisely the molecular interaction between chemotherapeutic and thermo-immunotherapeutic effect in our CTI therapy.

ABBREVIATIONS

DDS= drug delivery system
HSP=heat shock protein
AMF= alternating magnetic field
NPrCAP/M= N-propionyl 4S cysteaminylphenol/ magnetite nanoparticle
NPrCAP= N-propionyl 4S cysteaminylphenol
CTI therapy= Chemo-thermo-immunotherapy
MSH= melanocyte stimulating hormone
MITF= microphthalmia transcription factor
MC1R= melanocortin 1 receptor
NAcCAP= N-acetyl 4S cysteaminylphenol
BSO= buthionine sulfoxide
PEG= polyethylene glycol
NPrCAP/PEG/M= N-propionyl 4-S cysteaminylphenol/ polyethylene glycol/ magnetite nanoparticle
ML=non-cationic magneto-liposome
CML= cationic magneto-liposome

ACKNOWLEDGEMENTS

This work was supported by a Health and Labor Sciences Research Grant-in-Aid (H21-Nano-006) for

Research on Advanced Medical Technology from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

[1] Balch, C.M. Buzaid, A.C. Soong, S.J. et al ; Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J. Clin Oncol. 2001, 19, 3635-3648.

[2] Jimbow, K. Iwashina, T. Alena, F. Yamada, K. Pankovich, J. Umemura, T. ; Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. J. Invest Dermatol, 1993, 100, suppl. 2, pp、231S-238S.

[3] Alena, F. Ishikawa, T. Gili, A. Jimbow, K. ; Selective in vivo accumulation of N-acetyl-4-S-cysteaminylphenol in B16F10 murine melanoma and enhancement of its in vitro and in vivo antimelanoma effect by combination of buthionine sulfoximine. Cancer Res, 1994, 54, 2661-2666.

[4] Pankovich, J.M. Jimbow, K. ; Tyrosine transport in a human melanoma cell line as a basis for selective transport of cytotoxic analogues. Biochem, 1991, 15, 28, 721-725.

[5] Reszka, K. Jimbow, K. Electron donor and acceptor properties of melanin pigments in the skin. ; In : Fuchs J, Packer L. Oxidative Stress in Dermatology. Marcel Dekker, Inc. New York, 1993 ; pp.287-320.

[6] Tandon, M. Thomas, P.D. Shokravi, M. Singh, S. Samra, S. Chang, D. Jimbow, K. ; Synthesis and antitumour effect of the melanogenesis-based antimelanoma agent N-propionyl-4-S-cysteaminylphenol. Biochem Pharmacol, 1998, 15, 2023-2029.

[7] Thomas P.D. Kishi, H. Cao, H. Ota, M. Yamashita, T. Singh, S. Jimbow, K. ; Selective incorporation and specific cytocidal effect as the cellular basis for the antimelanoma action of sulphur containing tyrosine analogs. J. Invest Dermatol, 1999, 113, 928-934.

[8] Ito, A. Shinkai, M. Honda, H, et al. ; Medical application of functionalized magnetic nanoparticles. J. Biosci Bioeng, 2005, 100, 1-11.

[9] Kawai, N. Ito, A. Nakahara, Y. Futakuchi, M. Shirai, T. Honda, H. Kobayashi, T. Kohri, K. ; Anticancer effect of hyperthermia on prostate cancer mediated by magnetite cationic liposomes and immune-response induction in transplanted syngeneic rats. The Prostate, 2005, 64,373-381.

[10] Yanase, M. Shinkai, M. Honda, H. Wakabayashi, T. Yoshida, J. Kobayashi, T. ; Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. Japanese J. Cancer Res, 1998, 89, 775-782.

[11] Shinkai, M. Yanase, M, Honda, H. et al. ; Intracellular hyperthermia for cancer using magnetite cationic liposomes : in vitro study. Jpn J. Cancer Res, 1996, 87, 1179-1183.

[12] Menoret, A. Chandawarkar, R. ; Heat-shock protein-based anticancer immunotherapy : an idea whose time has come. Semin Oncol, 1998, 25, 654-660

[13] Srivastava, P.K. Menoret, A. Basu, S. Binder, R. Quade K. ; Heat shock proteins come of age : primitive functions acquired new roles in an adaptive world. Immunity, 1998, 8, 657-665.

[14] Tamura, Y. Tsuboi, N. Sato, N. Kikuchi, K. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. J. Immunol, 1993, 51, 5516-5524.

[15] Tamura, Y. Peng, P. Liu, K. Daou, M. Srivastava, P.K. ; Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. Science, 1997, 278, 117-120.

[16] Tamura, Y. Sato, N. ; Heat shock proteins : chaperoning of innate and adaptive immunities.

Japanese J. Hyperthermic Oncol, 2003, 19, 131-139.

[17] Srivastava, P.K. ; Immunotherapy for human cancer using heat shock protein-peptide complexes. Current Oncol Reports, 2005, 7, 104-108.

[18] Tamura, Y. Takashima, S. Cho, J.M. Qi, W. Kamiguchi, K. Torigoe, T. Takahashi, S. Hirai, I. Sato, N. Kikuchi, K. ; Inhibition of natural killer cell cytotoxicity by cell growth-related molecules. Jpn J. Cancer Res, 1996, 87, 623-630.

[19] Ueda, G. Tamura, Y. Hirai, I. Kamiguchi, K. Ichimiya, S. Torigoe, T. Hiratsuka, H. Sunakawa, a H. Sato, N. ; Tumor-derived heat shock protein 70-pulsed dendritic cells elicit tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity. Cancer Sci, 2004, 9, 248-253.

[20] Ito, A. Honda, H. Kobayashi, T. ; Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles : a novel concept of "heat-controlled necrosis" with heat shock protein expression. Cancer Immunol Immunother, 2006, 55, 320-328.

[21] Shi, H. Cao, T. Connolly, J.E. Monnet, L. Bennett, L. Chapel, S. Bagnis, C. Mannoni, P. Davoust, J. Palucka, A.K. Banchereau, J. Hyperthermia enhances CTL cross-priming. J. Immunol, 2006, 176, 2134-2141.

[22] Dakour, J. Vinayagamoorthy, M. Chen, H. Luo, D. Dixon, W. Jimbow, K. : Identification of A cDNA for Ca++-binding Calnexin-like phosphoprotein (p90) on melanosomes in normal and malignant human melanocytes. Exp Cell Res, 1993, 209, 288-300.

[23] Toyofuku, K. Wada, I. Hirosaki, K. Park, J.S. Hori, Y. Jimbow, K. : Involvement of calnexin in maturation of tyrosinase as a molecular chaperone. J. Biochem, 1999, 125, 82-89.

[24] Jimbow, K. Gomez, P.F. Toyofuku, K. Chang, D. Miura, S. Tsujiya, H. Park, J.S. : Biological role of tyrosinase related protein and its biosynthesis and transport from TGN to stage I melanosome, late endosome, through gene transfection study. Pigment Cell Res, 1997, 10, 206-213.

[25] Jimbow, K. Park, J.S. Kato, F. Hirosaki, K. Toyofuku, K. Hua, C. Yamashita, T. : Assembly, target signal and intracellular transport of tyrosinase gene family protein in the initial stage of melanosome biogenesis. Pigment Cell Res, 2000, 13, 222-229.

[26] Jimbow, K. Hua, C. Gomez, P.F. Hirosaki, K. Shinoda, K. Salopek, T.G. Matsusaka, H. Jin, H.Y. Yamashita, T. : Intracellular vesicular trafficking of tyrosinase gene family protein in eu- and pheomelanosome biogenesis. Pigment Cell Res, 2000, 13 (Suppl. 8), 110-117.

[27] Miura, T. JIMBOW, K. Ito, S. ; The in vivo antimelanoma effect of 4-S-cysteaminylphenol and its n-acetyl derivative. Int J. Cancer, 1990, 46, 931-934.

[28] Tandon, M. Thomas, P.D. Shokravi, M. Singh, S. Samra, S. Chang, D. Jimbow, K, ; Synthesis of the melanogenesis-based antimelanoma agent, N-propionyl-4-S-cysteaminylphenol, and screening of depigmenting and anti-tumour effects. Biol Pharmacol, 1998, 55, 2023-2029.

[29] Ito, S. Kato, T. Ishikawa, K. Kasuga, T. Jimbow, K. : Mechanism of selective toxicity of 4-S-cysteinylphenol and 4-S-cysteaminylphenol to melanocytes. Biochem Pharmacol, 1987, 36, 2007-2011.

[30] Gili, A. Thomas, P.D. Ota, M. Jimbow, K. ; Comparison of in vitro cytotoxicity of N-acetyl and N-propionyl derivatives fo phenolic thioether amines in melanoma and neuroblastoma cells and the relationship to tyrosinase and tyrosine hydroxylase enzyme activity. Melanoma Res, 2000, 10, 9-15.

[31] Parsons, P.G. Favier, F. McEwan, M. Takahashi, T. Jimbow, K. Ito, S. : Action of cysteaminylphenols on human melanoma cells in vivo and in vitro : 4-S-cysteaminylphenol binds protein disulphide isomerase. Melanoma Res, 1992, 1, 97-104.

[32] van Landeghem F.K., Maier-Hauff K., Jordan A., Hoffmann K.T., Gneveckow U., Scholz R., Thiesen B., Bruck W., von Deimling A. : Post-mortem studies in glioblastoma patients treated with thermotherapy using magnetic nanoparticles. Biomaterials. 2009, 30, 52-57.

[33] Thiesen B., Jordan A.. Clinical applications of magnetic nanoparticles for hyperthermia. Int J Hyperthermia. 2008, 24, 467-474.

[34] Johannsen, M. Gneveckow, U. Eckelt, L. Feussner, A. Waldofner, N. Scholz, R. Deger, S. Wust, P. Loening, S.A. Jordan, A. ; Clinical hyperthermia of prostate cancer using magnetic nanoparticles : presentation of a new interstitial technique. Int J. Hypertherm, 2005, 21, 637-647.

[35] Ito, A. Fujioka, M. Yoshida, T. Wakamatsu, K. Ito, S. Yamashita, T. Jimbow, K. Honda H : 4-S-cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma. Cancer Sci, 2007, 98, 424-430.

[36] Takada, T. Yamashita, T. Sato, M. Sato, A. Ono, I. Tamura, Y. Sato, N. Miyamoto, A. Ito, A. Honda, H. Wakamatsu, K. Ito, S. Jimbow, K. : Growth inhibition of re-challenge B16 melanoma transplant by conjugates of melanogenesis substrate and magnetite nanoparticles as the basis for developing melanoma-targeted chemo-thermo-immunotherapy. J. Biomed Biotechnol, , 2010 (in press)

[37] Sato, M. Yamashita, T. Ohkura, M. Osai, Y. Sato, A. Takada, T. Matsusaka, H. Ono, I. Tamura, Y. Sato, N. Sasaki, Y. Ito, A. Honda, H. Wakamatsu, K. Ito, S. Jimbow, K. : N-Propionyl-Cysteaminylphenol-Magnetite Conjugate (NPrCAP/M) Is a Nanoparticle for the Targeted Growth Suppression of Melanoma Cells. J. Invest Dermatol, 2009, 129, 2233-41.

[38] Lindquist, S. ; The heat-shock response. Ann Rev Biochem, 1986, 55, 1151-1191.

[39] Konno, A. Sato, N. Yagihashi, A. Torigoe, T. Cho, J.M. Torimoto, K. Hara, I. Wada, Y. Okubo, M. Takahashi, N. Kikuchi, K. ; Heat- or stress-inducible transformation-associated cell surface antigen on the activated H-ras oncogene-transfected rat fibroblast. Cancer Res, 1989, 49, 6578-6582.

[40] Suzuki, M. Shinkai, M. Honda, H. Kobayashi, T. Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes. Melanoma Res. 2003, 13, 129-135.

[41] Ito, A. Shinkai, M. Honda, H. Wakabayashi, T. Yoshida, J. Kobayashi, T. ; Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. Cancer Immunol Immunother. 2001, 50, 515-522.

[42] Ito, A. Shinkai, M. Honda, H. et al. ; Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles. Cancer Immunol Immunother, 2003, 52, 80-88.

[43] Mise, K. Kan, N. Okino, T. Et al. ; Effect of heat treatment on tumor cells and antitumor effector cells. Cancer Res, 1990, 50, 6199-6202.

[44] Ito, A. Matsuoka, F. Honda, H. et al. ; Heat shock protein 70 gene therapy combined with hyperthermia using magnetic nanoparticles. Cancer Gene Ther, 2003, 10, 918-925.

[45] Wells, A.D. Rai, S.K. Salvato, M.S. et al. ; Intracellular hyperthermia for cancer using magnetite cationic liposomes : an in vivo study. Jpn J. Cancer Res, 1998, 89, 463-469.

[46] Osai, Y. Ohkura, M. Tamura, Y. Sato, N. Ito, A. Honda, H. Wakamatsu, K. Ito, S. Yamashita, T. Jimbow, K. ; Intratumoral administration of melanoma targeting N-propionyl cysteaminylphenol induces in vivo anti-melanoma effect and tumor specific immunity. Pigment Cell Melanoma Res, 2008, 21,329 (Abstract).

[47] Minamitsuji, Y. Toyofuku, K. Sugiyama, S. Jimbow, K. ; Sulphur containing tyrosinase analogs can cause selective melanocytoxicity involving tyrosinase-mediated apoptosis. J. Invest Dermatol, 1999, 4, 130S-136S.

[48] Jimbow, K. Miyake, Y. Homma, K. Yasuda, K. Izumi, Y. Tsutsumi, A. Ito S, Characterization of melanogenesis and morphogenesis of melanosomes by physicochemical properties of melanin and melanosomes in malignant melanoma. Cancer Res, 1984, 44, 1128-1134.

JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 30, Number 6, 2010
© Mary Ann Liebert, Inc.
DOI: 10.1089/jir.2009.0015

Increased Caspase-2 Activity is Associated With Induction of Apoptosis in IFN-β Sensitive Melanoma Cell Lines

Takafumi Kamiya,[1] Tamaki Okabayashi,[2] Shin-ichi Yokota,[2] Yuji Kan,[1] Jiro Ogino,[1] Toshiharu Yamashita,[1] Nobuhiro Fujii,[2] and Kowichi Jimbow[1,3]

Interferon (IFN) is believed to be one of the most effective anti-melanoma agents. Specifically, IFN-β has the ability to induce apoptosis of melanoma cells. Induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has also been suggested to have a critical role in IFN-β-induced apoptosis. To characterize the signaling pathway involved in IFN-β-induced apoptosis, we analyzed the biological effects of IFN-β on the cell death and caspase activation of melanoma cells. IFN-sensitive cell lines, MM418, SK-mel-23, and SK-mel-118, showed increased apoptotic populations correlated with the activation of caspase-2 and caspase-3 by IFN-β. IFN-β-induced apoptosis was significantly suppressed by inhibitors for caspase-2 or caspase-3, but not by inhibitors for caspase-8 or caspase-9 in these cell lines. TRAIL expression was observed in IFN-β-treated cells of SK-mel-23 and SK-mel-118, but not in those cells of MM418, which showed massive IFN-β-induced apoptosis and resistance to exogenous TRAIL-mediated apoptosis. G361 was resistant to IFN-β-induced apoptosis but sensitive to exogenous TRAIL-mediated apoptosis. Furthermore, IFN-β pretreatment significantly increased the sensitivity against exogenous TRAIL-mediated apoptosis and activation of caspase-2 in G361. These results suggested that caspase-2 activation is commonly associated with induction of IFN-β-induced apoptosis in IFN-β-sensitive melanoma cells.

Introduction

THERAPY FOR MALIGNANT MELANOMA has been a difficult challenge for physicians. Among various therapeutic approaches, interferon (IFN) is believed to be an effective anti-melanoma agent.

Although the mechanisms of IFN-mediated cell death have not been fully elucidated, IFN is known as an effective anti-tumor agent (Fisher and others 1985; Pfeffer and others 1998; Stark and others 1998). It was reported that IFN-β induced apoptosis in melanoma cells more significantly than IFN-α and -γ did (Chawla-Sarkar and others 2001; Leaman and others 2003; Merchant and others 2004). It induces many biological responses by regulating IFN-stimulated genes (ISGs) (Chawla-Sarkar and others 2003). Several ISGs, such as double-stranded RNA-activated protein kinase (*PKR*), myxovirus resistance protein A (*MxA*), melanoma differentiation-associated gene-5 (*MDA-5*), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), are related to the anti-tumor effects of IFN (Leaman and others 2003; Kang and others 2004).

Recently, it has been clarified that IFN has apoptotic effects on several tumors. TRAIL, known as Apo2 ligand, is also a member of the TNF family of transmembrane proteins, which leads tumor cells to apoptosis by stimulating death receptors (DRs) (Griffith and others 1998; Kimberley and Screaton 2004; Zhang and Fang 2005). It has been suggested that the involvement of TRAIL is essential for the apoptotic cascade induced by IFNs in certain melanoma cell lines, as well as in other tumor cell lines (Chawla-Sarkar and others 2001; Chen and others 2001; Meng and El-Deiry 2001; Morrison and others 2005). Furthermore, other ISGs, such as Ras association domain family 1A (RASSF1A) and XIAP (X-linked inhibition of apoptosis protein)-associated protein (XAF1), also contribute to IFN-β-induced and TRAIL-induced apoptosis (Chawla-Sarkar and others 2003; Reu and others 2006).

[1]Department of Dermatology, and [2]Department of Microbiology, Sapporo Medical University School of Medicine, [3]Institute of Dermatology and Cutaneous Sciences, Sapporo, Hokkaido, Japan.

Apoptosis can be included by 2 signaling pathways. One is through apoptosis-inducing ligands such as Fas ligand, TRAIL, and TNF-α-stimulating DRs. The other is through cytochrome *c* release from mitochondria. In these pathways, it is evident that the functions of caspase-3, -8, and -9 are essential. Caspase-2, which is considered to be an initiator caspase and involved in upper stream of the apoptotic pathways, is located diversely in the cytoplasm and nuclei. However, the biological role of caspase-2 remains controversial compared with those of other caspases (Zhivotovsky and others 1999; van Loo and others 2002; Zhivotovsky and Orrenius, 2005). The correlation between TRAIL and caspase-2 has been indicated recently in TRAIL-mediated apoptotic pathways, in which caspase-2 processes procaspase-8 or cleaves Bid, a proapoptotic Bcl-2 family member (Wagner and others 2004; Shin and others 2005). Although the interaction among apoptosis-inducing ligands, IFNs, and caspase-2 has recently been clarified to some extent, the role of caspase-2 in IFN-induced apoptosis remains to be elucidated.

In this study we were interested in the activities of caspases, especially caspase-2 in the IFN-β-induced apoptotic pathway. Therefore, we examined 2 classes of human melanoma cell lines that were sensitive and resistant to apoptosis induced by IFN-β treatment.

Materials and Methods

Cell cultures and reagents

The human melanoma cell lines, SK-mel-118, SK-mel-23, MM418, and G361 (Yamashita and others 2001), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and antibiotics in 5% CO_2 incubators at 37°C. SK-mel-23 and MM418 are pigmented cell lines, and the others are non-pigmented cell lines. IFN-α2 was purchased from Serotec (Oxford, UK). IFN-β was supplied by Mochida Pharmaceutical (Tokyo, Japan). Cells were treated with 1,000 IU/mL of IFNs for different time periods depending on the experiment. Recombinant human TRAIL (R&D Systems, Minneapolis, MN, USA) was used at the final concentration of 100 ng/mL. Recombinant human TNF-α (R&D systems, Minneapolis, MN, USA) was used at the final concentration of 50 ng/mL. The anti-Fas (CD95) antibody (MBL, Nagoya, Japan) was used at the final concentration of 500 ng/mL.

Cell viability assay

Cells were plated in 100 mL of medium in 96-well plates in triplicate with 1,000 cells/well. After 24 h, cells were treated with IFN-α2, or IFN-β (72 h), or treated with IFN-β (24 h) followed by addition of TRAIL, TNF-α, or the anti-Fas (CD95) antibody (48 h) after PBS washing. At 48 h after plating, cells were also treated with TRAIL, TNF-α, or the anti-Fas (CD95) antibody (48 h) to be compared with IFN-β-pretreated cells. The viability of treated cells was determined with the MTT assay, which is a formazan-formation assay, using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using an RNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using Super Script™-III Rnase H (Invitrogen, Carlsbad, CA, USA) from total extracted RNA (50 ng). The quantitative PCR was validated by the linearity of the determination curves at various concentrations of cDNA. Specific primers (Sigma-Genosys, Ishikari, Japan) were constructed as follows: 5′-TTGGCTCAGGTGG ATTTGG-3′ and 5′-GGCTTTTCTTCCACACAGTC-3′ for PKR, 5′-GCATCCCACCCTCTATTACT-3′ and 5′-TGTCTT CAGTTCCTTTGTCC-3′ for MxA, 5′-GGAAGTACAATGAG GCCCTACAAA-3′,5′-TCCTCAGTCCTAGTATATTGCTCC-3′ for mda-5, 5′-GGCTATGATGGAGGTCCAGG-3′ and 5′-GGTCCATGTCTATCAAGTGCTC-3′ for TRAIL, 5′-AGC GTGCCAACGCGCTGCGCAT-3′ and 5′-CAGGCTCGTC CACGTTCGTGTC-3′ for RASSF1A, 5′-GCCTGCGGTTCCTG GTCCTG-3′ and 5′-GCTGGGCGAGCATGCGGTGC-3′ for XAF1, 5′-CCAACAAGACCTAGCTCCCCAGC-3′ and 5′-AA GACTACGGCTGCAACTGTGACTCC-3′ for DR4, 5′-GTCCT GCTGCAGGTCGTACC-3′ and 5′-GATGTCACTCCAGG GCGTAC-3′ for DR5, 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR using Taq DNA polymerase (Promega, Wisconsin, WI) as follows: denaturation, 94°C for 30 s; annealing, 55°C for 30 s; and extension, 72°C for 1 min. The number of cycles was 35.

Fluorescence-activated cell sorting analysis

Analysis of TUNEL assay of apoptotic cells was done by fluorescence-activated cell sorting (FACS). Cells were plated in 8 mL of medium in 10-cm dishes with 2×10^4 cells/mL. After 72 h, cells were harvested by scraping. For TUNEL assay, cells were assayed using the Apo-BrdU In situ DNA fragmentation Assay Kit (MBL, Nagoya, Japan) according to manufacturer's protocol. These stained cells were analyzed with a FACS Caliber and Cell Quest software (Becton Dickinson, San Jose, CA).

Caspase activity assay

Cells were plated in 8 mL of medium in 10-cm dishes with 2×10^4 cells/mL. After 24 h, cells were treated with IFN-α2 and IFN-β (48 h), or with IFN-β (24 h) followed by PBS washing and addition of TRAIL, TNF-α, and the anti-Fas (CD95) antibody (24 h). At 48 h after cell plating, cells were also treated with TRAIL, TNF-α, and the anti-Fas (CD95) antibody (24 h) to be compared with IFN-β-pretreated cells. Cells were harvested by scraping, and were assayed with a commercially available caspase-2, -3, -8, -9, and -10 fluorometric assay kit (MBL, Nagoya, Japan) as procedure was described. Caspase activity was measured by spectrofluorometer Fluoroskan Ascent FL (Labsystems, Helsinki, Finland). The excitation wavelength was 390 nm and the emission wavelength was 510 nm. Western blotting analysis of caspase protein was performed as described elsewhere (Yamashita and others 2001). Rabbit anti-caspase-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Caspase inhibitor treatment

Caspase inhibitors, Z-VDVAD-FMK (specific for caspase-2), Z-DQMD-FMK (specific for caspase-3), Z-IETD-FMK (specific for caspase-8 and granzyme B), and Z-LEHD-FMK (specific for caspase-9) were purchased from Calbiochem

(San Diego, CA). The cells were treated with 1,000 IU/mL IFN-β for 48 h in the presence or absence of the caspase inhibitor at a concentration of 100 mM. Cell viability was determined with MTT assay.

Statistical analysis

Statistical comparisons were made using Student's *t*-test.

Results

IFN-β inhibited the viability of melanoma cell lines SK-mel-118, SK-mel-23, and MM418, but not G361.

To assess the viability of IFN-α and -β-treated melanoma cell lines, MTT assay was performed (Fig. 1A). The cell viabilities of SK-mel-118, SK-mel-23, and MM418 were reduced by IFN treatments. Detached cell death and cell growth inhibition was microscopically detected in these cell lines. The reduction of cell viability by IFN-β was significantly higher than by IFN-α2. However, slight cell growth inhibition and no detached cell death of G361 were microscopically detected by IFN treatments. The cell viability was slightly inhibited by IFN-α2 or IFN-β but no significant difference was observed between untreated cell viability and IFN-treated cell viability. And more cell death was not induced by higher dose (>1,000 IU/mL) of IFN-β in G361 and SK-mel-118 (data not shown). These results indicated that these 4 melanoma cell lines were divided into 2 groups, one in which cell viability inhibition and detached cell death was observed by IFN, and the other in which slight cell viability inhibition and no detached cell death was observed.

Induction of ISGs after treatment with IFNs

To assess whether IFN treatment could transduce intracellular signaling in melanoma cell lines, we investigated

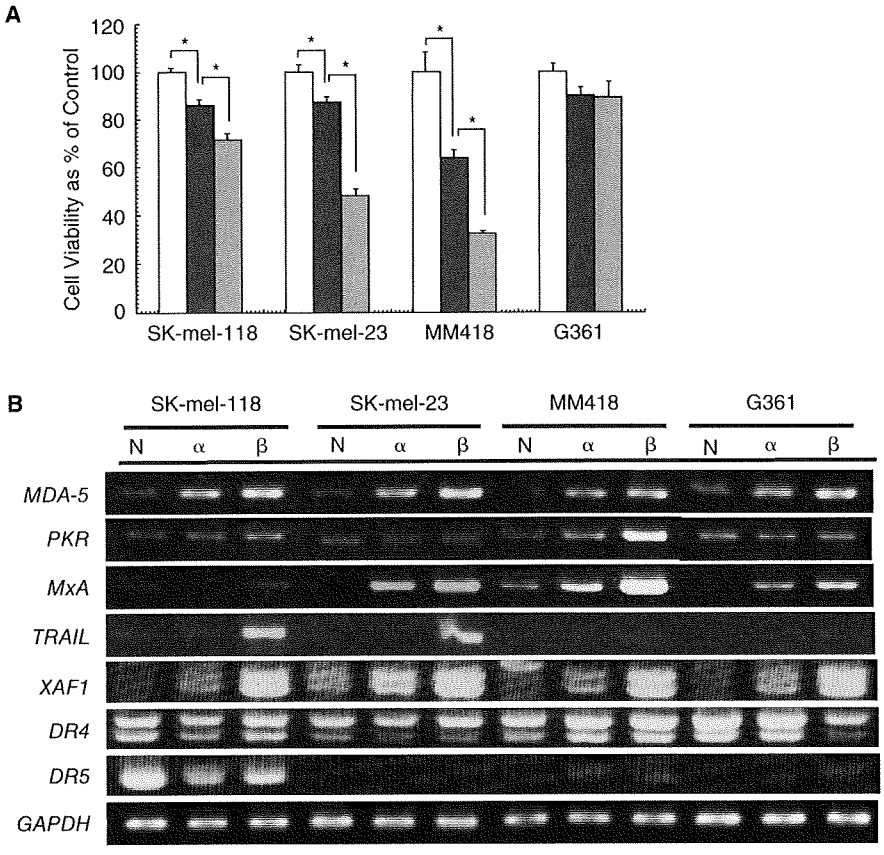

FIG. 1. Cell viability and mRNA expression of interferon (IFN)-stimulated genes after treatment with IFN-α2 and IFN-β. **(A)** Each cell line was plated in a 96-well plate with 1×10^3 cells/well. The cell lines were treated in the presence of 1,000 IU/mL of IFN-α2 and IFN-β for 72 h. The viability of treated cells was determined by the formazan-formation assay at 72 h in triplicate. The results are expressed as relative values (mean ± S.D.) to the untreated control. Untreated: empty column, IFN-α2: shaded column, IFN-β: dotted column. *Significant difference ($P < 0.01$). **(B)** The cell lines were plated in 24-well plate with 3×10^3 cells/well. After adding 1,000 IU/mL IFN-α2 and IFN-β for 24 h, total RNA was extracted. cDNA was synthesized from total extracted RNA (50 ng). The results of the semiquantitative RT-PCR were validated by the linearity of the determination of curves at various concentrations of cDNA. *MDA-5*, melanoma differentiation-associated gene-5; *PKR*, double-stranded RNA-activated protein kinase; *MxA*, myxovirus resistance protein A; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; *XAF1*, (X-linked inhibition of apoptosis protein)-associated protein; *DR4*, death receptor 4; and *DR5*, death receptor 5. *GAPDH* was determined as a control. N, untreated group; α, treatment with IFN-α2; and β, treatment with IFN-β.

mRNA expression of ISGs, which associated with cell death, such as MDA-5, PKR, MxA, TRAIL, RASSF1A, and XAF1 in IFN-treated cell lines (Fig. 1B). The mRNA expressions of MDA-5 were significantly up-regulated by IFN treatment in all the cell lines, including G361, which was resistant to the anti-viability effects of IFN. Those of PKR, MxA, and XAF1 also tended to be up-regulated in the tested cell lines. These results indicated that the IFN-signaling pathway was not suppressed in any of the tested cell lines. TRAIL-mRNA inductions of SK-mel-118 and SK-mel-23 by IFN-β treatment were markedly higher than those of MM418 and G361. RASSF1A mRNA was not detected in all cell lines. These results suggested that expression levels of any ISGs so far examined did not correlate with the degree of IFN-induced apoptosis. Furthermore, we examined expression levels of proapoptotic DRs for TRAIL. DR5 mRNA strongly expressed in SK-mel-118, which is highly sensitive to TRAIL, and decreased by treatment with IFN. DR4 mRNA was reduced by IFN treatment in G361 and it did not show any significant changes by IFN treatment in other cell lines. These results suggested that DRs for TRAIL had little to do with the effect of IFN-induced cell death.

Induction of apoptosis in melanoma cells by IFN-β treatment

We investigated the mechanism of cell death induced by IFN-β. We performed TUNEL assay in these 4 cell lines to assess whether the cell viability reduction and detached cell death by IFN-β occurred via apoptosis (Fig. 2). This assay showed that BrdU-FITC-positive cells corresponding to an apoptotic population, which underwent DNA fragmentation, increased in IFN-treated SK-mel-118, SK-mel-23, and MM418, whose cell viabilities were reduced by IFN-β in MTT assay. Apoptotic cells in G361, whose cell growth was slightly inhibited, were not increased by treatment with IFN-β (Fig. 2). It was reported that TRAIL is essential for the apoptosis cascade induced by IFN (Chawla-Sarkar and others 2001). In SK-mel-118 and SK-mel-23 that expressed TRAIL mRNA after IFN-β treatment, the induction of apoptosis seemed to be related to TRAIL expression. However, MM418 did not induce TRAIL-mRNA expression even if massive apoptosis was induced by IFN-β treatment. Thus the IFN-induced apoptotic pathway of MM418 was speculated to occur not through TRAIL induction by IFNs.

Up-regulation of caspase-2 activity was related to IFN-β-induced apoptosis in melanoma cell lines.

Activation of a caspase cascade is critical for initiation of apoptosis. We performed caspase fluorometric assay to detect up-regulation of caspase activity when apoptosis was induced in melanoma cells by IFN-β treatment. Caspase-3 activities in IFN-β-treated SK-mel-118, SK-mel-23, and MM418 showed about 2.5- to 7-fold increases, but not in G361 (Fig. 3). These results were consistent with the extent of apoptotic population of the TUNEL assay (Fig. 2). To characterize the related regulation of the caspase cascade, we furthermore analyzed activation of caspase-2, -8, -9, and -10 in IFN-β-treated cell lines. Activations of caspase-2, -3, and/or -9 were observed in IFN-β sensitive cell lines, SK-mel-118, SK-mel-23, and MM418. IFN-resistant G361 cells had no significant up-regulation of any caspase activity. Activation of caspase-2 was also confirmed by the finding that protein levels of procaspase-2 decreased by treatment with IFN-β as shown by Western blotting (Fig. 4). Active caspase-2 fragments were not detected, although degradation of procaspase-2 was able to be detected. Probably because of the sensitivity, Western

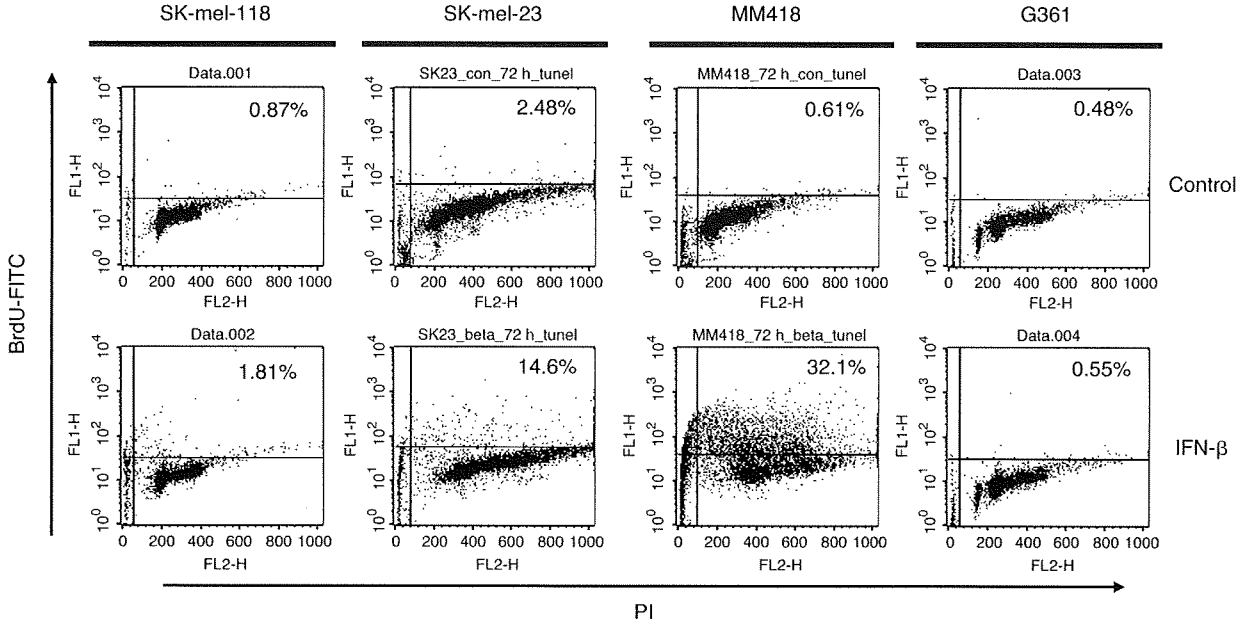

FIG. 2. FACS analysis of DNA fragmentation by TUNEL assay. Cells were plated on 10-cm dishes with 2×10^4 cell/mL. The cell lines were treated with 1,000 IU/mL IFN-β. After 72 h, cells were harvested and fixed. They were labeled with bromo-dUTP (BrdU) by the enzyme TdT, and then stained with an FITC-labeled anti-BrdU antibody. The percentage of FITC-positive cells was assessed by FACS.

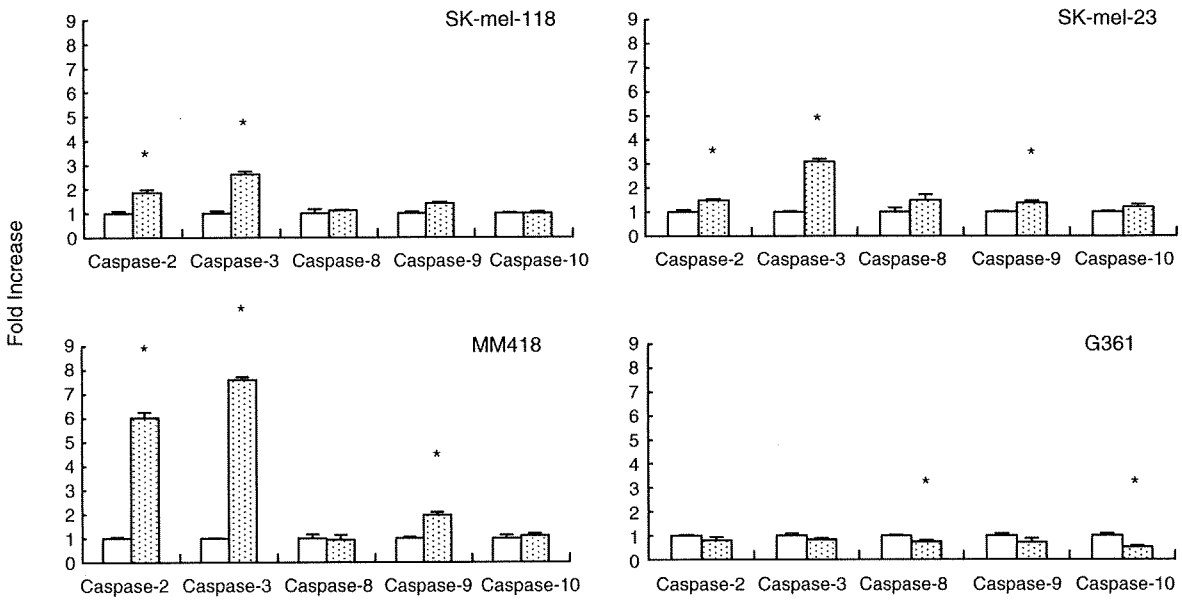

FIG. 3. Caspase assay after treatment with interferon (IFN)-β. Cells were plated on 10-cm dishes with 2×10^4 cells/mL. The cell lines were treated with 1,000 IU/mL IFN-β in triplicate. After 48 h, cells were harvested and caspase activity was assessed by caspase fluorometric assay as described in the manufacturer's protocol. The caspase activity is expressed as the fold increase compared with the untreated cells. The results are expressed as relative values (mean ± S.D.) to untreated controls. Untreated: empty column, IFN-β: dotted column. *Significant difference ($P < 0.01$) from the value in the untreated control.

blotting was not enough to detect the active caspase fragment in these IFN-β-treated cells. The levels of procaspase-2 and -3 were not up-regulated by treatment with IFN-β in all cell lines (data not shown). The IFN-β-induced apoptosis in MM418, SK-mel-23, and SK-mel-118 was significantly suppressed by the addition of inhibitor for caspase-2 or caspase-3, and these inhibitors showed equivalent anti-apoptotic effect against IFN-β-induced apoptosis. However, the inhibitors for both caspase-8 and caspase-9 did not show any anti-apoptotic effect by IFN-β (Fig. 5). These results indicated that caspase-2 and caspase-3 induced had critical role on IFN-β-induced apoptosis as an initiator and an effector for caspases, respectively.

Exogenous TRAIL caused apoptosis in melanoma cells

To elucidate the mechanism of IFN resistance in melanoma cells, we treated the 4 melanoma cell lines by adding exogenous apoptosis-inducing ligands (Fig. 4). When G361 and SK-mel-118 were treated with TRAIL, TNF-α, or an anti-Fas (CD95) antibody, TRAIL significantly reduced their cell viability. The anti-Fas (CD95) antibody also reduced the cell viability of SK-mel-118. The cell viabilities of SK-mel-23 and MM418 were not influenced with treatment of exogenous apoptosis-inducing ligands (Fig. 6). We also examined the effect of anti-TRAIL antibodies on the IFN-β-induced apoptosis. Anti-TRAIL antibody slightly suppressed IFN-β-induced apoptosis but did not show significant anti-apoptotic effect induced by IFN-β in SK-mel-118 and SK-mel-23. IFN-β-induced apoptosis was not suppressed by anti-TRAIL antibody in MM418 (data not shown). Furthermore, the cell viability of those tested cells was assessed after treatment with IFN-β, followed by apoptosis-inducing ligands treatment. IFN-β pretreatment followed by TRAIL stimulation significantly reduced the cell viability compared with TRAIL stimulation alone in G361 (Fig. 6). In IFN-sensitive melanoma cell lines, IFN-β pretreatment followed by TRAIL stimulation reduced the cell viability of SK-mel-23 compared with IFN-β treatment alone, although exogenous apoptosis-inducing ligands treatment did not reduce its cell viability. However, IFN-β pretreatment followed by any apoptosis-inducing ligands did not reduce the cell viability of MM418 compared with IFN-β treatment alone. In IFN-resistance G361 cells, IFN-β pretreatment followed by TRAIL stimulation significantly reduced cell viability compared with that of TRAIL treatment alone.

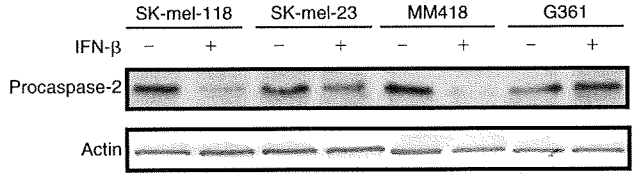

FIG. 4. Activation of caspase-2 detected with degradation of procaspase-2 protein by Western blotting. The cells were treated with interferon (IFN)-β at 1,000 IU/mL for 48 h. The treated cells were lysed, and processed to Western blotting.

IFN-β pretreatment enhanced up-regulation of caspase-2 activity in G361 treated with TRAIL.

We next analyzed the up-regulation of caspase activity of G361, which is an IFN-resistant cell line, when cells were treated with TRAIL, and with IFN-β followed by TRAIL. The cells treated with TRAIL showed up-regulation of caspase-2 and -3 activities as did IFN-sensitive cell lines, in which IFN-β induced apoptosis. Furthermore, IFN-β pretreatment

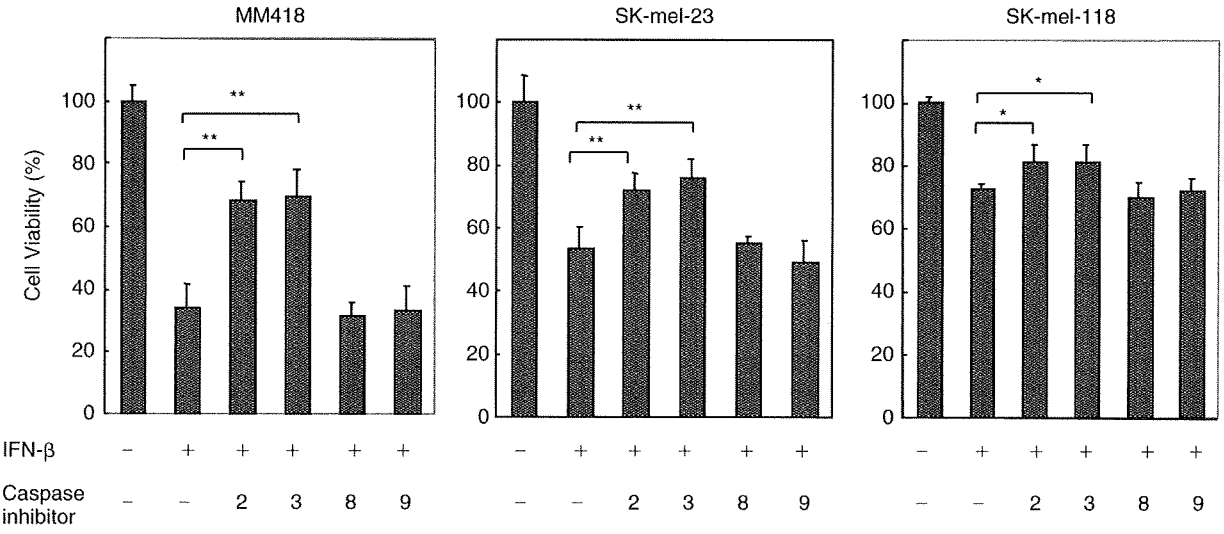

FIG. 5. Effect of caspase inhibitors on interferon (IFN)-β-induced apoptosis. The cells were treated with IFN-β at 1,000 IU/mL in the presence of caspase inhibitor (specific for caspase-2, -3, -8, or -9) at a concentration of 100 mM. After 48 h treatment, cell viability was determined by MTT assay. Each experiment was performed in quadraplicate. The results are expressed as relative values (mean ± S.D.) to untreated controls. $^{**}P < 0.01$, $^{*}P < 0.05$.

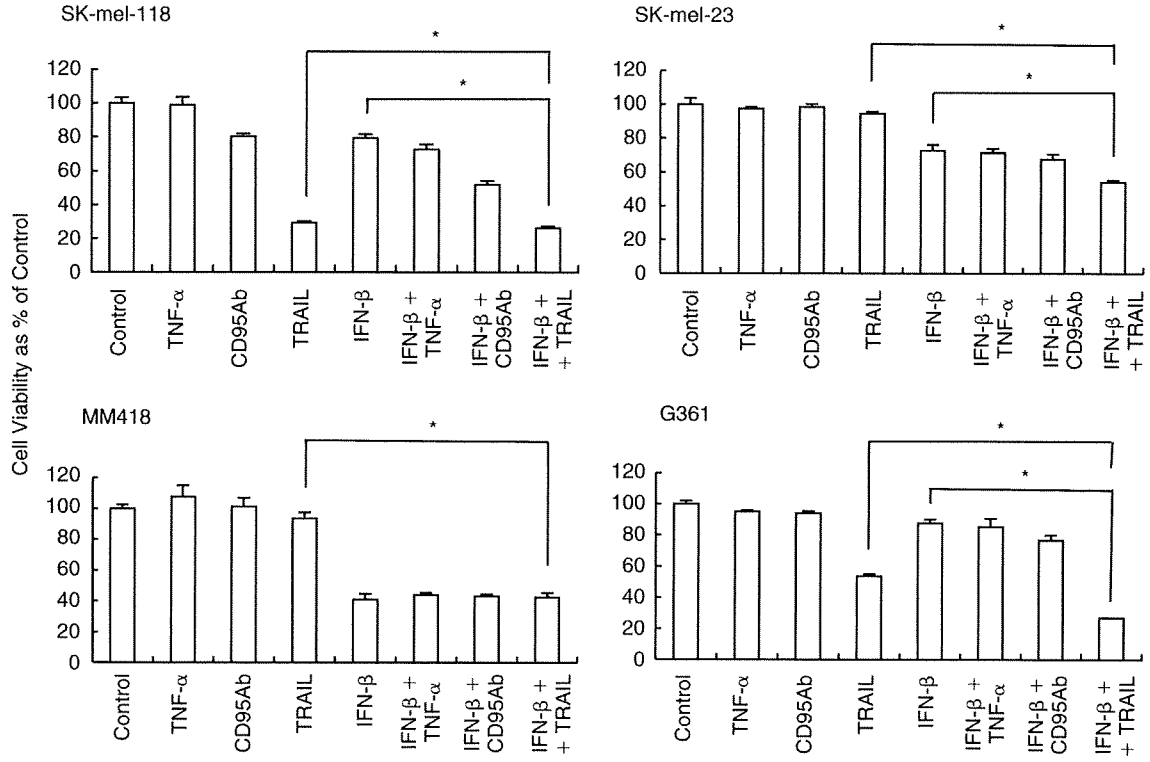

FIG. 6. Cell viability assay after treatment with exogenous apoptosis-inducing ligands and interferon (IFN)-β pretreatment followed by apoptosis-inducing ligands. Cells were plated in 96-well plates with 1×10^3 cells/well. Cells were treated with TNF-α, the anti-Fas (CD95) antibody, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for 48 h at the concentrations of 50, 500, and 100 ng/mL, respectively, or with 1,000 IU/mL IFN-β (24 h) pretreatment followed by apoptosis-inducing ligands for 48 h at the same concentrations as above. Cell viability was determined by the formazan-formation assay in triplicate. The results are expressed as relative values (mean ± S.D.) to untreated controls.

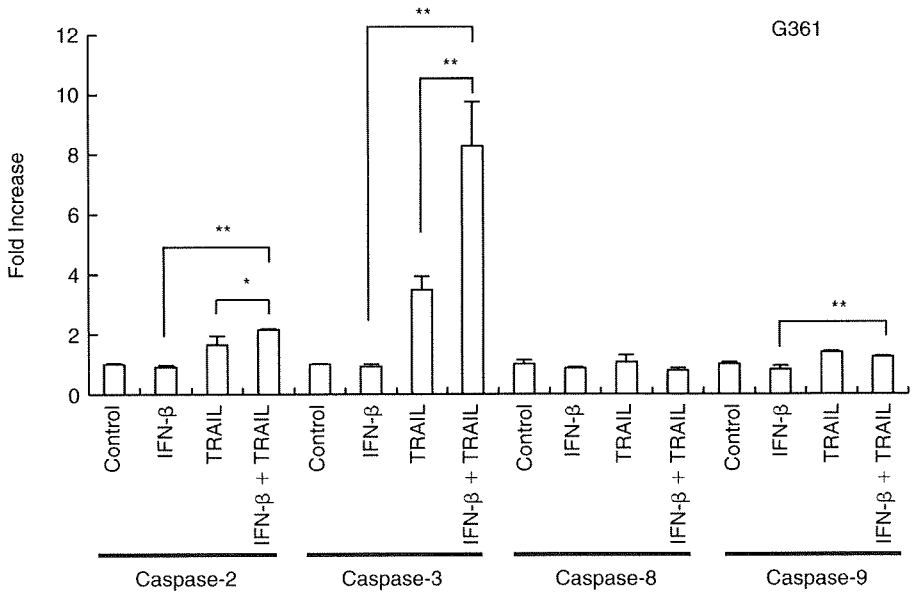

FIG. 7. Caspase assay after treatment with apoptosis-inducing ligands and interferon (IFN)-β pretreatment followed by apoptosis-inducing ligands. G361 cells were plated in a 10-cm dishes with 2×10^4 cells/mL. Cells were treated with TRAIL for 24 h at the concentration of 100 ng/mL, or with 1,000 IU/mL IFN-β (24 h) pretreatment followed by a PBS wash and addition of TRAIL for 24 h at the same concentration as above. Cells were harvested and caspase activity was assessed by caspase fluorometric assay as in the manufacturer's protocol. The caspase activity is expressed as the fold increase compared with the untreated cells. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF-α, tumor necrosis factor α; *significant (P <0.01) difference.

enhanced the up-regulation of caspase-2 and -3 (Fig. 7), correlated with inducibility of apoptosis (Fig. 6).

Discussion

In this study we found that cell death was promoted by IFN-α2 and -β treatments in human melanoma cell lines (SK-mel-118, SK-mel-23, and MM418), and that IFN-β preferentially induced cytotoxic effects compared to IFN-α2. We also found that this cell death induced by IFN-β was induced by apoptosis.

It has been reported that IFN-β treatment induces apoptosis that is dependent on TRAIL induction in melanoma cells. It was indicated that the cells that failed to express TRAIL after IFN-β treatment were resistant to IFN-induced apoptosis, whereas those that induced TRAIL by IFN-β were sensitive to IFN-induced apoptosis (Chawla-Sarkar and others 2001). The induction of TRAIL by IFN-β has been shown to initiate the apoptotic cascade in a wide variety of tumor cells (Shin and others 2005; Vogler and others 2007). It was shown in melanoma cells that TRAIL had a more significant tumoricidal effect than other members of the tumor necrosis factor family such as TNF-α and Fas ligand (Griffith and others 1998). We also showed a similar result that TRAIL had more significant apoptotic effect than the other apoptosis-inducing ligands, such as TNF-α and anti-Fas antibody, in TRAIL-sensitive cell lines, SK-mel-118 and G361 (Fig. 6). The induction of TRAIL by IFN-β was shown to be necessary, but still insufficient to induce apoptosis (Chawla-Sarkar and others 2002). In our study 2 melanoma cell lines, SK-mel-118 and SK-mel-23, exhibited such biological reactions in IFN-β-induced apoptosis, and markedly up-regulated TRAIL mRNA in response to IFN-β treatment. In contrast, in G361, in which ISGs were induced by IFN-β treatment, IFN-β did not induce apoptosis and significant expression of TRAIL mRNA, although exogenous TRAIL induced apoptotic cell death. However, anti-TRAIL antibody slightly suppressed IFN-β-induced apoptosis but did not show any significant anti-apoptotic effect by IFN-β on SK-mel-118 and SK-mel-23 cells. These results indicated that a TRAIL-independent pathway may exist, although TRAIL expression contributes to IFN-β-induced apoptosis to some extent.

Our study showed a new and unique finding in the study of MM418. MM418 significantly underwent apoptosis induced by IFN-β treatment but did not exhibit significant expression of TRAIL mRNA (Fig. 1B). Furthermore, exogenous TRAIL did not induce apoptosis in MM418, and caspase-2 activation of MM418 by IFN-β treatment was much higher than in SK-mel-118 and SK-mel-23, which are thought to undergo TRAIL-mediated apoptosis to some extent. These findings suggested that MM418 cells underwent apoptosis via an alternative pathway, such as a TRAIL-independent apoptotic pathway induced by IFN-β.

Several recent studies demonstrated TRAIL-mediated apoptosis through the caspase-2 pathway in other tumor cell lines (Wagner and others 2004; Shin and others 2005). They suggested that caspase-2 was required in the upstream of Bid, which leads to caspase-9 activation in the mitochondrial apoptotic pathway (Wagner and others 2004; Bonzon and others 2006). Samraj and others indicated that caspase-2 activation was absent in a mutant Jurkat T-cell line with a defect of caspase-9 when treated with an anticancer drug releasing cytochrome *c* (Samraj and others 2007). Shin and others

also indicated that caspase-2 processed procaspase-8 (Shin and others 2005). Thus caspase-2 appears to have diverse functions in apoptotic processes. Our study suggested the possible presence of an alternative IFN-induced apoptotic pathway that activates caspase-2 directly without induction of TRAIL. In a previous study, TRAIL was indicated to be a novel therapeutic modality useful for the management of melanomas (Ren and others 2004). We, however, identified TRAIL-independent IFN-β-induced apoptosis in one cell line, MM418.

Another new finding in this study was for G361. It did not show IFN-β-mediated apoptosis, but its IFN signal transduction seemed to be normal. Griffith and others previously reported that only TRAIL among apoptosis-inducing ligands had a significant cytotoxic effect (Griffith and others 1998). In this study TRAIL mRNA was not induced by IFN-β in G361, although other ISGs were expressed. Thus, this loss of TRAIL-mRNA induction may be a meaningful biological process for the resistance to IFN-β-induced apoptosis because exogenous TRAIL induced significant apoptosis in G361 (Fig. 6). It has also been suggested that IFN-β pretreatment sensitizes TRAIL-mediated apoptosis (Chawla-Sarkar and others 2002). In this study, we also showed that IFN-β pretreatment increased sensitivity against TRAIL-mediated apoptosis in G361 and SK-mel-23, SK-mel-118. When G361 was treated with IFN-β followed by additional TRAIL treatment, caspase-2 activity was up-regulated compared with that after TRAIL treatment alone. Thus not only TRAIL but also other factors induced by IFN seem to be important for apoptotic processes, because synergetic activity of TRAIL and IFN-β treatment was observed in this study after treatment with TRAIL and IFN-β in G361 and specifically in SK-mel-23, in which TRAIL alone did not induce apoptosis although IFN-β pretreatment enhanced apoptosis by TRAIL stimulation. Along with a previous report (Chawla-Sarkar and others 2002), we found in this study that the TRAIL/caspase-2 system was important for induction of apoptosis in 3 (SK-mel-118, SK-mel-23, G361) of the 4 melanoma cell lines tested. On the other hand, in MM418 IFN-induced caspase-2 and -3 activation and apoptosis occurred without TRAIL expression.

Our results further suggested that those melanoma cell lines (SK-mel-118, SK-mel-23, MM418), in which apoptosis was induced by IFN-β, commonly had up-regulated caspase-2 activity with (SK-mel-118, SK-mel-23) or without (MM418) involvement of a TRAIL-related pathway. Furthermore, caspase-2 and -3 inhibitors remarkably suppressed IFN-β-induced apoptosis that was equivalent to that in IFN-β-sensitive cell lines. The finding was a common phenomenon in these cell lines, and the increased apoptotic population had up-regulation on caspase-2 activity, but not on TRAIL induction.

Thus our present results clearly indicate that the extent of the IFN-β-induced apoptosis depends on up-regulation of caspase-2 activity more strongly than on induction of TRAIL. These findings suggest that measurement of caspase-2 activity in primary culture cells from excised melanoma tissues can be a novel marker for estimating the extent of the cytotoxic effect of IFN-β adjuvant therapy for melanoma. Caspase-2 activity may be useful for IFN-induced sensitization of chemotherapeutic drugs to melanoma cells.

Acknowledgments

We are indebted to Drs. Akinori Kawakami, Kenji Yanagisawa and Masae Okura of Sapporo Medical University for their valuable contributions. This study was supported by Grants-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Culture, Japan (16390319, 16024218) and the Ministry of Health, Labour and Welfare, Japan (H17-nano-004).

Author Disclosure Statement

No competing financial interests exist.

References

Bonzon C, Bouchier-Hayes L, Pagliari LJ, Green DR, Newmeyer DD. 2006. Caspase-2-induced apoptosis requires bid cleavage: a physiological role for bid in heat shock-induced death. Mol Biol Cell 17(5):2150–2157.

Chawla-Sarkar M, Leaman DW, Borden EC. 2001. Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. Clin Cancer Res 7(6):1821–1831.

Chawla-Sarkar M, Leaman DW, Jacobs BS, Borden EC. 2002. IFN-beta pretreatment sensitizes human melanoma cells to TRAIL/Apo2 ligand-induced apoptosis. J Immunol 169(2):847–855.

Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, Borden EC. 2003. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis 8(3):237–249.

Chen Q, Gong B, Mahmoud-Ahmed AS, Zhou A, Hsi ED, Hussein M, Almasan A. 2001. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. Blood 98(7):2183–2192.

Fisher PB, Prignoli DR, Hermo H, Jr., Weinstein IB, Pestka S. 1985. Effects of combined treatment with interferon and mezerein on melanogenesis and growth in human melanoma cells. J Interferon Res 5(1):11–22.

Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. 1998. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol 161(6):2833–2840.

Kang DC, Gopalkrishnan RV, Lin L, Randolph A, Valerie K, Pestka S, Fisher PB. 2004. Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, mda-5: a novel type I interferon-responsive apoptosis-inducing gene. Oncogene 23(9):1789–1800.

Kimberley FC, Screaton GR. 2004. Following a TRAIL: update on a ligand and its five receptors. Cell Res 14(5):359–372.

Leaman DW, Chawla-Sarkar M, Jacobs B, Vyas K, Sun Y, Ozdemir A, Yi T, Williams BR, Borden EC. 2003. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. J Interferon Cytokine Res 23(12):745–756.

Meng RD, El-Deiry WS. 2001. p53-independent upregulation of KILLER/DR5 TRAIL receptor expression by glucocorticoids and interferon-gamma. Exp Cell Res 262(2):154–169.

Merchant MS, Yang X, Melchionda F, Romero M, Klein R, Thiele CJ, Tsokos M, Kontny HU, Mackall CL. 2004. Interferon gamma enhances the effectiveness of tumor necrosis factor-related apoptosis-inducing ligand receptor agonists in a xenograft model of Ewing's sarcoma. Cancer Res 64(22):8349–8356.

Morrison BH, Tang Z, Jacobs BS, Bauer JA, Lindner DJ. 2005. Apo2L/TRAIL induction and nuclear translocation of inositol hexakisphosphate kinase 2 during IFN-beta-induced apoptosis in ovarian carcinoma. Biochem J 385(Pt 2):595–603.

Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Bordens R, Walter MR, Nagabhushan TL, Trotta PP, Pestka S. 1998. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. Cancer Res 58(12):2489–2499.

Ren DH, Mayhew E, Hay C, Li H, Alizadeh H, Niederkorn JY. 2004. Uveal melanoma expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors and susceptibility to TRAIL-induced apoptosis. Invest Ophthalmol Vis Sci 45(4):1162–1168.

Reu FJ, Leaman DW, Maitra RR, Bae SI, Cherkassky L, Fox MW, Rempinski DR, Beaulieu N, MacLeod AR, Borden EC. 2006. Expression of RASSF1A, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. Cancer Res 66(5):2785–2793.

Samraj AK, Sohn D, Schulze-Osthoff K, Schmitz I. 2007. Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization. Mol Biol Cell 18(1):84–93.

Shin S, Lee Y, Kim W, Ko H, Choi H, Kim K. 2005. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. EMBO J 24(20):3532–3542.

Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. 1998. How cells respond to interferons. Annu Rev Biochem 67:227–264.

van Loo G, Saelens X, Matthijssens F, Schotte P, Beyaert R, Declercq W, Vandenabeele P. 2002. Caspases are not localized in mitochondria during life or death. Cell Death Differ 9(11):1207–1211.

Vogler M, Dürr K, Jovanovic M, Debatin KM, Fulda S. 2007. Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. Oncogene 26(2):248–257.

Wagner KW, Engels IH, Deveraux QL. 2004. Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. J Biol Chem 279(33):35047–35052.

Yamashita T, Tokino T, Tonoki H, Moriuchi T, Jin HY, Omori F, Jimbow K. 2001. Induction of apoptosis in melanoma cell lines by p53 and its related proteins. J Invest Dermatol 117(4):914–919.

Zhang L, Fang B. 2005. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 12(3):228–237.

Zhivotovsky B, Orrenius S. 2005. Caspase-2 function in response to DNA damage. Biochem Biophys Res Commun 331(3):859–867.

Zhivotovsky B, Samali A, Gahm A, Orrenius S. 1999. Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ 6(7):644–651.

Address correspondence to:
Dr. Kowichi Jimbow
Institute of Dermatology & Cutaneous Sciences 1-27
Odori W-17
Chuoku, Sapporo 0600042
Japan

E-mail: jimbow@sapmed.ac.jp

Received 6 February 2009/Accepted 21 September 2009