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Induction of ICAM-1 and HLA-DR Expression by IFN-γ in Malignant Melanoma Cell Lines

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Two human malignant melanoma cell lines, Malme-3M and SK-Mel-28, were analyzed for their ability to induce the expression of intercelluar adhesion molecule 1 (ICAM-1) and human leukocyte antigen (HLA)-DR molecules on their cell surfaces as well as at the transcriptional level before and after treatment with interferon (IFN)-γ. Both cell lines demonstrated a high percentage (>99%) of ICAM-1 expression regardless of IFN-γ treatment. Before IFN-γ treatment, Malme-3M cells barely expressed HLA-DR molecules (<2%) and SK-Mel-28 cells demonstrated a relatively high percentage (>50%) of HLA-DR expression. Both cell lines displayed elevated levels of HLA-DR expression in a time dependent manner after IFN-γ treatment. However, these two cell lines have been shown to respond differentially to IFN-γ. The molecular mechanism underlying such a differential behavior was investigated, and HLA-DR gene regulation was studied at the transcriptional level. Treatment with IFN-γ led to the steady-state mRNA augmentation of the HLR-DR gene. The HLA-DRA mRNA augmentation was similar in both cell lines, whereas in Malme-3M, IFN-γ did not augment the rate of transcription of the HLA-DRB gene as much as in SK-Mel-28. Data from this study established the fact that the melanoma cell lines displayed a differential susceptibility to IFN-γ on the modulation of HLA-DR molecules, and this modulation was transcriptionally regulated.

Key Words: Malignant melanoma cell lines, ICAM-1, HLA-DR expression, IFN-γ, transcriptional regulation

Intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin gene superfamily and human leukocyte antigen (HLA)-DR molecules can be induced or up-regulated by IFN-γ (Rothlein *et al.* 1988; Maio *et al.* 1989). In terms of the expression level, it has been suggested that the differential susceptibility of various tumor cells to cytokines may influence the interaction with the host immune system, the malignant metastatic potentials, and the clinical prognosis of a tumor (Maio *et al.* 1989).

It has been considered that the increased expression of ICAM-1 on melanoma cells may positively correlate with a higher risk of metastasis (Natali *et al.* 1990) and both the expression and shedding of ICAM-1 from malignant tumor cells were increased by IFN-γ treatment (Tsujisaki *et al.* 1991). On the contrary, it has been also considered that the expression of ICAM-1 on tumor cells may facilitate recognition by autologous lymphocytes and may reduce metastasis (Vanky *et al.* 1990). Thus, the role of ICAM-1 in the malignant progression and the metastatic process of tumor cells is uncertain.

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An unexpected but frequent expression of HLA class II gene has been observed in neoplastic cells of various histologic origins (Natali *et al.* 1981). As for melanomas, it is well established that both primary and metastatic human melanomas may constitutively express HLA class II molecules.

In some HLA class II-negative melanomas, phenotypic modulation of HLA class II molecules can be achieved by stimulation with IFN-γ (Parmiani *et al.* 1990). In the human system, it was demonstrated that IFN-γ acts on HLA class II genes at the level of transcription. However, a differential susceptibility of HLA class II gene to induction by IFN-γ has been reported in melanoma, in neuroblastoma, and in breast adenocarcinoma cell lines (Anichini *et al.* 1988; Maio *et al.* 1988; Sugimoto *et al.* 1989; Jabrane-Ferrat *et al.* 1990). These systems represent valuable models to investigate transcriptional processes, leading to class II gene expression.

It has been considered that transcriptional control is a major mode of regulation of the HLA class II genes; the HLA-DR mRNA transcription rate is responsible for the cell surface expression of HLA-DR molecules. Variable induction of these molecules by IFN-γ in tumor cells is transcriptionally regulated in connection with the metastatic capacity of tumor cells (Fertsch *et al.* 1987). The study of ICAM-1 expression and MHC class-II gene regulation in tumor cells is thus of interest to understand and to control the immune response to metastasis.

We have examined the expression of ICAM-1 and HLA-DR in two human malignant melanoma cell lines, Malme-3M and SK-Mel-28, and investigated their susceptibility to modulation by IFN-γ to determine whether the differential susceptibility to IFN-γ modulation is transcriptionally regulated.

MATERIALS AND METHODS

Maintenance of cell lines

Two human malignant melanoma cell lines, Malme-3M (ATCC HTB64) and SK-Mel-28 (ATCC HTB72) were used. These cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

SK-Mel-28 cells were maintained in the Eagle's minimum essential medium or McCoy's 5a medium supplemented with 15 mM N-2-hydroxyethylpiperazine-N-ethane sulfonic acid (HEPES, Hazelton, Lenexa, KS, USA), penicillin (120 mg/L), streptomycin (270 mg/L) and 10% heat-inactivated fetal calf serum (Gibco BRL, Gaithersburg, MD, USA) as recommended. Malme-3M cells were cultured in L-15 (Sigma Chemical Co., St. Louis, MO, USA) containing 15% fetal calf serum.

Monoclonal antibodies

Monoclonal antibody, 84H10 (mouse IgG1, Makgoba *et al.* 1988) to ICAM-1, MAb B8.12.2 (mouse IgG2b kappa) to HLA-DR (Hansen *et al.* 1978) and FITC-conjugated goat anti-mouse antibody, all obtained from Immunotech S.A. (Marseilledex, France) were used to detect the surface expression of ICAM-1 and HLA-DR on tumor cells.

Flow cytometric analysis

Surface expression of ICAM-1 and HLA-DR molecules was analyzed by indirect immunofluorescence and flow cytometry (Kim *et al.* 1993). Cells were cultured for 4 hr, 24 hr, and 48 hr intervals in the presence of recombinant human IFN-γ at a concentration of 200 U/ml. In order to determine constitutive ICAM-1 and HLA-DR expression, culture medium without IFN-γ was added to the cultures. Cell suspensions were washed with cold PBS three times, and then incubated with the anti-ICAM-1 or anti-HLA-DR monoclonal antibody for 30 min at 4℃. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody for another 30 min. After two washes, cell pellet was resuspended in PBS. Flow cytometric analysis of ICAM-1 and HLA-DR molecules was performed using a FACStar (Becton Dickinson, Mountainview, CA, USA).

Probes

cDNA probes for HLA-DRA (Gustafsson *et*

al. 1984) and -DRB gene (Long *et al.* 1983) were obtained from Dr. SY Yang, Memorial Sloan-Kettering Cancer Center, NY. Probes were radiolabeled at a specific activity of $>10^8$ cpm/μg with [α-^{32}P]dCTP using random primer labeling kit (Amersham Co., Arlington Heights, IL, USA).

Northern blot hybridization

Total cellular RNA was extracted by guani-

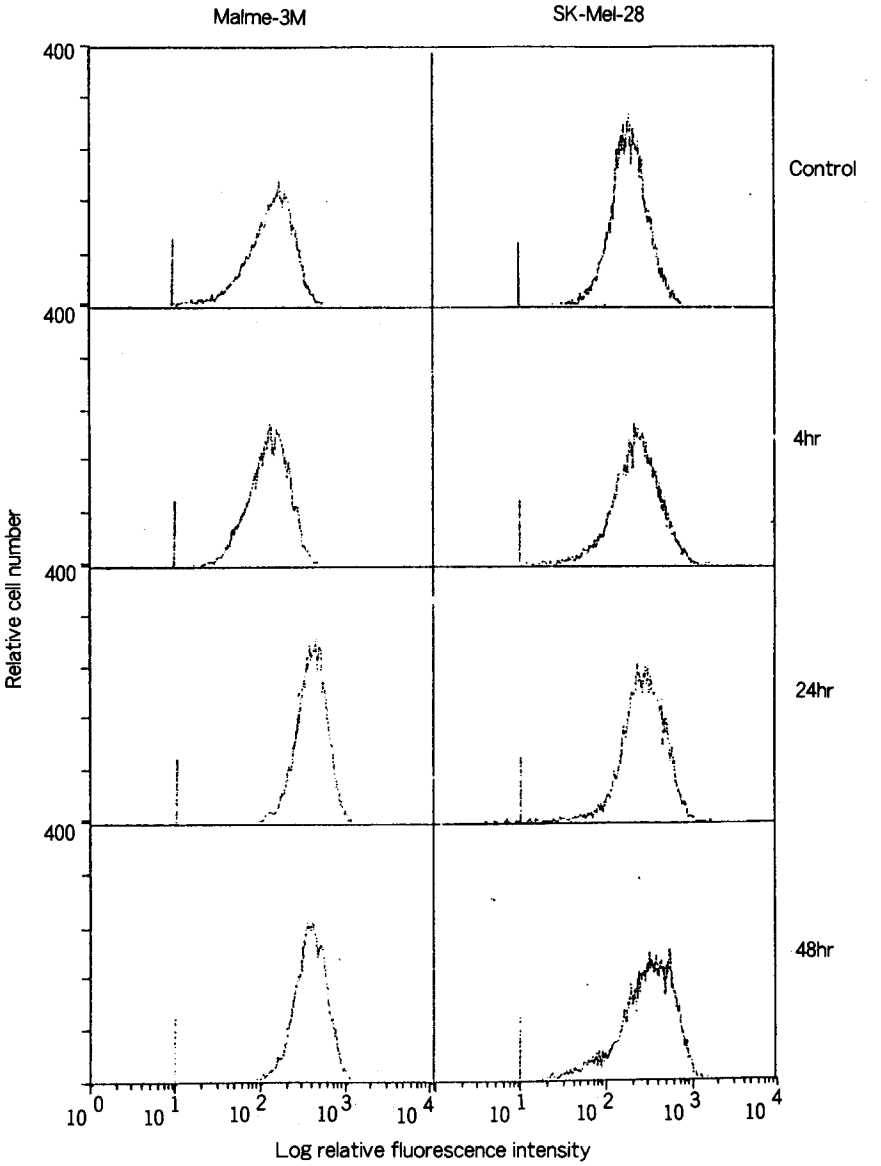

Fig. 1. *Effect of IFN-γ on the expression of ICAM-1 in the Malme-3M (left) and SK-Mel-28 (right) melanoma cells. Cells were incubated with 200U/ml of IFN-γ for 4hr, 24hr, and 48hr intervals, and the control was incubated only with culture media. The expression of ICAM-1 was tested with relevant mAb. Results were analyzed by FACS histograms plotting fluorescence intensity vs. cell number.*

dium isothiocyanate/cesium chloride method (Chirgwin *et al.* 1979). Twenty μg of total cellular RNA was electrophoresed through 1.2% agarose-formaldehyde denaturating gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) using 20× SSPE (Sambrook *et al.* 1989). Transferred RNA was fixed onto the membrane by UV cross linking and prehybridized at 42℃ for 4 hr using a prehybridization buffer containing 5×SSPE, 50% formamide, 5×Denhardt's solution and 100 μg/ml of heat-denatured herring sperm DNA. The blot was hybridized overnight at 42℃ in the hybridization buffer containing a ^{32}P-labeled HLA-DRA or -DRB specific cDNA probe. Following two washes with washing buffer A (2×SSPE, 0.5% SDS), once with washing buffer B (0.5×SSPE, 0.5% SDS) and then once with washing buffer C (0.2× SSPE, 0.5% SDS), each wash for 15 min at 65℃, the blot was dried and autoradiographed at −70℃ for several days using Super HR-G film (Fuji Co., Tokyo, Japan). γ-actin cDNA probe was used for the internal control.

RESULTS

Effect of IFN-γ on the percentage of melanoma cell lines bearing the ICAM-1

The rate and the degree of ICAM-1 expression in Malme-3M, and SK-Mel-28 human malignant melanoma cell lines were measured by flow cytometric analysis before and after IFN-γ treatment and are shown in Figures 1 and 2. The proportion of both cell lines expressing ICAM-1 was not increased by IFN-γ. More than 99% of cells from both cell lines expressed ICAM-1 before treatment. Therefore, an increased rate of ICAM-1 expression could not be anticipated after IFN-γ treatment (Fig. 1). However, IFN-γ treatment increased the degree of ICAM-1 expression level (Fig. 2). Mean fluorescence intensity (MFI) of both cell lines was enhanced by IFN-γ. In Malme-3M, IFN-γ treatment increased the MFI from 169.7 at constitutive expression level, *i.e.*, before IFN-γ treatment, to 402.3 and 438.0 after 24 hr and 48 hr respectively. In SK-Mel-28, IFN-γ treatment increased the MFI from 409.9 at constitutive level to 566.1 and 662.3 after 24 hr and 48 hr respectively.

Modulation of the expression of HLA-DR molecules on melanoma cells by IFN-γ

Malme-3M and SK-Mel-28 were treated with IFN-γ for 4 hr, 24 hr and 48 hr at a concentration of 200 U/ml, and the expression of HLA-DR on their surfaces was evaluated by flow cytometric analysis. Control cells, receiving no treatment, showed a relatively high rate (57.5%) of SK-Mel-28 cells expressing HLA-DR; in contrast, Malme-3M lacked the constitutive expression of HLA-DR (1.8%). The expression of HLA-DR molecules on both cell lines after IFN-γ treatment was gradually induced, but there were marked differences in their susceptibility to modulation by IFN-γ. After a 24 hr treatment to the cells with IFN-γ, the induced expression of HLA-DR on SK-Mel-28 cells was much higher than that of Malme-3M; while 96.3% of SK-Mel-28 cells expressed HLA-DR, only 31.9% of Malme-3M cells expressed HLA-DR. After a 48 hr treatment to the cells with IFN-γ, the induced expressions of HLA-DR were 99.1% and 93.1% on SK-Mel-28 cells and Malme-3M cells res-

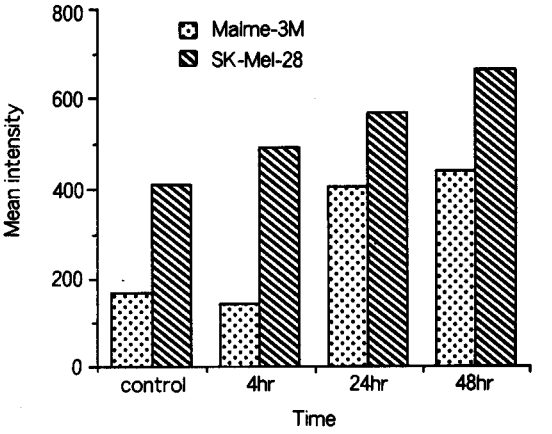

Fig. 2. *Histogram of mean fluoresence intensity of ICAM-1 expression by the same experiment shown in Fig. 1. Intensities were plotted as mean linear channel number vs. incubation time.*

pectively (Fig. 3). MFI was enhanced by IFN-γ in both cell lines (Fig. 4).

Transcriptional activation of class II genes by IFN-γ in melanoma cell lines

Since it has been considered that steady-state levels of mRNA are affected by the rate of gene transcription and indeed the surface expression of HLA-DR was dependent upon the mRNA transcription of HLA-DRA and -DRB genes. Further analysis of HLA-DR expression at the mRNA level was performed

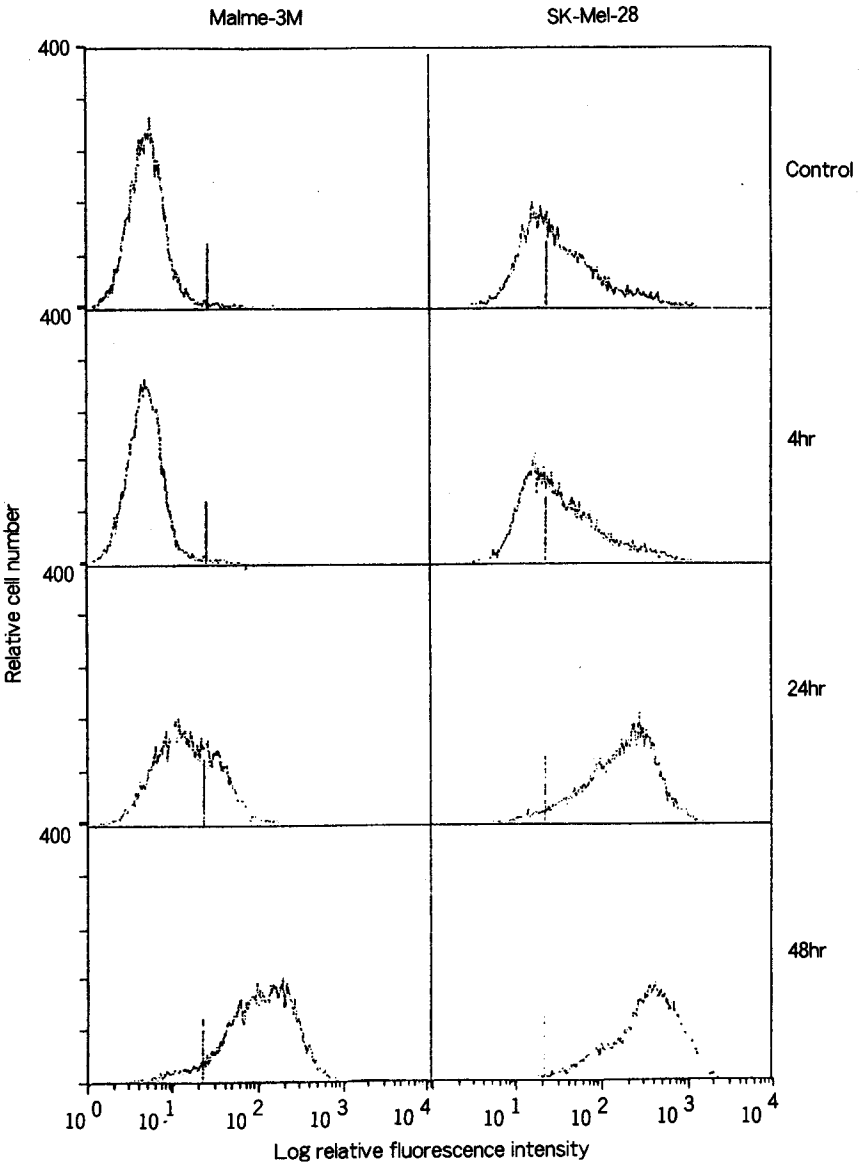

Fig. 3. *Effect of IFN-γ on the expression of HLA-DR in the Malme-3M (left), and SK-Mel-28 (right) melanoma cells. Cells were incubated with 200U/ml of IFN-γ for 4hr, 24hr, and 48hr intervals, and the control was incubated only with culture media. The expression of HLA-DR was tested with relevant mAb and results were analyzed by FACS histograms plotting fluorescence intensity vs. cell number.*

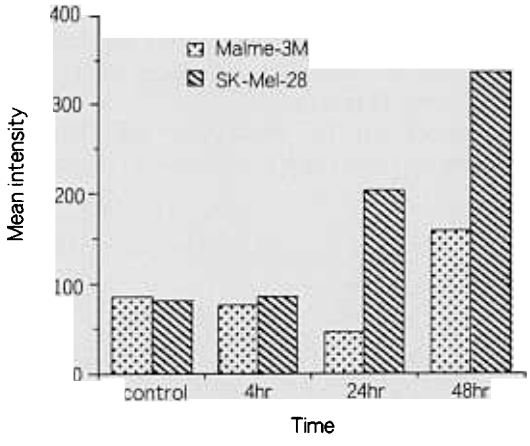

Fig. 4. *Histogram of mean fluoresence intensity of HLA-DR by the same experiment shown in Fig. 3. Intensities were plotted as mean linear channel number vs. incubation time.*

by Northern blot hybridization (Fig. 5). IFN-γ could act on the transcription of these genes, and induce *de novo* expression of HLA-DR. To determine whether the differential susceptibility to IFN-γ in Malme-3M and SK-Mel-28 cells occurred at the transcriptional level, Northern blot was performed. Cells from each cell line were cultured for 4 hr, 24 hr, and 48 hr in a medium with (200 U/ml) or without IFN-γ.

There was no detectable expression of HLA-DR mRNA in the control sample of both cell lines. However, after IFN-γ treatment, HLA-DRA and -DRB mRNA gradually increased in a time dependent manner. While the increase of HLA-DRA mRNA transcription was similar in both cell lines, the extent of the increase of HLA-DRB mRNA tran-

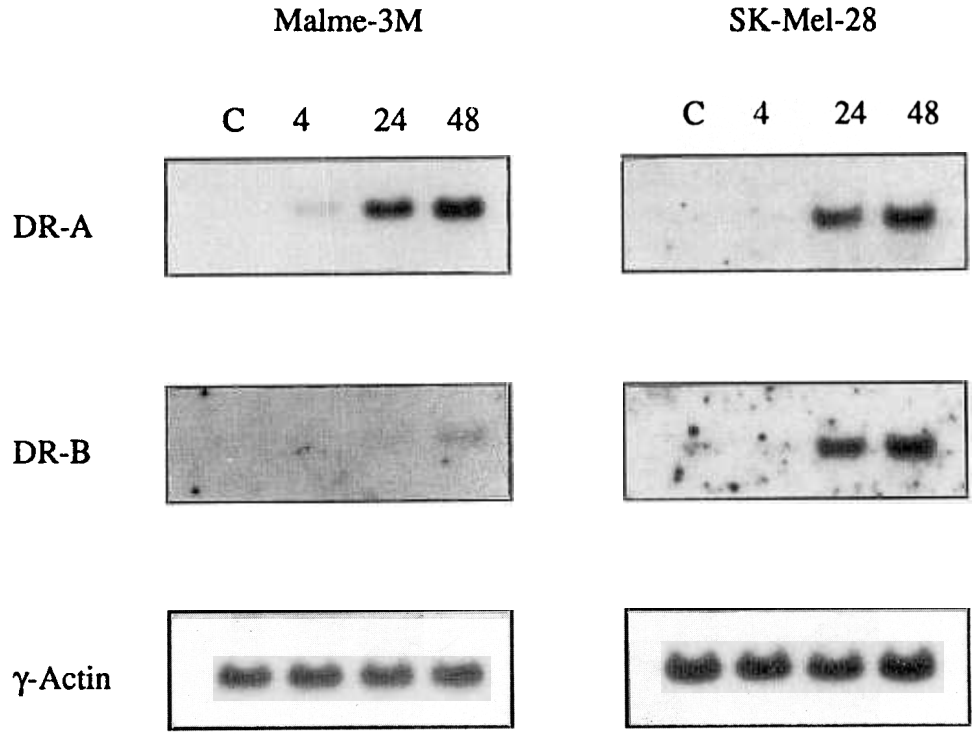

Fig. 5. *Northern blot analysis of HLA-DR mRNA expression in cultured Malme-3M (left), and SK-Mel-28 (right) melanoma cells. The cells were incubated for 4hr, 24hr, and 48hr intervals with 200U/ml of IFN-γ , and then from them the total RNA was extracted. The control cells (C) were incubated only with culture media. Twenty μg of total RNA was electrophoresed, and transferred to NC membrane. The blot was hybridized with ^{32}P-labeled HLA-DRα (upper panel) or -DRβ specific cDNA probe (middle panel). γ-actin cDNA probe was used for internal control (lower panel) on the same blot.*

scription in SK-Mel-28 was greater than that of Malme-3M (Fig. 5). These results suggested that the differential expression of HLA-DR molecules on the cell surface in these two cell lines was transcriptionally controlled, especially in HLA-DRB mRNA transcription.

DISCUSSION

In vivo and *in vitro* comparisons between normal cells as well as benign tumor cells and malignant tumor cells suggest that there are marked phenotypic changes in malignant tumor cells; the alterations of cell surface molecules accompany the neoplastic transformation (Varmus and Weinberg, 1993). Many attempts have been made to clarify the correlation between these phenotypic changes in tumor cells and their malignancy. It is likely that the expression level of various cell surface molecules on tumor cells may influence metastatic potential, progression and prognosis of tumors (Ruiter *et al.* 1984). Understanding the heterogeneous expression of cell surface antigenic markers and their relation to malignancy may allow us to more effectively modulate tumor cell activity.

Several cell adhesion molecules (CAMs) including laminin and its receptors, very late antigen (VLA) family, CD44 and intercellular adhesion molecule-1 (ICAM-1) and human leukocyte antigen (HLA) class I and class II molecules have been thought to be related with tumor cell malignancy. Among these, ICAM-1 and HLA-DR molecules, are thought to play an important role in establishing the intercellular interaction of immune responses against tumor cells (Larson and Springer, 1990), and in the specific steps of the metastasis process (Maio *et al.* 1988, 1989). Thus, these molecules have been considered to be related with tumor malignancy. However, the exact roles of ICAM-1, HLA-DR, and the cytokine effects for their expressions in malignant progression remain to be evaluated. Since variation of cell surface molecules such as ICAM-1 and HLA-DR influences immune regulation, quantitative differences in immune response gene expression are important for host defense against tumor and *vice versa* (Horoszewicz and Murphy, 1989). The expressions of ICAM-1 and HLA-DR are highly controlled. Therefore, a comprehension of the molecular mechanism responsible for the control of their expressions will contribute to understanding the respective roles of ICAM-1 and HLA-DR in tumor invasion and/or tumor metastasis.

Class II genes of the major histocompatibility complex (MHC) play a key role in regulating and restricting the immune response. Probably as a reflection of their regulatory role, class II gene products exhibit specific and limited cell-type distribution. Class II molecules are expressed in a tissue-specific manner, and the constitutive expression of class II molecules are mainly confined to cells of the immune system such as B lymphocytes, macrophages, dendritic cells, and some epithelial cells (Benoist and Mathis, 1990). IFN-γ has been known to be the most potent inducer of HLA class II molecules. Cytokines such as IFN-γ can increase the constitutive expression and induce *de novo* class II expression in several class II negative nonhematopoietic cell types, for example astrocytes (Pober *et al.* 1983). However, several studies have shown that cells having no immune function such as melanoma cells express HLA class II molecules (Winchester *et al.* 1978; Wilson *et al.* 1979). These expressions are increased by various soluble factors, such as IFN-γ and IL-4 (Halloran *et al.* 1985).

In this paper we report the effect of human recombinant IFN-γ on the expression of ICAM-1 and HLA-DR in human malignant melanoma cell lines. IFN-γ induced a time-dependent increase of the level of expression in both molecules. Two human malignant melanoma cell lines, Malme-3M and SK-Mel-28, which are known to have different malignant potential, were used in this study. Malme-3M originated from a malignant melanoma with lung metastasis (Fogh *et al.* 1977), and SK-Mel-28 originated from a recurrent melanoma (Carey *et al.* 1976). These two cell lines demonstrated a highly expressed level of ICAM-1 regardless of IFN-γ treatment. The reason why IFN-γ could not increase the percentage

of melanoma cells bearing ICAM-1 might be explained by the fact that the initial proportions were near 100%. However, IFN-γ induced an increase in the MFI of ICAM-1 in these cells.

IFN-γ not only increases rate of gene transcription, but may also stabilize MHC class II mRNA. Since treatment of tumor cells with IFN-γ has been reported to cause an increased expression of HLA-DR (Hokland *et al.* 1988), we conducted experiments to determine the effect of IFN-γ on HLA-DR expression in human melanoma cell lines. After IFN-γ treatment both cell lines, Malme-3M and SK-Mel-28, displayed increased surface expression of HLA-DR in a time-dependent manner. Through Northern blot analysis, we demonstrate here that the differential induction of HLA-DR molecules by IFN-γ on different melanoma cell lines is transcriptionally regulated. These results demonstrate that these melanoma cell lines demonstrated a differential susceptibility to IFN-γ in HLA class II gene modulation, detected as steady-state mRNA augmentation. However, SK-Mel-28 demonstrated greater expression of HLA-DR after treatment with IFN-γ than did Malme-3M. These differences in increased expressions of HLA-DR on the cell surfaces of the two melanoma cell lines coincided with the HLA-DR mRNA level in Northern hybridization. DRβ chain mRNA transcription is exceptionally higher in SK-Mel-28 than in Malme-3M. After treatment with IFN-γ, SK-Mel-28 expressed high levels of both DRA and DRB mRNA, while a high mRNA level could not be induced for the DRB gene in Malme-3M. These suggest that the differential expression of HLA-DR molecules resulted from the differential steady-state mRNA augmentation of the DRB gene.

Evidences suggests that IFN-γ plays a role in the induction of HLA class II molecules on melanoma cells (Houghton *et al.* 1984; Herlyn *et al.* 1985; Tsujisaki *et al.* 1987), and in the metastasis inhibition of melanoma to lung (Saiki *et al.* 1992). It has been suggested that class II genes could be differentially regulated in some tumors such as melanomas, neuroblastomas, and breast adenocarcinomas (Anichini *et al.* 1988; Maio *et al.* 1988; Sugimoto *et al.* 1989; Jabrane-Ferrat *et al.* 1990). This observation was based on an increase in steady-state mRNA and the cell surface molecule expression of HLA class II genes following IFN-γ induction in HLA-DR. Our results coincided with the study where IFN-γ enhanced the expression of HLA-DR by cells with low and intermediate malignant potential than by cells with high metastatic and invasive potential (Hendrix *et al.* 1990). Other studies using IFN-γ for modulating in melanoma cells have found; 1) only small differences in the number and/or affinity of IFN-γ receptors on the cell surface (Maio *et al.* 1989); 2) the increase in HLA-DR expression by IFN-γ was independent from the cell cycle of the tumor cells (Taramelli *et al.* 1986); and 3) IFN-γ (at 1000U/ml) caused an anti-proliferative effect (Maio *et al.* 1989). The differential susceptibility to the induction of HLA class II molecules on the two cell lines cannot account for the differences in the number and affinity of IFN-γ receptors, or in the sensitivity of IFN-γ, but most likely reflects an intrinsic property in each cell line. The regulatory mechanism(s) that control(s) the expression of HLA class II molecules appear(s) to be different between the two cell lines tested. The expression of class II molecules was induced in melanocytes that only expressed class II molecules during an early stage of melanocyte differentiation (Houghton *et al.* 1984), suggesting that this expression in melanomas might be a consequence of events occurring during malignant transformation. Thus, in different melanoma cells, the differential modulation of HLA-DR expression by IFN-γ is transcriptionally regulated, and HLA-DR expression might be correlated with tumor malignancy.

Several hypotheses can be proposed to account for the differential transcriptional activation of DRB gene in the two melanoma cell lines studied. Malme-3M and SK-Mel-28 could derive from tumor cells at different stage of differentiation, with a different program for class II expression. In fact, it has been shown that class II antigen expression relates to the differentiation program of melanoma cells (Houghton *et al.* 1982, 1987).

Alternatively, Malme-3M might display mutation or deletion in the IFN-γ responsive sequences shown to function either as positive or negative regulatory elements in HLA-DR gene expression. Changes in chromatin structure that have been associated with class II gene expression (Wright and Ting, 1992) could also account for the difference observed between our two cell lines. Since we were not able to rule out the different stability of mRNA in this experiment, post-transcriptional regulation should be considered, and further experiments are needed to gain more insight into the fine mechanism of the differential transcriptional activation described here for HLA-DR expression of melanoma cell lines.

In conclusion, our study might suggest that the primary control of the differential susceptibility of HLA-DR gene induced by IFN-γ in melanoma cell lines lies at the level of transcription. The HLA-DR-positive and -negative human melanoma cell lines might represent a useful model to further investigate the relationship between the malignant potential and the IFN-γ induced HLA-DR expression.

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