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Recombinant gamma interferon induces HLA-DR expression on squamous cell carcinoma, trichilemmoma, adenocarcinoma cell lines, and cultured human keratinocytes

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Summary. We investigated the effects of recombinant human gamma interferon on the induction of HLA-DR expression by two human squamous cell carcinoma, three trichilemmoma, one eccrine carcinoma, two adenocarcinoma cell lines, and cultured human keratinocytes in vitro. None of eight epithelial cell lines or keratinocytes expressed HLA-DR without gamma interferon treatment. In contrast, pure gamma interferon (500 IU/ml, 72-h treatment) induced HLA-DR expression on 1/2 squamous cell carcinoma, 3/3 trichilemmoma, 2/2 adenocarcinoma cell lines, and 4/4 keratinocyte cell lines, as determined using a fluorescence-activated cell sorter. A maxillary squamous cell carcinoma line and an eccrine carcinoma cell line failed to express HLA-DR with gamma interferon treatment; however, the growth of cells was inhibited by gamma interferon treatment. By indirect immunoperoxidase techniques, tumor cells such as Bowen's disease and squamous cell carcinoma were found to express HLA-DR. Since HLA-DR expression has been shown to be important for various immune responses, these findings suggest that gamma interferon plays important roles in various immune-related skin diseases.

Key words: Gamma interferon – HLA-DR induction – Epithelial tumor cells – Keratinocytes

Introduction

Although only Langerhans cells [21] and acrosyringial epithelium [16] express HLA-DR in normal human epidermis, cells in immune-related skin diseases such as allergic contact dermatitis [2], graft-vs.-host disease [12], and malignant epithelial tumor cells, such as basal cell carcinoma and squamous cell carcinoma [17] have been demonstrated to express HLA-DR. Gamma interferon treatment has recently been shown to induce HLA-DR biosynthesis and expression in a variety of cell types such as keratinocytes, melanocytes [1], and melanoma cells [3]. These findings suggest that gamma interferon may also be capable of inducing HLA-DR expression by various epithelial tumor cells. We report here that gamma interferon induces HLA-DR biosynthesis and expression not only by cultured human epithelial cell lines, but also by cultured human keratinocytes in the absence of Langerhans cells.

Gamma interferon has antiproliferative effects on keratinocytes [18], melanoma cells [9], human epithelial, and hematopoietic cell lines [22]. It is assumed that the first step in gamma interferon action is its binding to specific receptors on the plasma membrane [24]. Therefore the question arises whether the gamma interferon insensitive cell lines have gamma interferon receptors or not. In order to approach this question, we investigated the antiproliferative effects of gamma interferon on gamma interferon insensitive cell lines. The results obtained reveal that two gamma interferon insensitive cell lines, as well as a gamma interferon sensitive cell line, are cell growth inhibited by gamma interferon treatment.

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Materials and methods

Interferon

Recombinant human gamma interferon from *E. coli* was supplied by Japan Roche (Tokyo, Japan). The titer of gamma interferon determined by the virus inhibition plaque assay was $5-9 \times 10^6$ IU/mg.

Cell culture

The following cell lines were used in the present study: squamous cell carcinoma, maxillary squamous cell carcinoma, three

Table 1. HLA-DR expression on epithelial tumor cells with gamma interferon treatment

Cell lines	Stained cells (%)	
	Before	After
Squamous cell carcinoma (epidermal)	0	32
Squamous cell carcinoma (maxillary)	0	0
Trichilemmoma (K-TL-1)	0	9
Trichilemmoma (IK-TL-2)	0	38
Trichilemmoma (TK-TL-3)	0	44
Eccrine carcinoma	0	0
Gastric adenocarcinoma	0	64
Mammary adenocarcinoma (MCF7)	0	36

Tumor cells were treated by gamma interferon (500 IU/ml, 72 h) and percent of cells with detectable staining were analyzed by FACS

trichilemmoma (K-TL-1, IK-TL-2, TK-TL-3), eccrine carcinoma, gastric adenocarcinoma, and mammary adenocarcinoma (MCF7) cell lines. The origins and characteristics of most of these cell lines have been described [8, 10, 11]. All these cell lines except eccrine carcinoma and MCF7 were established in our laboratory. The cells (1×10^6) were plated on 35-mm dishes (Falcon Plastics, Oxnard, CA) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 100 units/ml penicillin. Gamma interferon was added 48 h after cell seeding. Single cell suspensions of normal skin were prepared from foreskins obtained at surgery as described by Eisinger [7]. Cells were seeded on 24-well dishes (Nunc, Roskilde, Denmark) at a density of 2×10^6 cells/well. The cells were maintained at 37°C in a humidified incubator with 5% CO_2 in air. Gamma interferon was added 1 week after seeding.

Panning

The panning technique followed was the method of Morhenn [15]. Briefly, the 35-mm dishes were coated with goat antimouse immunoglobulin (Tago, Burlingame, CA) at 10 µg/ml in 0.05 *M* Tris buffer, pH 9.5, and incubated for 40 min, swirled, and incubated for another 30 min. The supernatants containing the unattached cells were removed, and seeded exactly as described above.

Fluorescein labeling and FACS analysis

The cultured epithelial tumor cells and epidermal cells were collected by trypsinization for 3–10 min using 0.025% trypsin plus 0.014 *M* ethylenediaminetetraacetate (EDTA) (Denka-seiken, Tokyo, Japan) and washed with PBS. Then 5×10^5 to 10^6 cells were incubated with 50 µl culture medium L243 [13] (Monoclonal HLA-DR antibody) or 5 µl OKT6 (Langerhans cells monoclonal antibody, Ortho Diagnostic Systems), diluted in 20 µl PBS, for 30 min on ice. The cells were washed twice with PBS and incubated with 2 µl fluorescein isothiocyanate (FITC)-conjugated goat antimouse immunoglobulin (Tago) diluted in 20 µl PBS, for 30 min on ice, and they were then washed twice in PBS, and finally suspended in PBS containing 0.02% sodium azide. The fluorescence per cell was analyzed with a fluorescence-activated cell sorter (FACS 420; Becton-Dickinson); nonspecific background staining was determined by omitting the first antibody.

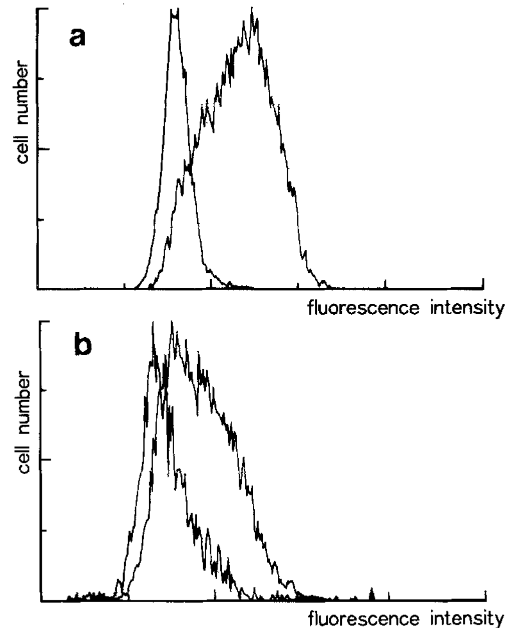

Fig. 1 a, b. Histogram of gamma interferon-treated keratinocytes labeled with monoclonal HLA-DR antibody plus FITC-conjugated goat antimouse immunoglobulin antibody. The *vertical axis* shows cell numbers per channel, and the *horizontal axis* shows fluorescence intensity in logarithmic units. The *curves* on the left are the untreated controls. **a** Experiment 1, panning (−): 64% of unfractionated keratinocytes express HLA-DR. **b** Experiment 1, panning (+): 21% of keratinocytes depleted of Langerhans cells express HLA-DR

Effect of gamma interferon on cell growth

Three epithelial cell lines, an eccrine carcinoma, a maxillary squamous cell carcinoma, and a squamous cell carcinoma were used in this study. The cells (5×10^5) were plated in 35-mm dishes in DMEM with 10% FCS, gamma interferon (500 IU/ml) was added at 24 h after cell seeding, and the cells were fed with fresh gamma interferon containing culture medium every other day. Cells were counted with a hemocytometer and each cell count determination represents the average of means obtained for duplicate or triplicate dishes. The total cell number per dish represents the cumulative sum of the number of attached and floating cells.

Tumor tissue study

The various skin tumors used in this study were: Bowen's disease (1), eccrine epithelioma or carcinoma (3), basal cell carcinoma (4), and metastatic mammary-adenocarcinoma of the skin (2). These tumors and normal skin specimens were embedded in OCT compound, snap frozen in liquid nitrogen, and stored at −80°C until use. Approximately 6-µm thick sections were cut and mounted on glass slides, air-dried, and fixed in cold acetone for 10 min.

Immunoperoxidase study

A two-step indirect immunoperoxidase method was employed. In the first step, sections were incubated with 20 µl culture medium L243 for 30 min at room temperature. After washing with PBS, the sections were incubated with 20 µl 1:20 diluted horseradish peroxidase (HRP) conjugated goat antimouse

Table 2. Effect of gamma interferon on cultured human keratinocytes

Experiment	Stained cells (%)		
	OKT6 staining	HLA-DR staining	
		Interferon (−)	Interferon (+)
Experiment 1			
Panning (−)	4	4	64
Panning (+)	0	0	21
Experiment 2			
Panning (−)	4	4	54
Panning (+)	0	0	6

Keratinocytes were treated by gamma interferon (500 IU/ml, 72 h) and analyzed by FACS

immunoglobulin antibody (Tago). After washing with PBS, peroxidase activity was detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB). The culture medium of Sp2/0, a BALB/c mouse myeloma cell line, was used as the control antibody.

Results

HLA-DR expression on epithelial cell lines and keratinocytes

After the treatment with gamma interferon (500 IU/ml, 72 h), the culture cells were harvested and analyzed for the fluorescence intensity per cell and the percent of HLA-DR positive cells. None of eight epithelial cell lines expressed HLA-DR without gamma interferon treatment. However, gamma interferon induced HLA-DR expression by one out of two squamous cell carcinoma, three out of three trichilemmoma, and two out of two adenocarcinoma cell lines; HLA-DR was found to have been expressed by 9% – 64% of these cells (Table 1). However, one eccrine carcinoma and one maxillary squamous cell carcinoma failed to express HLA-DR in spite of gamma interferon treatment. Next we studied the effect of gamma interferon on primary cultures of keratinocytes from two different adult patients. Gamma interferon enhanced the mean expression of HLA-DR and increased the percent of HLA-DR positive cells to 54% – 64% on the unfractionated keratinocytes as seen in the histogram of FACS analysis (Fig. 1a). To eliminate the possibility that HLA-DR was shed from Langerhans cells and subsequently absorbed by keratinocytes, a panning technique was applied to remove the Langerhans cells. The histogram of OKT6 positive cells in the keratinocytes could then be superimposed quite accurately on the histogram of untreated control cells, indicating that these keratinocytes contained no significant number of residual Langerhans cells (data not shown). The induction of HLA-DR by gamma interferon was observed in 6% – 21% cells, and the peak of specific fluorescein decreased from channel number 126 to 78 (Fig. 1b). These results are summarized in Table 2.

Kinetics of HLA-DR expression

The effects of gamma interferon (5 IU/ml to 10000 IU/ml, 72 h) on HLA-DR expression were dose-dependent in all cell lines (Table 3). The gastric adenocarcinoma cells were induced to express HLA-DR by as low as 5 IU/ml with 59% of the cells HLA-DR positive. Other cell lines required 5 – 50 IU/ml gamma interferon to induce expression of HLA-DR; the maximal level of HLA-DR expression was observed at approximately 50 IU/ml on the gastric adenocarcinoma cell line, but 500 IU/ml was required for other cell lines.

In a time course study, the cells were treated with 500 IU/ml gamma interferon and fed fresh culture medium every other day. The gastric adenocarcinoma cells began to express HLA-DR as rapidly as 6 h after treatment. In other cell lines, HLA-DR expression could be detected only after 24 – 48 h, but after 96 h incubation there was no further significant increase of HLA-DR expression in most of the cell lines. These results are summarized in Table 4.

On keratinocytes depleted of Langerhans cells, the effects of gamma interferon on HLA-DR expression were also dose-dependent. The cells began to express HLA-DR after 5 IU/ml gamma interferon treatment, and the maximal level of HLA-DR expression was observed at approximately 2000 IU/ml (Table 5).

Antiproliferative effects on epithelial cell lines

Gamma interferon inhibited the growth of all epithelial cell lines (Fig. 2). The cell numbers per dish were 27% – 76% of controls 5 days after seeding, and 29% – 60% of control after 9 days. However, cell viability was not altered by the treatment (over 95%).

Immunohistochemical study

By the indirect immunoperoxidase technique, HLA-DR positive tumor cells were detected on one out of one Bowen's disease cells, two out of two Paget's disease cells, one out of three squamous cell carcinomas, two out of three eccrine carcinomas or epithelioma, and one out of two skin metastases of mammary adenocarcinoma, but were not detected on four basal cell carcinomas. There was a tendency noted that as the tumor cells were surrounded by

Table 3. Kinetics of gamma interferon induction of HLA-DR expression: dosage

Cell lines	Gamma interferon (IU/ml)							
	0	5	10	50	100	500	2,000	10,000
Squamous cell carcinoma (epidermal)	0	0	1	9	15	32	43	37
Trichilemmoma (K-TL-1)	0	3	2	8	8	9	5	10
Trichilemmoma (IK-TL-2)	0	2	1	3	8	38	46	70
Trichilemmoma (TK-TL-3)	0	2	2	27	38	44	41	40
Gastric adenocarcinoma	0	59	51	75	72	64	64	82
Mammary adenocarcinoma (MCF7)	0	0	1	13	23	36	32	36

Data are expressed as % stained cells; tumor cells were treated with gamma interferon for 72 h

Table 4. Kinetics of gamma interferon induction of HLA-DR expression: duration of treatment

Cell lines	Gamma interferon treatment (h)								
	0	6	12	24	48	72	96	120	144
Squamous cell carcinoma (epidermal)	0	0	0	4	16	14	18	17	11
Trichilemmoma (K-TL-1)	0	0	0	0	2	7	6	5	8
Trichilemmoma (IK-TL-2)	0	0	0	4	32	61	60	73	77
Trichilemmoma (TK-TL-3)	0	0	0	0	2	7	17	13	21
Gastric adenocarcinoma	0	4	2	6	35	64	74	78	73
Mammary adenocarcinoma (MCF7)	0	0	0	30	50	28	35	42	50

Data are expressed as % stained cells; tumor cells were treated with 500 IU/ml gamma interferon

Table 5. Kinetics of HLA-DR expression on cultured human keratinocytes

Experiment	Gamma interferon (IU/ml)							
	0	5	10	50	100	500	2,000	10,000
3	0	2	3	7	15	32	38	37
4	0	3	3	6	7	21	28	29

Data are expressed as % stained cells; Langerhans cells depleted keratinocytes were treated with gamma interferon for 72 h

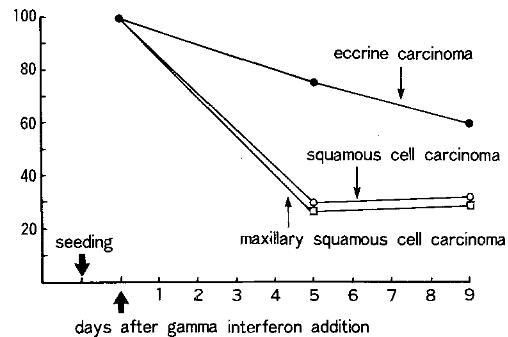

Fig. 2. Antiproliferative effect of gamma interferon. The tumor cells (5×10^5/35-mm dish) were treated with 500 IU/ml gamma interferon for the time noted. Number of tumor cells per dish were compared to untreated controls (%)

more infiltrative cells, they expressed more HLA-DR, especially in the case of squamous cell carcinoma (Fig. 3).

Discussion

This study demonstrates that gamma interferon induces HLA-DR expression by six out of eight cultured epithelial cell lines and by four out of four cultured keratinocyte lines. It further shows that gamma interferon inhibits cell growth of three out of three epithelial cell lines. Recent advances in immunology have revealed the importance of HLA-DR expression to the immune response, and a further understanding of the pathophysiological role of HLA-DR has been achieved. HLA-DR on the surface of antigen presenting cells (APC) has been reported to play an important role in the initial stage of the immune response. HLA-DR molecules are crucially involved in the recognition of antigens by helper T cells [20]. On macrophages, HLA-DR is necessary for effective antigen presentation by cells that (a) express Ia antigen in mice and (b) produce lymphokines [6]. It has been reported that keratinocytes have phagocytic activity [23] and produce an IL-1 like substance, epidermal cell derived

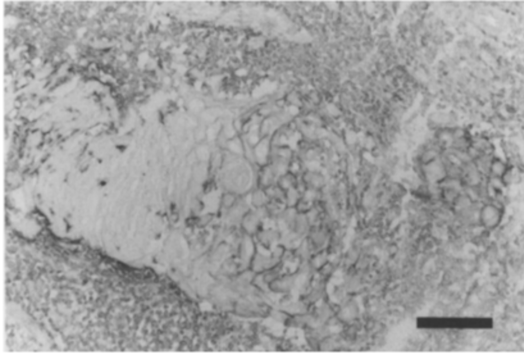

Fig. 3. Indirect immunoperoxidase staining of a squamous cell carcinoma with monoclonal HLA-DR antibody. Tumor cells which are surrounded by many infiltrating cells express HLA-DR on their cell surfaces (×200); *bar*, 50 μm

thymocyte activating factor ETAF [19], and also that a squamous cell carcinoma cell line produces ETAF [14]. These findings suggest the possibility that HLA-DR bearing keratinocytes and epidermal tumor cells may function as APC.

In our histochemical study, epidermal tumor cells expressed HLA-DR. We noted that as tumor cells were surrounded by more infiltrative cells, they expressed more HLA-DR. It has been reported that in various immune related skin diseases such as allergic contact dermatitis and graft-vs.-host disease keratinocytes express HLA-DR. These diseases have a common histological feature – they have infiltrative mononuclear cells. Gamma interferon has been reported to be produced during human mixed leukocyte-tumor cell reactions in vitro [4]. Our results and the data from that report suggest that infiltrative cells make the tumor cells and keratinocytes express HLA-DR by gamma interferon secreted in vivo.

On cultured keratinocyte lines 1 and 2, HLA-DR was found to be expressed by 54%–64% of these cells before panning. However, after keratinocytes were depleted of Langerhans cells by panning, the induction of HLA-DR expression by gamma interferon was observed on only 6%–21% of the cells. Although it has been reported that gamma interferon increases the expression of HLA-DR by Langerhans cells [5], only 4% of the cells had OKT6 antigen on epidermal cells 1 and 2, and the peak of specific fluorescence was remarkably decreased with the panning technique. Therefore, these differences in the percentage of HLA-DR positive cells and the fluorescence intensity between unfractionated keratinocytes and Langerhans cells depleted keratinocytes, may be explained by (a) the shedding of HLA-DR from Langerhans cells and subsequent adsorption by keratinocytes, or (b) the possibility that gamma interferon treated Langerhans cells induce keratinocytes to produce an expression of HLA-DR.

Eccrine carcinoma and maxillary squamous cell carcinoma cell lines could not be induced to express HLA-DR with gamma interferon treatment. However, gamma interferon had antiproliferative effects on both these cell lines. These findings suggest that these lines possess the gamma interferon receptor.

Our data demonstrate that HLA-DR expression is inducible by gamma interferon in a variety of epithelial tumor cell and keratinocyte cell lines. This may imply that the expression of HLA-DR by tumor cells and keratinocytes is necessary for the immune responses, including antigen presentation, induction of cytolytic T-lymphocyte responses, and host immune system defense mechanisms, in various immune-related skin diseases.

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