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Differential Effects of Protein Tyrosine Kinase Inhibitors on Interferon-γ-Induction of Major Histocompatibility Complex Class II and Intercellular Adhesion Molecule-1 Expression in Human Corneal Epithelial Cells

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Purpose: Interferon (IFN)-γ induces major histocompatibility complex (MHC) class II and intercellular adhesion molecule-1 (ICAM-1) expression on human corneal epithelial (HCE) cells. So far, it has not been clarified whether both inductions by IFN-γ use the same signal transduction pathway. Therefore, in the present study, we tried to determine the significance of the protein tyrosine kinase (PTK)-dependent signaling pathway in the induction of both MHC class II and ICAM-1 expression by IFN-γ in cultured HCE cells.

Methods: Cultured HCE cells were treated with human recombinant IFN-γ. The induction of protein tyrosine phosphorylation of proteins including PTKs, janus kinase (JAK)1, and JAK2, was examined by Western blotting and immunoprecipitation. The effects of treatment of HCE cells with specific PTK inhibitors on IFN-γ-induction of MHC class II and ICAM-1 expression were examined by flow cytometry.

Results: IFN 1 (Interferon) induced tyrosine phosphorylation of multiple substrates, particularly that of 75,000; 90,000; 130,000; and 160,000 molecular weight proteins including JAK1 and JAK2 in cultured HCE cells. The PTK inhibitors, herbimycin A and genistein, inhibited tyrosine phosphorylation of those proteins. Also, these PTK inhibitors prevented IFN-γ-induction of MHC class II synthesis and surface expression. However, neither herbimycin A nor genistein had any effect on IFN-γ-induction of ICAM-1 expression.

Conclusions: Tyrosine phosphorylation of proteins including JAK1 and JAK2 is essential for IFN-γ-induction of MHC class II expression, but not critical for that of ICAM-1 expression in cultured HCE cells. In addition, it is suggested that the IFN-γ-induction of MHC class II requires PTK activities not only in the primary JAK-signal transducers and activators of transcription (STAT) pathway but also in the subsequent pathway mediated by IFN-γ-induced intermediate proteins. **Jpn J Ophthalmol 2001;45:13–21** © 2001 Japanese Ophthalmological Society

Key Words: Human corneal epithelial cell, intercellular adhesion molecule-1, interferon-γ, major histocompatibility complex class II, tyrosine phosphorylation.

Introduction

Interferon (IFN)-γ, a major pro-inflammatory cytokine produced by activated T cells, plays a key role in regulating T-cell immune responses through its pleiotropic activities.[1] IFN 1 (Interferon) induces and enhances the expression of major histocompatibilty complex (MHC) class II and intercellular adhesion molecule-1 (ICAM-1, CD54) on various cell types. The MHC class II is a pivotal surface molecule for T-cell antigen recognition. On the other hand, ICAM-1 is one of the main adhesion molecules in various cell-to-cell interaction, such as that between antigen-presenting cells and T cells. Moreover, the interaction between ICAM-1 and its counter-recep-

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tor, such as lymphocyte function antigen (LFA)-1 (CD11a/CD18), delivers a co-stimulatory signal with the critical signal mediated by the T-cell receptor/CD3 complex, which cooperatively activates T cells.[2] Many previous studies have shown that aberrant MHC class II expression and strong expression of ICAM-1 are observed on various cells at the inflammatory sites in tissue-specific autoimmune diseases or allograft rejection.[3–5] In corneal epithelium, co-expression of MHC class II and ICAM-1 are found on inflamed corneal epithelial cells such as those with herpetic keratitis and allograft rejection,[6,7] although neither MHC class II nor ICAM-1 is normally expressed.[8,9] In addition, it has been demonstrated that IFN-γ induces the expression of MHC class II and ICAM-1 on cultured human corneal epithelial (HCE) cells;[10,11] IFN-γ-treated MHC class II-bearing HCE cells have a potential to stimulate lymphocytes[12]; the ICAM-1 LFA-1 pathway plays a crucial role in adhesive interaction between HCE cells and lymphocytes.[13] Therefore, elucidation of the mechanisms of IFN-γ-induction of MHC class II and ICAM-1 expression on each of various cell types, including corneal epithelial cells, may contribute to further understanding of local immune responses and might lead to the development of new clinical approaches to immune diseases. Signal transduction pathways for many biological responses induced by IFN-γ are well documented by the janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway. The interaction between IFN-γ and its receptors triggers tyrosine phosphorylation of JAK1 and JAK2, protein tyrosine kinases (PTKs) associated with IFN-γ receptors physically and functionally, resulting in activation of these PTKs.[14] The phosphorylated JAK1 and JAK2 recruit and phosphorylate tyrosine residues of a target protein, such as STAT1.[15] Subsequently, a homodimer of STAT1 (STAT1α) moves to the nucleus and binds to the IFN-γ-activated site of an IFN-γ-stimulated gene, such as the guanylate binding protein gene. The requirement of STAT1 for IFN-γ-induction of MHC class II and ICAM-1 has been demonstrated in some cell types.[16–18] However, STAT1 is frequently activated in response to a wide variety of extracellular signals.[19] Also, there is little evidence to explain a direct interaction between the JAK-STAT pathway and the IFN-γ-induction of ICAM-1 expression. In addition, it has not been clarified whether IFN-γ induces the expression of MHC class II and ICAM-1 using the same signaling pathway. In the present study, we tried to determine if tyrosine phosphorylation of proteins including JAK1 and JAK2 is essential in the signal transduction pathway for IFN-γ-induction of both MHC class II and ICAM-1 expression in cultured HCE cells.

Materials and Methods

Human Corneal Epithelial Cell Culture

Human donor eyes were provided by domestic local eye banks. The portion of donor cornea remaining after corneal transplantation was used in this study, which was approved by the Ethics Committee for Human Research in Nihon University. Primary cultures of HCE cells were performed using the procedure described previously.[20]

Briefly, limbal explants without endothelium were incubated in 35-mm tissue culture dishes (Falcon 3001; Becton Dickinson, Lincoln Park, NJ, USA) with modified SHEM, which consists of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) (1:1; Gibco BRL, Grand Island, NY, USA), containing mouse epidermal growth factor (10 ng/mL), bovine insulin (5 μg/mL; Gibco BRL), cholera toxin (0.1 μg/mL), dimethylsulfoxide (0.5%; Sigma, St. Louis, MO, USA), gentamicin (40 μg/mL; Schering-Plough, Osaka), penicillin G (100 U/mL; Banyu, Tokyo), and 10% fetal bovine serum (FBS; Gibco). The cultures were incubated at 37°C under 5% CO_2 for 2 to 3 weeks. The medium was changed twice a week. The explants were removed after the cells had become confluent. By the methods described previously,[12] these cultured cells were confirmed as epithelial cells by keratin expression, and contamination in the cultured cells by Langerhans cells or by corneal fibroblasts was ruled out. Some primary HCE cell cultures were transferred to 12-well plates by 0.25% trypsin and 0.5% ethylenediaminetetraacetic acid (EDTA; Sigma) treatment for flow cytometry.

Treatment of Cultured HCE Cells with Protein Tyrosine Kinase Inhibitors and IFN-γ

The HCE cell cultures in 35-mm dishes and in 12-well-plates were divided into four groups to investigate the effect of PTK inhibitors on IFN-γ-induction of MHC class II and ICAM-1 expression and on IFN-γ-induction of tyrosine phosphorylation. These groups were treated with PTK inhibitor, herbimycin A or genistein (Sigma), and recombinant human IFN-γ (Genzyme, Boston, MA, USA) in different ways, as follows: (1) the culture was pretreated with PTK-inhibitor for 16 hours, then incubated with 1000 U/mL of IFN-γ at 37°C under 5% CO_2; (2) the culture without PTK-inhibitor pretreatment was in-

cubated with 1000 U/mL of IFN-γ; (3) the culture pretreated with PTK-inhibitor was incubated without IFN-γ; and (4) the culture was neither pretreated with PTK-inhibitor nor incubated with IFN-γ. The incubation period was the same in each group as indicated in figure legends and tables.

Anti-phosphotyrosine Immunoblot Analysis

Cultured HCE cells (5×10^5) were washed with DMEM thoroughly, then incubated with DMEM containing 1% FBS (1% FBS-DMEM) for 2 days. Subsequently, the cells were treated with and without 5 μg/mL of herbimycin A for 16 hours, followed by incubation with 1,000 U/mL of IFN-γ for 1 hour. The stimulation was terminated by the addition of sodium dodecyl sulphate (SDS) sample buffer (Sigma). The whole cell lysates were subjected to anti-phosphotyrosine (pTyr) immunoblot analysis by the previously described procedure.[21] Protein contents of the lysates were measured using a protein assay kit (BioRad, Hercules, CA, USA), then 20 μg of protein was separated by SDS-polyacrylamide gel electrophoresis using a 10% separation gel according to the method of Laemmli.[22] The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 0.5% gelatin in phosphate-buffered saline (PBS) at 4°C overnight, the membranes were incubated with anti-pTyr monoclonal antibody (mAb) (4G10; Upstate Biotechnology, Lake Placid, NY, USA) at room temperature for 1 hour, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG at room temperature for 1 hour. After extensive washing with PBS containing Tween-20, immunoreactive protein on the membranes was visualized by using ECL Western blotting kit (Amersham, Bucks, UK).

Immunoprecipitation

Cultured HCE cells (4×10^6) were washed with DMEM thoroughly. Then, the cells were incubated with 1% FBS-DMEM for 2 days, followed by incubation with 1,000 U/mL of IFN-γ for 1 hour. The stimulation was terminated by washing the cells with 1 mmol of sodium orhovanadate (Na_3VO_4) in PBS. These cells were suspended in PY-lysis buffer containing 20 mmol of Tris-HCl (pH 7.4), 137 mmol of NaCl, 10% glycerol, 1% Nonidet P-40, 1 mmol of Na_3VO_4, 2 mmol of EDTA, 0.2 mmol of phenylmethylsulphonyl fluoride, 20 μmol of leupeptin, and 0.15 U/mL of aprotinin, and allowed to stand on ice for 20 minutes. The whole cell lysates were incubated with protein A-sepharose at 4°C overnight. These samples were incubated with anti-JAK1 or -JAK2 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour; then protein A-sepharose was added to the samples and incubation continued at 4°C for 1 hour. After washing with PY-lysis buffer, the immunoprecipitates were subjected to anti-P-Tyr immunoblot analysis and also to anti-JAK1 or anti-JAK2 immunoblot analysis to ascertain the amount of specific protein immunoprecipitated as described above.

Flow Cytometry

Cultured HCE cells treated with PTK-inhibitors and IFN-γ as described above were converted to cell suspension with 0.25% trypsin and 0.5% EDTA treatment. Then, the cells were settled in 10% FBS-DMEM at room temperature for 2 hours for cellular recovery. The cells were stained by the following immunofluorescence technique: the cells were washed twice with PBS containing 2% bovine serum albumin and 0.1% NaN_3 (Sigma); then incubated with fluorescein isothiocyanate-conjugated (FITC) anti-MHC class II mAb (CR3/43; DAKO, Denmark), or FITC-conjugated anti-ICAM-1 mAb (B-C14: Serotec, Oxford, UK) at 4°C for 45 minutes; then washed twice with PBS.

More than 90% of the cells were alive after the entire treatment followed by the staining procedure, as determined by trypan blue exclusion. The cells were analyzed by flow cytometry (Ortho Cytoron; Ortho Diagnostic System, Tokyo).

Analysis of MHC Class II Synthesis by Immunoblotting

Cultured HCE cells (5×10^5) were pretreated with and without herbimycin A for 16 hours, then incubated with 1,000 U/mL of recombinant human IFN-γ for 3 days. The stimulation was terminated by the addition of SDS sample buffer (Sigma). Twenty micrograms of protein of the whole cell lysates from these treated HCE cells was subjected to anti-MHC class II immunoblot analysis using mAb (CR3/43, DAKO) as described above.

Results

IFN-γ-Induction of Tyrosine Phosphorylation of Multiple Proteins Including JAK1 and JAK2 in Cultured HCE Cells

Cultured HCE cells were preincubated with 1% FBS-DMEM for 2 days, then incubated with and without 1,000 U/mL of IFN-γ for 1 hour. Cell lysates

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from these treated HCE cells were analyzed by immunoblotting with the anti-pTyr mAb. Figure 1 shows the results of the study on induction of tyrosine phosphorylation. The IFN-γ induced tyrosine phosphorylation of multiple proteins, particulary that of 75,000; 90,000; 130,000; and 160,000 molecular weight proteins (pp75, pp90, pp130, and pp160, respectively).

Next, JAK1 and JAK2 in cultured HCE cells (4×10^6) treated as described above were immunoprecipitated with the specific antibodies, and their tyrosine phosphorylation was determined by immunoblotting with the anti-pTyr mAb. As shown in Figure 2, in the IFN-γ-nontreated cells, no tyrosine phosphorylation band was observed for JAK1 or JAK2; while in the cells with IFN-γ treatment, both tyrosine phosphorylated JAK1 and JAK2 were found at 130,000 molecular weight.

Effects of PTK Inhibitors on IFN-γ-Induction of Tyrosine Phosphorylation

Cultured HCE cells were pretreated with 5 μg/mL of herbimycin A, a PTK-specific inhibitor,[23] for 16 hours; then incubated with 1,000 U/mL of IFN- for 1 hour. Tyrosine phosphorylation in these treated HCE cells was determined. As shown in Figure 1, pretreatment of HCE cells with herbimycin A dramatically inhibited IFN-γ-induced pp75, pp130, pp160, and also reduced pp90 to that in IFN-γ-nontreated HCE cells.

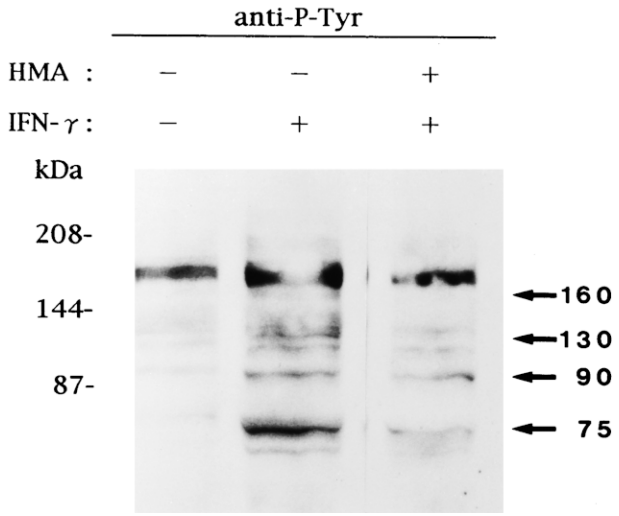

Figure 1. Interferon (IFN)-γ-induction of tyrosine phosphorylation and effect of protein tyrosine kinase inhibitor on IFN-γ-induction of tyrosine phosphorylation in cultured human corneal epithelial (HCE) cells. Cultured HCE cells were washed with Dulbecco's Modified Eagle Medium (DMEM) thoroughly, then incubated with DMEM containing 1% fetal bovine serum (FBS; 1% FBS-DMEM) for 2 days. Next, cells were treated with and without 5 μg/mL of herbimycin A (HMA) for 16 hours, followed by incubation with 1,000 U/mL of IFN-γ for 1 hour. Subsequently, whole cell lysates from these cells were subjected to anti-phosphotyrosine (P-Tyr) immunoblot analysis. Arrows indicate IFN-γ-induced tyrosine phosphorylated proteins.

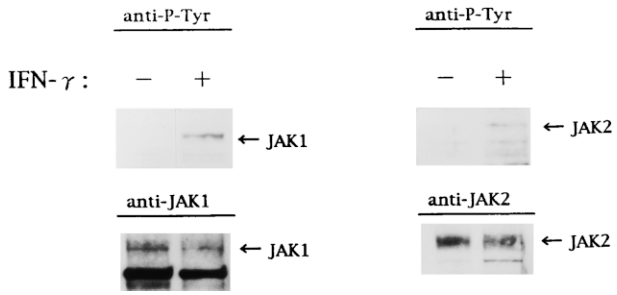

Figure 2. Interferon (IFN)-γ-dependent tyrosine phosphorylation of janus kinase (JAK)1 and JAK2 molecules. Cultured human corneal epithelial (HCE) cells were washed with Dulbecco's Modified Eagle Medium (DMEM) thoroughly, then incubated with 1% fetal bovine serum-DMEM for 2 days. Subsequently, cells were incubated with 1,000 U/mL of IFN-γ for 1 hour. Samples from 4×10^6 of cells were immunoprecipitated with anti-JAK1 or anti-JAK2 polyclonal Ab. Subsequently, immunoprecipitates were subjected to anti-phosphotyrosine (P-Tyr) immunoblot analysis (upper panel) and also to anti-JAK1- or anti-JAK2- immunoblot analysis (lower panel) to ascertain amount of specific protein immunoprecipitated.

We performed the same experiments using genistein, another PTK-specific inhibitor.[24] Cells were treated with 50 μg/mL of genistein for 16 hours followed by incubation with 1,000 U/mL of IFN-γ for 1 hour. Primarily, the same results were obtained with genistein (data not shown).

Effects of PTK Inhibitors on IFN-γ-Induction of MHC Class II and ICAM-1 Expression on Cultured HCE Cells

We examined the effects of PTK inhibitors on the IFN-γ-induction of MHC class II and ICAM-1 expression on cultured HCE cells to determine the role of tyrosine phosphorylation in this induction effect of IFN-γ. Cultured HCE cells were pretreated with herbimycin A at variable concentrations up to 5 μg/mL for 16 hours, then incubated with 1,000 U/mL of IFN-γ for 3 days. These cells were immunostained with anti-MHC class II mAb or anti-ICAM-1 mAb, then analyzed by flow cytometry. As demonstrated previously,[13,20] both MHC class II and ICAM-1 expression are induced dramatically by the IFN-γ treatment. However, pretreatment of HCE cells with

herbimycin A inhibited IFN-γ-induction of MHC class II expression in a dose- dependent fashion (Table 1, Figure 3). In contrast, herbimycin A had no effect on ICAM-1 expression (Figure 3). Experiments with either a higher dose (10 μg/mL) of herbimycin A showed no inhibitory effect on IFN-γ-induction of ICAM-1 expression (data not shown). Treatment with herbimycin A alone neither induced the expression of MHC class II nor had any effect on the basal level of ICAM-1 expression on cultured HCE cells. Although we examined cultured HCE cells from different donors, we obtained basically the same results in five separate experiments (Table 2).

We also performed the same experiments with genistein. Cultured HCE cells were pretreated with 50–100 μg/mL of genistein for 16 hours, then incubated with 500–1,000 U/mL of IFN-γ for 3 days. The genistein treatment had a remarkable inhibitory effect on MHC class II induction by IFN-γ, whereas genistein had little effect on IFN-γ-induction of ICAM-1 expression (Table 3). We could not obtain any reliable result with a dose of genistein higher than 100 μg/mL because of its cytotoxity.

Effect of PTK Inhibitor on MHC Class II Synthesis in IFN-γ-Treated Cultured HCE Cells

We attempted to determine the point in the pathway where PTK inhibitor inhibits IFN-γ-induction of MHC class II expression. Cultured HCE cells were treated with and without herbimycin A (5 μg/mL, for 16 hours) followed by incubation with IFN-γ (1,000 U/mL, for 3 days). Whole cell lysates from these treated cells were examined by immunoblotting with the anti-MHC class II common β chain mAb (CR3/43). A strong band of MHC class II β chain was found in the IFN-γ-treated HCE cells without herbimycin A pretreatment. However, pretreatment of HCE cells with herbimycin A dramatically reduced the band of the MHC class II β chain to the nondetectable level of HCE cells without IFN-γ treatment (Figure 4).

Table 1. Effect of Herbimycin A on Interferon (IFN) γ Induction of Major Histocompatibility Complex (MHC) Class II Expression on Cultured Human Corneal Epithelial (HCE) Cells*

Concentration of Herbimycin A (μg/mL)	IFN-γ Induction of MHC Class II Expression
	Positive Cells (%)
0	77.0
0.5	52.5
1.0	47.1
2.0	33.7
5.0	21.4

*There was no positive staining on herbimycin A-treated HCE cells without IFN-γ treatment.

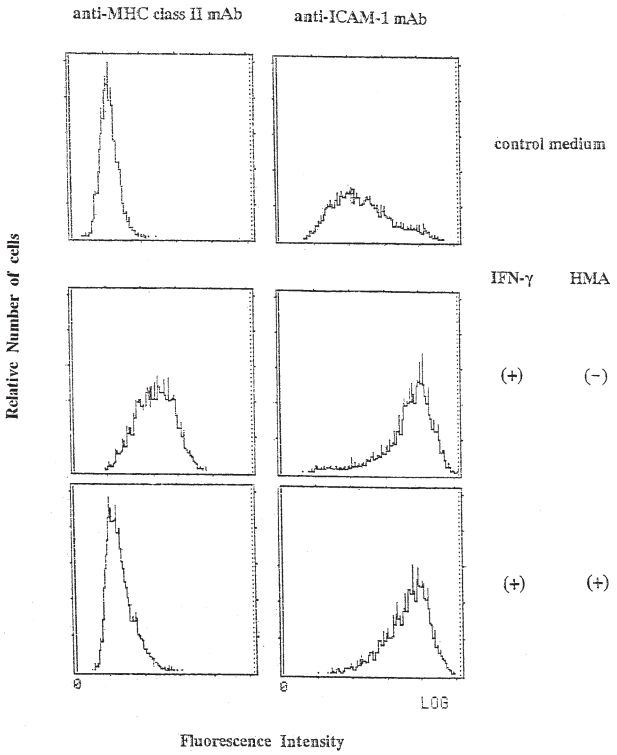

Figure 3. Effects of herbimycin A (HMA) on interferon (IFN)-γ-induction of major histocompatibility complex (MHC) class II and intercellular adhesion molecule-1 (ICAM-1) expression on cultured human corneal epithelial (HCE) cells. Cultured HCE cells were treated with and without 5.0 μg/mL of HMA for 16 hours, then incubated with 1,000 U/mL of IFN-γ for 3 days. These cells were immunostained with anti-MHC class II or anti-ICAM-1 mAb, then analyzed by flow cytometry.

Time Course of the Effect of PTK Inhibitor on IFN-γ-Induction of MHC Class II and ICAM-1 Expression

An IFN responsive element such as the IFN-γ-activation site that participates in the direct immediate gene transcription by IFN-γ has not been defined in MHC class II genes.[25,26] For IFN-γ-activation of MHC class II genes, intermediate proteins have been shown to be required. Recently, IFN-γ-induced intermediate proteins such as the MHC class II transactivator (CIITA) and the IFN-γ-enhanced factor X (IFNEX) have been demonstrated to interact with and directly bind MHC class II genes, respec-

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Table 2. Effects of Herbimycin A on Interferon (IFN)-γ Induction of Major Histocompatibility Complex (MHC) Class II and Intercellular Adhesion Molecule-1 (ICAM-1) Expression*

	MHC Class II Expression	
	Positive Cells (%)	
	IFN-γ	
Experiment	Herbimycin A: −	+ (% Inhibition)
1	59.0	6.8 (88.5)
2	60.4	19.6 (67.6)
3	79.1	37.2 (53.0)
4	71.7	26.4 (63.2)
5	54.1	21.1 (61.0)

	ICAM-1 Expression			
	Positive Cells (%)			
	IFN-γ:	+	−	
Experiment	Herbimycin A:	− +	−	+
1		96.2 97.0	54.7	57.5
2		99.3 97.3	58.5	64.0
3		99.6 98.2	65.8	71.4
4		87.5 86.8	45.9	39.7
5		97.7 95.6	73.2	69.8

*Five separate experiments were performed using cultured human corneal epithelial cells from five donors. Percent inhibition was calculated as follows: {1 − MHC class II $^+$ cells with herbimycin A treatment (%)/MHC class II $^+$ cells without herbimycin A treatment (%)} × 100. Treatment with herbimycin A alone neither induced expression of MHC class II nor had any significant effect on basal level of ICAM-1 expression on cultured human corneal epithelial cells.

tively.[27–29] The maximal response by either protein occurs 12–24 hours after IFN-γ stimulation. Therefore, we tried to determine whether tyrosine phosphorylation is required not only in the primary JAK-STAT pathway but also in the subsequent pathway where IFN-γ-induced intermediate proteins activate the MHC class II genes. Cultured HCE cells were divided into five groups, depending on the timing of the addition of herbimycin A (5 μg/mL) at −16, and 0, 12, 24, and 36 hours after the addition of IFN-γ (groups 1 to 5, respectively). Each group was incubated with 1,000 U/mL of IFN-γ for 3 days. As shown in Figure 5, in groups 1 to 3, the IFN-γ-induction of MHC class II expression was markedly inhibited. However, in groups 4 and 5, there was no inhibitory effect. Significant differences were found in the inhibitory effect of herbimycin A between groups 1 to 3 and groups 4 and 5. There was no effect of herbimycin A on the IFN-γ-induction of ICAM-1, irrespective of the timing of the addition of herbimycin A (data not shown).

Table 3. Effects of Genistein on Interferon (IFN)-γ Induction of Major Histocompatibiltiy Complex (MHC) Class II and Intercellular Adhesion Molecule-1 (ICAM-1) Expression*

	Positive Cells (%)	
Experiment	MHC Class II	ICAM-1
1		
IFN-γ (500 U/mL)	49.0	96.2
+ Genistein (50 μg/mL)	8.7	95.8
+ Genistein (100 μg/mL)	6.1	97.0
2		
IFN-γ (1000 U/mL)	60.4	93.4
+ Genistein (50 μg/mL)	10.7	97.4
+ Genistein (100 μg/mL)	7.5	97.8

*Two experiments were performed using cultured human corneal epithelial cells from two donors. Treatment with genistein alone neither induced expression of MHC class II nor had any significant effect on basal level of ICAM-1 expression on cultured human corneal epithelial cells.

Discussion

Interferon-γ-induction of the expression of both MHC class II and ICAM-1 is regulated at the transcriptional level of these genes.[30–32] So far, it is not clear whether IFN-γ uses the same signal transduction pathway when inducing MHC class II and ICAM-1 expression. Therefore, in the present study, we tried to determine the significance of the JAK-dependent signaling pathway in the induction of both MHC class II and ICAM-1 expression by IFN-γ in cultured HCE cells.

We demonstrated that in cultured HCE cells IFN-γ induced multiple tyrosine-phosphorylated proteins, especially pp75, pp90, pp130, and pp160. The apparent molecular weight of pp130 corresponds to those of JAK1 and JAK2, and the molecular weight of pp90 is thought to correspond to that of STAT1. These are three critical components in IFN-γ triggered signal transduction. Indeed, IFN-γ-dependent tyrosine phosphorylation of JAK1 and JAK2 were observed in cultured HCE cells. These findings indicate that IFN-γ triggers the activation of JAK1 and JAK2 and the intracellular signaling cascade including STAT1 activation in cultured HCE cells, as occurs in other cell types. We also demonstrated that PTK-specific inhibitors, herbimycin A and genistein, inhibited IFN-γ-induction of MHC class II expression remarkably, but had no effect on ICAM-1 expression, although these PTK-inhibitors dramatically inhibited IFN-γ-induced pp75, pp130, pp160, and also reduced pp90 to the same level as in cells not treated with IFN-γ. We performed the same experiments using cultured HCE cells from different

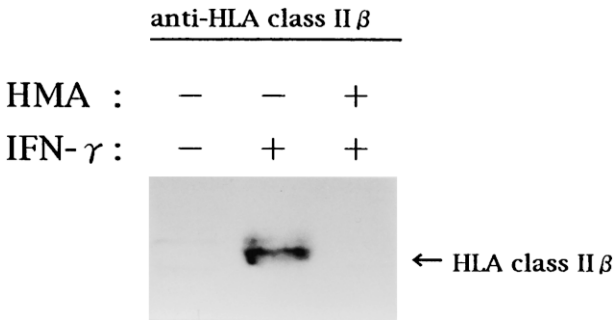

Figure 4. Effect of herbimycin A (HMA) on interferon (IFN)-γ-induced major histocompatibility complex (MHC) class II protein in cultured human corneal epithelial (HCE) cells. Cultured HCE cells were treated with HMA (5 g/mL, for 16 hours), then incubated with and without 1,000 U/mL of IFN-γ for 3 days. These cells were subjected to anti-MHC class II chain immunoblot analysis. First lane shows result without HMA and IFN-γ treatment. HLA (human leukocyte antigen), the human MHC.

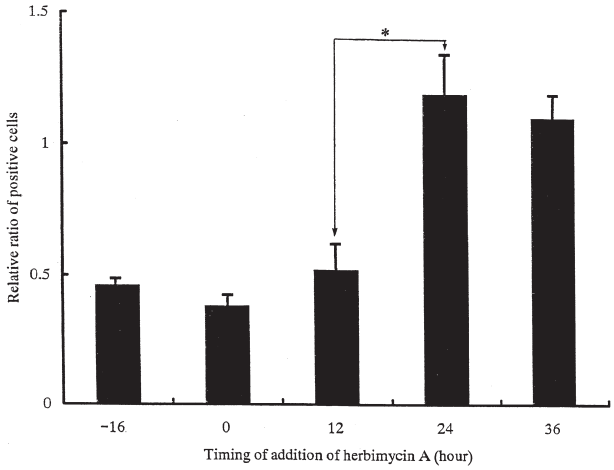

Figure 5. Time course of effect of protein tyrosine kinase inhibitor on interferon (IFN)-γ-induction of major histocompatibility complex (MHC) class II expression. Cultured human corneal epithelial (HCE) cells were divided into five groups with different timing of herbimycin A (HMA) addition (5.0 g/mL); −16, and 0, 12, 24, and 36 hours after addition of IFN-γ (1,000 U/mL). Incubation with IFN-γ was for 3 days in each group. These cells were immunostained with anti-MHC class II mAb, then analyzed by flow cytometry. Relative ratio of positive cells was calculated as follows: MHC class II-positive percent in IFN-γ-treated HCE cells with HMA pretreatment (as indicated) that in IFN-γ-treated HCE cells without HMA pretreatment. Data are expressed as mean relative ratio and standard deviation for three separate experiments with cultured HCE cells from three different donors. $*P <$ .02, for statistical difference between two indicated groups; Student *t*-test.

donors and we could obtain consistent results in each separate experiment. Taken together, our results indicate that in cultured HCE cells tyrosine phosphorylation of proteins including JAK1 and JAK2 is essential for the induction of MHC class II expression but not critical for the induction of ICAM-1 expression by IFN-γ .

Many proinflammatory cytokines including IFN-γ induce ICAM-1 expression. However, the signal transduction pathway and intracellular regulatory elements remain largely unknown. Several studies have demonstrated the importance of STAT1 protein in IFN-γ induction of ICAM-1 expression.[16,18,33,34] However, to our knowledge, there is no report demonstrating that PTK inhibitors prevent IFN-γ-induction of ICAM-1 expression, although they could inhibit IFN-γ-induction of MHC class II expression on an astrocyte-derived glioblastoma cell line, T98G.[35] It has been shown that genistein enhances rather than inhibits IFN-mediated upregulation of ICAM-1 expression on the endothelial cell line EA.Hy 926 through an undefined mechanism.[36] Our results with a similar concentration of genistein did not agree with their results. However, we obtained identical results with genistein and herbimycin A. A possible explanation for the difference between our results and theirs might be the difference in cell type. The dependency on protein kinase C (PKC) in the induction of ICAM-1 by IFN-γ has been studied previously. However, those results varied with cell types; the activation of PKC is crucial in vascular endothelial cells,[37] but not in keratinocytes[38] and human renal carcinoma cell line CaKi-1.[39] The above knowledge strongly suggests the existence of multiple pathways to mediate IFN-γ-induction of ICAM-1 expression, and that the critical pathway could differ among cell types. Therefore, in further studies we plan to seek an essential signaling pathway other than the PTK (JAK)-dependent pathway for IFN-γ-induction of ICAM-1 expression in cultured HCE cells.

Interferon-γ causes immediate transcriptional activation of several genes including guanylate binding protein gene through binding of a γ-activation factor to an IFN-γ response element, the IFN-γ-activation site. However, MHC class II genes exhibit a secondary response to IFN-γ requiring IFN-γ-induced intermediate activators. Since induction of MHC class II genes by IFN-γ is characterized by a long lag period and is dependent upon de novo protein synthesis by IFN-γ,[30,31] the IFN-γ-activation site has not been defined in MHC class II genes.[25,26] Recently, two different intermediate proteins, the MHC class II transactivator (CIITA) and the IFN-γ-enhanced factor X (IFNEX), have been demonstrated to be responsible for the inducible expression of MHC class

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II genes by IFN-γ.[27–29] The CIITA is found in a number of cell lines and the IFNEX is reported to be expressed in astrocytes. Regarding the characteristic features of the CIITA and the IFNEX, the CIITA does not bind to DNA directly but acts as a coactivator through a protein-to-protein interaction. On the other hand, the IFNEX binds to the X element of MHC class II genes directly. However, the kinetics of the CIITA and the IFNEX responses are similar, with the maximal response occurring at 12 to 18 hours for the CIITA and at 12 to 24 hours for the IFNEX, after IFN-γ stimulation.

Recently, the CIITA expression has been shown to be mediated by the JAK-STAT pathway.[40] However, the pathway that mediates the IFNEX expression is still not clear. In the present study, we obtained the intriguing result that the inhibitory effect of PTK inhibitor on the IFN-γ-induction of MHC class II expression depends on the timing of the addition of the inhibitor to IFN-γ-treated cultured HCE cells. In detail, when herbimycin A was added together with or at 12 hours after the addition of IFN-γ, the inhibitory effect was still as strong as when the inhibitor was added at 16 hours before the addition of IFN-γ. However, when the inhibitor was added at more than 24 hours after the addition of IFN-γ, an inhibitory effect was no longer observed. In addition, we demonstrated that PTK inhibitor blocks the cell surface expression of MHC class II through its inhibition of IFN-γ-induced MHC class II protein. Taken together, our results raise the possibility that PTK-activities are required for the MHC class II gene transcription in both the induction and the activation phases of IFN-γ-induced intermediate proteins. In contrast to MHC class II, we detected no inhibitory effect of PTK inhibitor on the IFN-γ-induction of ICAM-1 expression, although we performed similar experiments changing the timing of the addition of PTK inhibitor.

We concluded that the induction of ICAM-1 by IFN-γ is mediated by a pathway other than the PTK (JAK1 and JAK2)-dependent pathway. In addition, IFN-γ-induction of MHC class II may require PTK activities not only in the primary JAK-STAT pathway but also in the subsequent pathway where IFN-γ-induced intermediate proteins activate the expression of MHC class II genes.

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