Interleukin 4–Induced Proliferation in Normal Human Keratinocytes Is Associated with c-*myc* Gene Expression and Inhibited by Genistein

Young Yang,*† Hyun Mi Yoo,† Inpyo Choi,† Kwang Ho Pyun,† Si Myung Byun,* and Hyunjung Ha†

*Department of Biological Sciences, Korea Advanced Institute of Science and Technology; Research Center for New Bio-Materials in Agriculture, Seoul National University, Suwon; and †Immune Cell Signal Transduction RU, Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, Taejon, Republic of Korea

We studied the effect of IL-4 on the proliferation of cultured normal human keratinocytes. Keratinocyte proliferation was stimulated by IL-4 and inhibited by anti–IL-4 antibody in a concentration-dependent manner. Anti-IL-6 antibody did not inhibit normal human keratinocyte proliferation, suggesting that the IL-4 could directly induce proliferation of these cells. IL-4 significantly induced cell cycle G_0/G_1 to S phase progression. The keratinocyte proliferation by IL-4 was mediated through one of the growth control genes, c-*myc* protooncogene. The expression of c-*myc* mRNA was significantly increased after IL-4 treatment of the keratinocytes, suggesting that c-*myc* plays a key role in the control of proliferation. The signal transduction pathways induced by IL-4 in the keratinocytes were studied with inhibitors of signal transduction. Genistein, a tyrosine kinase inhibitor, suppressed the level of the induced c-*myc* mRNA expression, but H7, a serine/threonine kinase inhibitor, and okadaic acid, a protein phosphatase 1 and 2A inhibitor, did not block the induced c-*myc* gene expression. Taken together, these results suggest that IL-4 stimulates the proliferation of keratinocytes *in vitro* by promoting a transition from G_0/G_1 to S phase of the cell cycle. Induction of c-*myc* after IL-4 treatment could indicate an important role for c-*myc* in the proliferation of keratinocytes. Our observations also suggest that tyrosine kinases may be involved in IL-4–induced proliferation. *Key words: cell cycle/tyrosine kinase. J Invest Dermatol 107:367–372, 1996*

IL-4 is a T-cell–derived cytokine that displays pleiotropic biologic effects not only on many hematopoietic cells, including B cells, T cells, mast cells, macrophages, and basophils (Spits, 1992), but also on nonhematopoietic cells (Tushinski *et al*, 1991; Fertin *et al*, 1991). c-*myc* appears to play a key role in cellular proliferation, and expression of c-*myc* is tightly associated with proliferation in nontransformed cells (Luscher and Eisenman, 1990; Spencer and Groudine, 1991). Indeed, c-*myc* activation may commit the G_1 to S phase of cell division (Eilers *et al*, 1991). Deregulation of c-*myc*, a protooncogene and an early response gene to mitogen or growth factor stimulation, is characteristic of many tumor cells, suggesting that its product plays a major role in growth control.

IL-4 upregulates IL-6 production in endothelial cells, skin fibroblast cells, and keratinocytes (Howells *et al*, 1991; Feghali *et al*, 1992; Derocq *et al*, 1994). The observation that IL-6 stimulates the proliferation of normal human keratinocytes in culture prompted us to investigate the role of IL-4 in the process. In the current study, we analyzed the effect of IL-4 on the proliferation of normal human keratinocytes by examining [^{3}H]thymidine incorporation, the expression of cell cycle–regulating nuclear factors, such as c-*myc*, and the cell cycle. As yet, there is little direct evidence for the involvement of a specific tyrosine kinase in response to IL-4 signaling through the IL-4 receptor (IL-4R), and much less is known about the tyrosine kinase cascade evoked by IL-4. We therefore sought to determine the signaling pathway triggered by IL-4 in keratinocytes. Our results provide evidence that IL-4 activates a tyrosine kinase cascade that mediates IL-4–induced cell growth and is an important positive regulator of the cell cycle in keratinocytes. These results suggest that IL-4, like IL-6, may play a potentially important role in the immune and inflammatory responses of the skin, stimulating proliferation of both epidermal cells and lymphoid cells.

Manuscript received November 13, 1995; revised May 9, 1996; accepted for publication May 23, 1996.

Reprint requests to: Dr. Hyunjung Ha, Immune Cell Signal Transduction RU, Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, P.O. Box 115, Yusong, Taejon 305-600, Republic of Korea.

Abbreviations: KBM, keratinocyte basal medium; KGM, keratinocyte growth medium.

MATERIALS AND METHODS

Reagents Keratinocyte basal medium (KBM) and keratinocyte growth medium (KGM) supplemented with epidermal growth factor and bovine pituitary extracts were purchased from Clonetics Corp. (San Diego, CA). [^{3}H]thymidine (83 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Recombinant human IL-4 and anti-human IL-6 antibody were the product of Genzyme (Boston, MA). We also used recombinant IL-4 prepared from *Escherichia coli* BL21 (λDE3) carrying the pET-3b/IL-4 plasmid by induction with 1 mM isopropyl-β-D-thiogalactopyranoside, carboxymethyl-cellulose column (1.5 × 10 cm; Sigma Chemical Co., St. Louis, MO), and capillary electrophoresis (P/ACE System 2000; Beckman, Fullerton, CA). The recombinant IL-4 showed a biologic activity with a calculated specific activity of 1×10^8 U/mg in B-cell proliferation assay. Anti-human IL-4 antibody, transforming growth factor–α (TGF-α), okadaic acid, and dispase (neutral protease grade II) were purchased from Boehringer Mannheim

0022-202X/96/$10.50

(Indianapolis, IN), and protein kinase inhibitors, H7 and genistein, were from GIBCO (Grand Island, NY). RNAzol B was obtained from Biotecx Laboratories Inc. (Houston, TX). Insulin-like growth factor-I (IGF-I) was a gift from Y.I. Lee (Korea Institute of Science and Technology, Taejon, Korea). The cDNA for glyceraldehyde-3-phosphate dehydrogenase was purchased from the American Type Culture Collection (Rockville, MD). The c-*myc* hybridization probe (1.4 kb, third exon) was obtained from Oncor Inc. (Gaithersburg, MD), and actinomycin D, cycloheximide, and propidium iodide were from Sigma.

Culture of Keratinocytes Human keratinocytes were obtained from fresh foreskin biopsies of adult donors and cultured as described previously (Saunders and Jetten, 1994). Briefly, the foreskin specimens were cut into 1- × 5-mm pieces and incubated in 0.1% dispase solution for 90 min at 37°C. The epidermal layer was detached from the dermis with sharp forceps and incubated in solution A (30 mM HEPES, pH 7.4, 10 mM glucose, 132 mM NaCl, 1 mM Na_2HPO_4, and 3 mM KCl) supplemented with 0.05% trypsin and 0.53 mM EDTA for 10 min at 37°C. Keratinocytes separated from the trypsinized epidermis were cultured in KGM. The cells were grown to 80–90% confluence, trypsinized, and subcultured in 10-cm dishes (Costar, Cambridge, MA). The fourth to sixth passage keratinocytes were used for the experiments.

Proliferation Assay Proliferative growth was assessed either by [^{3}H]thymidine incorporation or by cell counts as previously described (Turka *et al*, 1990). Keratinocytes grown in KGM were plated in 96- or 24-well flat-bottomed microplates (Costar, Cambridge, MA) at a concentration of 1×10^4 cells per well in a final volume of 200 μl or 1 ml, respectively, and cultured for 24–48 h. To arrest the keratinocyte growth in G_0/G_1 phase, cultures were washed twice with solution A and incubated in KBM for 18 h. IL-4 was then added at the indicated concentrations, and the cells were incubated for 48 h with [^{3}H]thymidine (0.5 μCi/well) added for the last 6 h. After the cells were harvested with trypsin, the amount of [^{3}H]thymidine uptake was counted by liquid scintillation spectrometry (Beckman LS6000A), or the number of viable cells was counted with a hemocytometer after trypan blue exclusion. Results were calculated as the means ± SEM of triplicate cultures.

Cell Cycle Analysis Keratinocytes grown in KGM were plated on 10-cm dishes at a concentration of 1×10^6 cells per dish and maintained for 24–48 h. Cells were treated with 1 ng IL-4 per ml after starvation in KBM for 18 h. The cells were washed with phosphate-buffered saline, trypsinized, pelleted by centrifugation at 800 rpm for 5 min, and resuspended in 1 ml of phosphate-buffered saline at the indicated times. The cells were immediately fixed by the addition of 3 ml of cold 95% ethanol at 4°C for at least 12 h followed by centrifugation at 1500 rpm for 10 min. The supernatant was carefully removed, and 1 ml of PI solution (3.8 mM sodium citrate, 1 mg RNase A per ml, 50 μg propidium iodide per ml) was added to each pellet. The cells were gently mixed and incubated at room temperature in the dark for at least 30 min, and the number of cells was measured. Cells (1×10^6) were subjected to FACScan. The percentages of cells in each phase of cell cycle were calculated by using Cellfit software (Becton Dickinson, San Jose, CA).

Northern Blot Analysis Keratinocytes grown in KGM were plated on 10-cm dishes at a concentration of 1×10^6 cells per dish and cultured for 24–48 h. Keratinocytes were treated with 1 ng IL-4 per ml after incubation in KBM for 18 h and washed with cold phosphate-buffered saline at the indicated time intervals. Northern blot analysis and extraction of total cellular RNA were performed as described previously, with modifications (Ha *et al*, 1992). In brief, the total cellular RNA was prepared with RNAzol B under conditions recommended by the manufacturer. Approximately 30 μg of total RNA were electrophoresed through 1% agarose-formaldehyde gel, transferred to Genescreen Plus nylon membrane (DuPont-NEN, Boston, MA), and vacuum-dried at 80°C for 2 h. The membranes were then hybridized to random primer–labeled cDNA probes in 50% formamide, 10% dextran sulfate, 7% sodium dodecyl sulfate, 0.25 M $NaHPO_4$, pH 7.2, 0.25 M NaCl, 1 mM ethylenediamine tetraacetic acid, and 100 μg denatured salmon sperm DNA per ml at 42°C for 18 h. The membranes were washed with 1 × sodium citrate/sodium chloride buffer and 0.2% sodium dodecyl sulfate at room temperature for 15 min, 0.5 × sodium citrate/sodium chloride buffer and 0.2% sodium dodecyl sulfate at room temperature for 15 min, and 0.25 × sodium citrate/sodium chloride buffer and 0.2% sodium dodecyl sulfate at 60°C for 15 min. Autoradiograms were prepared using Kodak XAR-5 film with intensifying screens at −80°C for 24–48 h. The same membranes were hybridized to ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA to verify equal loading of RNA.

Reverse Transcriptase-Polymerase Chain Reaction (PCR) Human keratinocytes incubated for 18 h in KBM were treated with 2.5 ng IL-4 per ml for the indicated times. Total cellular RNA was isolated as previously described and converted to cDNA under the following conditions: the RNA (1 μg) was solubilized at 65°C for 15 min and incubated for 1 h at 37°C with 19 μl of reverse transcription mixture containing 1 × reverse transcriptase buffer (GIBCO BRL, Gaithersburg, MD), 1 mM deoxynucleoside triphosphate, 0.25 μg of oligo deoxythymidylic cellulose (Promega Biotec), 5 mM dithiothreitol, and 200 U of murine moloney leukemia virus reverse transcriptase (GIBCO BRL). PCR was performed with 5 μl of first-strand cDNA on a DNA thermal cycler (Ericomp Inc, San Diego, CA) in 50 μl containing 1 × PCR buffer (50 mM KCl, 10 mM Tris(hydroxymethyl)-aminomethane, pH 9.0, 5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100), 0.4 μM of each primer, and 2.5 U of *Taq* polymerase (Korea Biotech, Taejon, Korea) for 40 cycles. Each PCR cycle consisted of annealing at 55°C for 30 s, extension at 72°C for 1 min, and denaturation at 94°C for 30 s. The amplified PCR products with the expected sizes were electrophoresed on a 1.5% agarose gel (319 bp for IL-4, 617 bp for IL-6, 548 bp for β-actin). The sequences of the oligonucleotides are as follows: for IL-4, sense oligonucleotide, 5′-GTGCGATATCACCTTACAGG-3′; antisense oligonucleotide, 5′-ACGTACTCTGGTTGGCTTCC-3′; for IL-6, sense oligonucleotide, 5′-ATGAACTCCTTCTCCACAAGC-3′; antisense oligonucleotide, 5′-GAAGAGCCCTCAGGCTGGACTG-3′; for β-actin (Butch *et al*, 1993), sense oligonucleotide, 5′-GTGGGGCGCCCCAG-GCACCA-3′; antisense oligonucleotide, 5′-CTCCTTAATGTCACG-CACGATTTC-3′.

Statistical Analysis Statistical analysis was carried out using the Student's t test, and all values are represented as the mean ± SEM.

RESULTS

IL-4 Stimulates Proliferation of Cultured Keratinocytes To evaluate the possible effect of IL-4 on keratinocyte proliferation, we treated cells with 0, 0.01, 0.1, 1, or 10 ng IL-4 per ml for 48 h in KBM. As shown in **Fig 1*d***, IL-4 significantly increased [^{3}H]thymidine uptake by keratinocytes in a concentration-dependent manner (maximal at 10 ng per ml), whereas, as with the cell growth rate shown in **Fig 1*e***, we observed a plateau or slight decrease at concentrations of more than 1 ng per ml in seven of 15 independent experiments (data not shown). To eliminate possible contamination of the recombinant IL-4 with other mitogens, we also tested the recombinant IL-4 before use in the proliferation assay. Heating IL-4 to 90°C for 30 min or 70°C for 1 h abrogated its proliferative activity on keratinocytes (data not shown). The property of IL-4 to stimulate keratinocyte proliferation was confirmed in experiments determining the number of cells, with trypan blue exclusion to ensure viability, after addition of graded amounts of IL-4. The growth-stimulatory effect of IL-4 also increased in a concentration-dependent manner and reached a peak at 1 ng per ml **(Fig 1*e*)**. A comparison of the keratinocyte proliferation induced by IL-4 and potent keratinocyte mitogens such as IGF-I and TGF-α showed a significant increase, although to a somewhat lesser extent, in keratinocyte proliferation with IL-4 treatment. As shown in **Fig 1 (*a,b,d*)**, IL-4, IGF-I, and TGF-α increased [^{3}H]thymidine uptake by approximately 2.5-, 3.1-, and 4-fold, respectively.

IL-4 Stimulates Keratinocyte Proliferation Distinct from That Induced by IL-6 We examined the possibility that the IL-4 effect on the proliferation of human keratinocytes could be mediated by induction of IL-6. A proliferation assay was performed in the presence of neutralizing antibody to determine whether or not the IL-4 effect was direct. As shown in **Fig 2*a***, we did not detect changes in growth in response to anti-IL-6 antibody (0–10 μg per ml) in IL-4–treated keratinocytes, but, when the cells were treated with IL-4 (2.5 ng per ml) combined with the indicated concentrations of anti–IL-4 antibody, antibody concentration-dependent inhibition of keratinocyte growth was observed (>60% growth inhibition at anti–IL-4 antibody concentration of 10 μg per ml). To further investigate whether IL-4 could increase the level of IL-6 mRNA, we performed reverse transcriptase-PCR to rule out the influence of IL-4 on IL-6 production in normal human keratinocytes. We did not detect a significant increase in the levels of IL-6 message and did not find a detectable level of IL-4 message after IL-4 treatment **(Fig 2*b*)**. Taken together, the data indicate that the IL-4–induced proliferation in human keratinocytes occurs

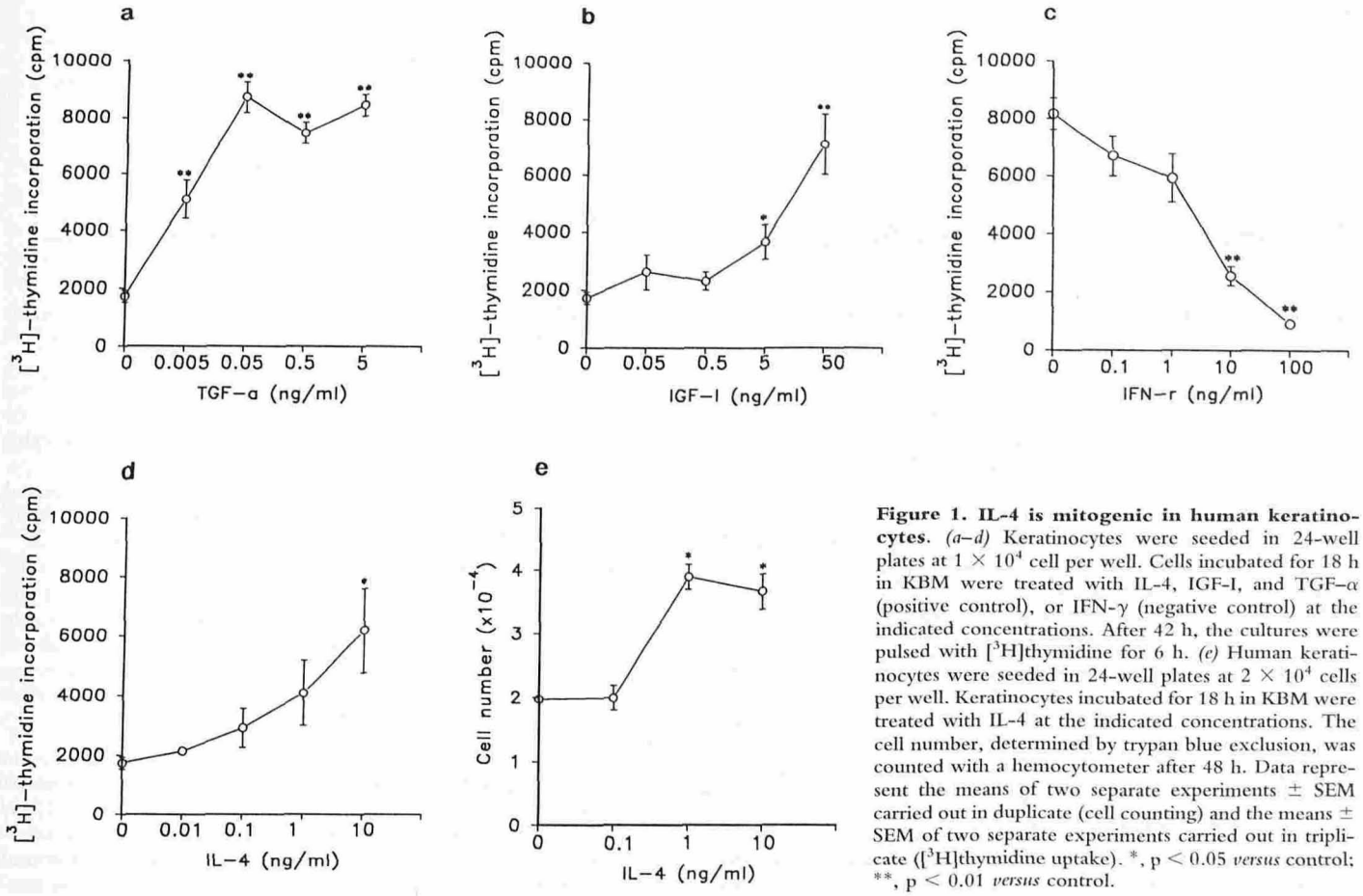

Figure 1. IL-4 is mitogenic in human keratinocytes. *(a–d)* Keratinocytes were seeded in 24-well plates at 1×10^4 cell per well. Cells incubated for 18 h in KBM were treated with IL-4, IGF-I, and TGF–α (positive control), or IFN-γ (negative control) at the indicated concentrations. After 42 h, the cultures were pulsed with [^{3}H]thymidine for 6 h. *(e)* Human keratinocytes were seeded in 24-well plates at 2×10^4 cells per well. Keratinocytes incubated for 18 h in KBM were treated with IL-4 at the indicated concentrations. The cell number, determined by trypan blue exclusion, was counted with a hemocytometer after 48 h. Data represent the means of two separate experiments ± SEM carried out in duplicate (cell counting) and the means ± SEM of two separate experiments carried out in triplicate ([^{3}H]thymidine uptake). *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control.

through the effect of IL-4 alone, not IL-6 production induced by IL-4, suggesting the existence of an IL-4–mediated paracrine growth loop in human keratinocytes.

IL-4 Promotes Cell Cycle from G_0/G_1 to S Phase Concentration-dependent stimulation of the growth of normal human keratinocytes by IL-4 in a 48-h proliferation assay and the sequential expression of c-*myc* following exposure of cells to IL-4 (see below, **Fig 4*a***) indicate IL-4 is a positive regulator of cell cycle progression and growth of these cells. To directly assess the effect of IL-4 on cell cycle progression, keratinocytes were precultured in basal medium for 18 h, then treated with 1 ng IL-4 per ml and incubated for the indicated times (0, 12, 24, or 48 h) before DNA distribution analysis by flow cytometry. As shown in **Fig 3**, a markedly increased S-phase population (compared to the untreated control cultures) was observed when the cells were treated with IL-4, while the G_0/G_1 phase of population was correspondingly decreased. These results indicate that IL-4 activation was responsible for progression from G_0/G_1 to S phase.

c-*myc* Gene Transcription Is Induced by IL-4 To better understand the mechanism by which IL-4 stimulated keratinocyte growth and cell cycle progression, we investigated the level of transcripts for several factors such as c-*fos*, c-*jun*, and c-*myc*, which are known to regulate cell cycle progression from G_0/G_1 to S phase. Expression of c-*myc* increased in the treated cells in a time-dependent manner after treatment with IL-4 at a concentration of 1 ng per ml **(Fig 4*a*)**, as early as 30 min after stimulation, and

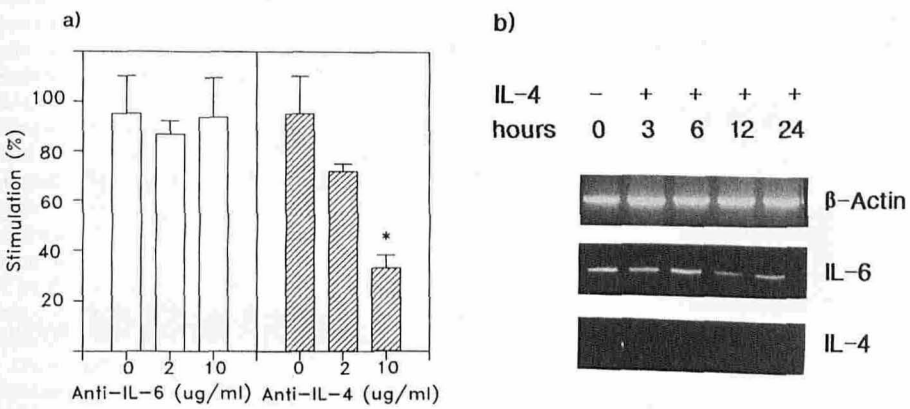

Figure 2. IL-4 directly stimulates keratinocyte proliferation. *(a)* Effect of mAbs to IL-4 and IL-6 on IL-4-induced proliferation. Cells were treated with IL-4 (2.5 ng per ml) that was preincubated in the presence of various concentrations of IL-4 and IL-6 mAbs (0, 2, or 10 μg per ml) for 1 h. *Bars* represent mean percentages of [^{3}H]thymidine uptake induced by IL-4 and the means ± SEM of three separate experiments carried out in triplicate. *(b)* Effect of IL-4 on IL-4 and IL-6 mRNA expression. Cells incubated for 18 h in KBM were cultured in the presence of IL-4 (2.5 ng per ml) for the indicated times. Total cellular RNA was isolated, and reverse transcriptase-PCR was carried out for 40 cycles as described in *Materials and Methods*. *, $p < 0.05$ *versus* control.

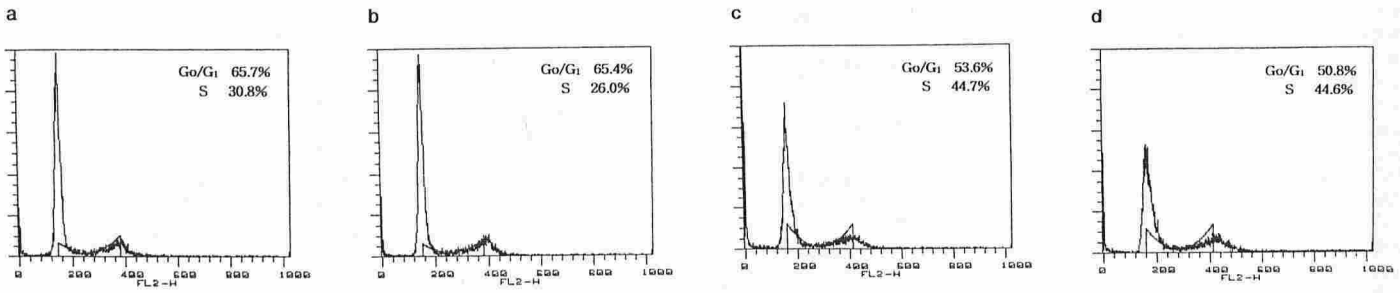

Figure 3. IL-4 triggers promotion of G_0/G_1 to S phase. Cells incubated for 18 h in KBM were treated with or without IL-4 (1 ng per ml) and further cultured for 0 *(a)*, 12 *(b)*, 24 *(c)*, or 48 h *(d)*. Cells were harvested at the indicated times, fixed with ethanol, incubated with PI solution, and analyzed by flow cytometry. Percentages of cells in G_0/G_1 and S phases of the cell cycle are indicated in each panel.

maximum expression of c-*myc* was observed approximately 6 h after IL-4 treatment (data not shown). Although there was no significant difference in c-*myc* mRNA expression throughout the time course (1–24 h), a decline in c-*myc* mRNA expression was observed after 24 h treatment **(Fig 4*a*)**. To determine how the induction of c-*myc* mRNA expression was regulated, keratinocytes were treated either with IL-4 alone or with IL-4 together with actinomycin D or cycloheximide. As shown in **Fig 4*b***, the treatment with actinomycin D (5 μg per ml) completely eliminated detectable c-*myc* mRNA in both control and IL-4–treated cultures. In addition, when the cells were treated with cycloheximide (20 μg per ml), IL-4–induced upregulation of c-*myc* mRNA expression was slightly decreased. These observations suggested not only that IL-4 regulation of c-*myc* expression occurs at the transcriptional or post-transcriptional levels, but that this induction requires, at least partially, the participation of *de novo* protein synthesis.

Genistein Inhibits the Induction of c-*myc* mRNA and Proliferation Induced by IL-4 Because IL-4 is known to induce tyrosine phosphorylation, we analyzed the possible involvement of tyrosine kinases in c-*myc* mRNA expression and cell proliferation. After treatment of cultured keratinocytes with IL-4 (5 ng per ml) and genistein (15 μM), we observed a marked reduction in c-*myc* mRNA expression **(Fig 5*a*)**. In contrast, H7 (20 μM), a serine/threonine kinase inhibitor, did not influence expression of c-*myc* mRNA in the presence of IL-4 (5 ng per ml). Okadaic acid (50 ng per ml), however, slightly increased c-*myc* mRNA induction by IL-4. These findings suggest that IL-4–induced c-*myc* mRNA expression is mediated by tyrosine kinase. In addition, as demonstrated in **Fig 5*a***, neither basal nor IL-4–induced levels of c-*myc* mRNA expression were altered by H7 and okadaic acid treatment, but a comparison of the c-*myc* mRNA levels in control (**Fig 5*a***, *lane 1*) and genistein-treated cells (**Fig 5*a***, *lane 5*) reveals that genistein downregulated the basal levels of c-*myc* mRNA, suggesting that the constitutive c-*myc* mRNA expression in cultured keratinocytes might be specifically regulated by tyrosine kinase.

To assess whether inhibition of c-*myc* by genistein was reflected in cell proliferation, cultured keratinocytes were treated with 5 ng IL-4 per ml in the presence of genistein ranging from 10^{-8} to 10^{-4} M. As shown in **Fig 5*b***, [^{3}H]thymidine incorporation induced by IL-4 was blocked in a concentration-dependent manner by genistein. Taken together, these data suggest that genistein is able to inhibit IL-4–induced c-*myc* mRNA expression and cell proliferation in human keratinocytes.

DISCUSSION

In addition to the possible involvement of tyrosine kinases in IL-4–induced proliferation, we show that IL-4 induces the growth of normal human keratinocytes, c-*myc* gene expression, and G_0/G_1 to S phase progression. Compared with potent keratinocyte mitogens such as IGF-I and TGF-α, IL-4 showed a less profound growth-promoting effect, but IL-4–induced proliferation was consistently observed from all donors tested. The IL-4–induced proliferation was not mediated by production of IL-6, which was shown to induce the proliferation of keratinocytes **(Fig 2*a,b*)**. Recently, Derocq *et al* (1994) reported that IL-4 stimulates IL-6 production in keratinocytes, but we did not detect IL-4–mediated IL-6 production. This discrepancy of IL-4 responsiveness in keratinocytes may be explained by the difference in culture conditions, especially in the culture medium.

Recent studies demonstrated that c-*myc* gene expression is necessary for proliferation of the murine keratinocytes (Pietenpol *et al*, 1990) and other mammalian cells (Heikkila *et al*, 1987; Goodrich *et al*, 1991). Our studies clearly demonstrate that IL-4 induced c-*myc* mRNA expression in association with proliferation *in vitro*, in agreement with previous reports on the role of c-*myc* in G_0-G_1 cell cycle progression. The c-*fos* protooncogene has been shown to be

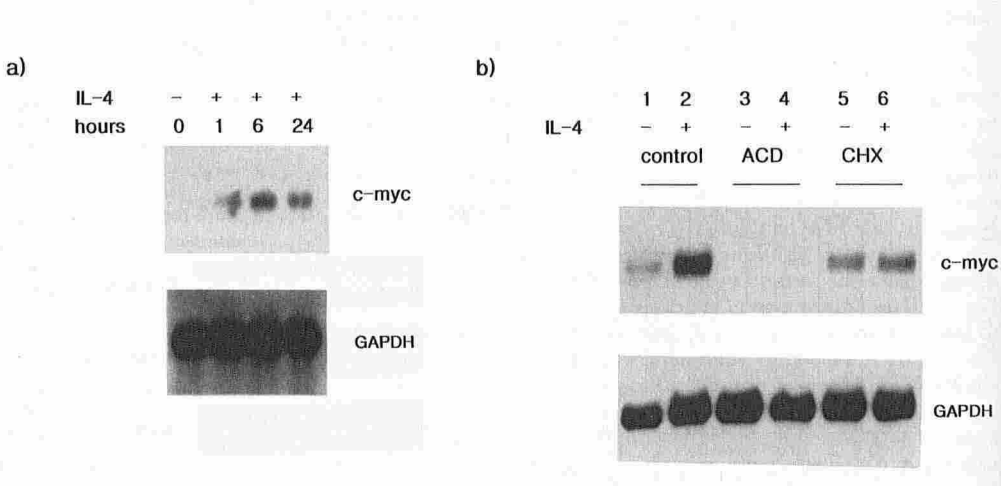

Figure 4. IL-4 induces c-*myc* mRNA expression. *(a)* Effect of IL-4 on c-*myc* mRNA expression. Keratinocytes were cultured for 18 h in KBM prior to stimulation with IL-4 (1 ng per ml) for the indicated times (0, 1, 6, or 24 h). *(b)* Effect of cycloheximide and actinomycin D on the induction of c-*myc* mRNA by IL-4. The cells were pre-treated for 30 min with cycloheximide (CHX, 20 μg per ml) or actinomycin D (ACD, 5 μg per ml) prior to stimulation with IL-4 (5 ng per ml) for 1 h. *Odd numbered lanes* represent the unstimulated cells, and *even numbered lanes* represent the IL-4–stimulated cells. Total RNA (approximately 30 μg/lane) was assayed by northern blot for hybridization to c-*myc* (2.2 kb) and glyceraldehyde-3-phosphate dehydrogenase (1.3 kb) cDNA probes.

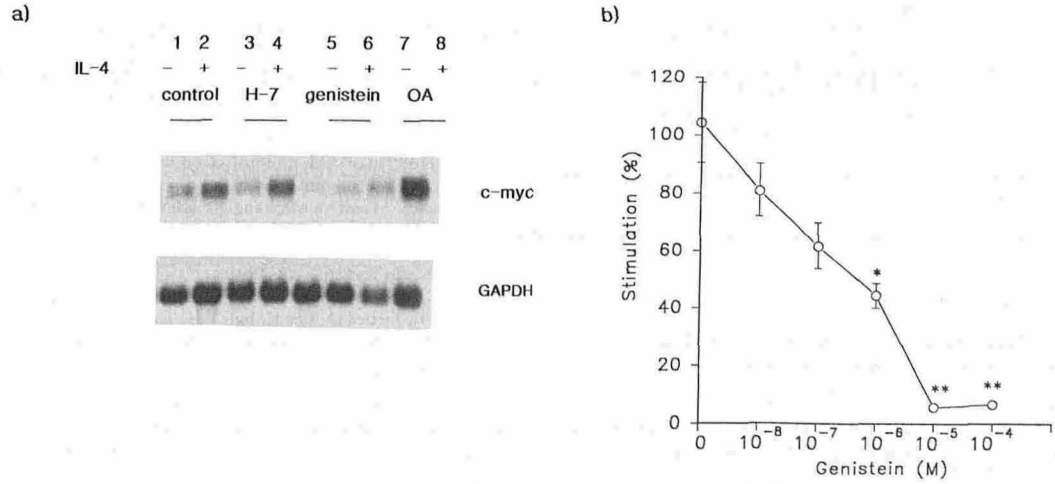

Figure 5. IL-4 induces c-*myc* mRNA expression and cell proliferation in a tyrosine kinase-dependent manner. *(a)* Inhibition of c-*myc* mRNA induction by genistein, a tyrosine kinase inhibitor. Human keratinocytes grown in KBM for 18 h were incubated in the presence of 20 μM H7, 15 μM genistein (Gen), or 50 ng okadaic acid (OA) per ml for 30 min prior to stimulation with either medium alone or medium with IL-4 (5 ng per ml) for 1 h. *Odd numbered lanes* show the unstimulated cells, and *even numbered lanes* show IL-4–stimulated cells. Even loading and integrity of RNA per lane was confirmed by ultraviolet visualization of ethidium-bromide staining and hybridization with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA (*lower panel*). Total cellular RNA was isolated and examined by northern blot analysis as described in *Materials and Methods*. *(b)* Suppression of the IL-4–induced proliferative response by genistein. The cells were incubated as in panel A and treated with 5 ng IL-4 per ml in the presence or absence of genistein from 10^{-8} to 10^{-4} M. Each point indicates the means ± SEM of two separate experiments carried out in triplicate, and the results are presented as the percentages of [^{3}H]thymidine uptake induced by IL-4 alone. *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control.

highly expressed in human epidermal cells (Basset-Seguin *et al*, 1990). In murine keratinocytes, it has been suggested that c-*fos* might be involved in the induction of cornification, a characteristic feature of squamous cell differentiation (Fisher *et al*, 1991; Basset-Seguin *et al*, 1994). In our experiments, expression of c-*fos* was not detected even after longer exposure, whereas c-Jun was more readily detectable without induction throughout IL-4 treatment for 2 h (data not shown). It seems that differential regulation of c-*fos* and c-Jun gene expression may occur in IL-4–treated human keratinocytes, raising the possibility that c-*myc* gene expression may be the primary regulator of keratinocyte growth. We cannot exclude other possibilities, however, such as the involvement of cyclin-dependent tyrosine kinases and cyclin D1.

Previous studies showed that the activation of tyrosine kinases plays a pivotal role in mediating signal transduction through a variety of cytokine receptors. Recently it was demonstrated that IL-4 induces tyrosine phosphorylation of several substrates in various cells (Keegan *et al*, 1994) and is a unique growth-stimulating cytokine in that it does not employ cellular p21^{ras}-guanosine triphosphate complexes or mitogen-activated protein kinase (Satoh *et al*, 1991; Welham *et al*, 1992). Despite extensive efforts over the last few years, signal transduction induced by IL-4 remains poorly understood and somewhat controversial (Mizuguchi *et al*, 1986; Chaikin *et al*, 1990; Finney *et al*, 1990). It is tempting to speculate that the tyrosine kinases may be involved in proliferation and c-*myc* gene expression in keratinocytes in response to IL-4. Although genistein, a tyrosine kinase inhibitor, nearly completely prevented IL-4–induced c-*myc* mRNA expression as well as growth of the cultured keratinocytes, the increase in c-*myc* gene expression by IL-4 was not reduced by either H7, a Ser/Thr kinase inhibitor, or okadaic acid, a protein phosphatase inhibitor **(Fig 5*a*)**. Thus, our studies provide evidence that activation of the tyrosine kinase pathway in keratinocytes can lead to the generation of signals for induction of c-*myc* mRNA and eventually cause the enhancement of proliferation. Nevertheless, we cannot rule out the possibility that other signal pathways are involved in transducing proliferation-related signals by IL-4 in keratinocytes.

Our studies provide evidence that the proliferation of human keratinocytes induced by IL-4 may relate in a direct or indirect manner to c-*myc* gene expression. Moreover, it seems reasonable to speculate that the putative tyrosine kinase activation may lead to growth stimulation induced by IL-4, possibly through c-*myc* gene expression, but it still remains to be elucidated whether human keratinocytes utilize the same or different signal transduction pathways in the process of proliferation and differentiation in response to different stimuli.

We thank Dr. Peter D. Burrows, Division of Clinical and Developmental Immunology, University of Alabama at Birmingham, for helpful discussions and critical review of the manuscript; and Dr. Hueng Sik Choi, Min Chan Gil, and Yoo Mi Choi for manuscript preparation. This work was partially supported by Grants N81330 and E72010 from the Ministry of Science and Technology and Korea Research Institute of Bioscience and Biotechnology. Dr. S. M. Byun was partially supported by a grant from Research Center for New Bio-Materials in Agriculture, Republic of Korea.

REFERENCES

Basset-Seguin N, Escot C, Blanchard JM, Kerai C, Verrier B, Mion H, Guilhou JJ: High levels of c-fos proto-oncogene expression in normal human adult skin. *J Invest Dermatol* 94:418–422, 1990

Basset-Seguin N, Demoly P, Moles JP, Tesnieres A, Gauthier Rouviere C, Richard S, Blanchard JM, Guilhou JJ: Comparative analysis of cellular and tissular expression of c-fos in human keratinocytes: evidence of its role in cell differentiation. *Oncogene* 9:765–771, 1994

Butch AW, Chung GH, Hoffmann JW, Nahm MH: Cytokine expression by germinal center cells. *J Immunol* 150:39–47, 1993

Chaikin E, Ziltener HJ, Razin E: Protein kinase C plays an inhibitory role in interleukin 3- and interleukin 4-mediated mast cell proliferation. *J Biol Chem* 265:22109–22116, 1990

Derocq JM, Segui M, Poinot Chazel C, Minty A, Caput D, Ferrara P, Casellas P: Interleukin-13 stimulates interleukin-6 production by human keratinocytes Similarity with interleukin-4. *FEBS Lett* 343:32–36, 1994

Eilers M, Schirm S, Bishop JM: The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J* 10:133–141, 1991

Feghali CA, Bost KL, Boulware DW, Levy LS: Human recombinant interleukin-4 induces proliferation and interleukin-6 production by cultured human skin fibroblasts. *Clin Immunol Immunopathol* 63:182–187, 1992

Fertin C, Nicolas JF, Gillery P, Kalis B, Banchereau J, Maquart FX: Interleukin-4 stimulates collagen synthesis by normal and scleroderma fibroblasts in dermal equivalents. *Cell Mol Biol* 37:823–829, 1991

Finney M, Guy GR, Michell RH, Gordon J, Dugas B, Rigley KP, Callard RE: Interleukin 4 activates human B lymphocytes via transient inositol lipid hydrolysis

and delayed cyclic adenosine monophosphate generation. *Eur J Immunol* 20:151–156, 1990

Fisher C, Byers MR, Iadarola MJ, Powers EA: Patterns of epithelial expression of Fos protein suggest important role in the transition from viable to cornified cell during keratinization. *Development* 111:253–258, 1991

Goodrich DW, Wang NP, Qian YW, Lee EY, Lee WH: The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* 67:293–302, 1991

Ha HJ, Kubagawa H, Burrows PD: Molecular cloning and expression pattern of a human gene homologous to the murine mb-1 gene. *J Immunol* 148:1526–1531, 1992

Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R, Neckers LM: A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature* 328:445–449, 1987

Howells G, Pham P, Taylor D, Foxwell B, Feldmann M: Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-gamma. *Eur J Immunol* 21:97–101, 1991

Keegan AD, Nelms K, Wang LM, Pierce JH, Paul WE: Interleukin 4 receptor: signaling mechanisms. *Immunol Today* 15:423–432, 1994

Luscher B, Eisenman RN: New light on Myc and Myb Part I. Myc. *Genes Dev* 4:2025–2035, 1990

Mizuguchi J, Beaven MA, Ohara J, Paul WE: BSF-1 action on resting B cells does not require elevation of inositol phospholipid metabolism or increased $[Ca^{2+}]_i$. *J Immunol* 137:2215–2219, 1986

Pietenpol JA, Holt JT, Stein RW, Moses HL: Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. *Proc Natl Acad Sci USA* 87:3758–3762, 1990

Satoh T, Nakafuku M, Miyajima A, Kaziro Y: Involvement of ras p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor, but not from interleukin 4. *Proc Natl Acad Sci USA* 88:3314–3318, 1991

Saunders NA, Jetten AM: Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon gamma: antagonism by retinoic acid and transforming growth factor beta 1. *J Biol Chem* 269:2016–2022, 1994

Spencer CA, Groudine M: Control of c-myc regulation in normal and neoplastic cells. *Adv Cancer Res* 56:1–48, 1991

Spits H: *IL-4: Structure and Function*. CRC Press, Boca Raton, FL, 1992

Turka LA, Ledbetter JA, Lee K, June CH, Thompson CB: CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J Immunol* 144:1646–1653, 1990

Tushinski RJ, McAlister IB, Williams DE, Namen AE: The effects of interleukin 7 (IL-7) on human bone marrow *in vitro*. *Exp Hematol* 19:749–754, 1991

Welham MJ, Duronio V, Sanghera JS, Pelech SL, Schrader JW: Multiple hemopoietic growth factors stimulate activation of mitogen-activated protein kinase family members. *J Immunol* 149:1683–1693, 1992