

The Expression of Toll-Like Receptors (TLRs) in Cultured Human Skin Fibroblast is Modulated by Histamine

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Fibroblasts are responsible for the synthesis and degradation of various connective tissue components and soluble mediators of extracellular matrix metabolism. Few studies have been conducted concerning the expression of toll-like receptors (TLRs) in fibroblasts until now. This study aimed first to determine the quantitative expression of TLRs 1 to 10 in human skin fibroblasts and secondarily to explore any influence of expression by histamine, which is a well-known factor engaged in dermal inflammation. It was found that all 10 TLRs were expressed in fibroblasts. Interestingly, the expression of TLRs 4, 5, and 10 was increased after 2 and 6 hours of histamine treatment during culture. However, the expression of TLRs 2, 3, 6, 7, 8, and 9 was decreased after 6 hours of histamine treatment. Among the TLRs with a decreasing expression pattern, TLRs 7 and 8 showed a persistent tendency to decrease. All of these changes in TLR expression with histamine treatment were antagonized by treatment with diphenhydramine, a well-known antihistamine. Thus, these results suggest a role of histamine in the early phase of the dermal inflammatory reaction mediated by TLRs.

Key Words: *Skin; Toll-like receptor; Fibroblast; Histamine*

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INTRODUCTION

Fibroblasts maintain a key role in extracellular matrix metabolism, and their diverse cellular interactions in the dermal inflammatory reaction are important in various cutaneous disorders. Fibroblasts are essential in producing many connective tissue matrix proteins (collagen, mucopolysaccharides, etc) as well as in the secretion of various soluble factors (cytokines, growth factors, etc). In the skin, dermal fibroblasts are known to process pathologic fibrous changes via unknown pathways when inflammation is overexpressed in some conditions.¹

Histamine is also a well-known mediator of allergic inflammation in which it performs various functions including vascular dilation, activation of inflammatory cells, gastric secretion regulation, and nerve signal transmission. Four types of histamine receptors have been discovered (H1, H2, H3, H4), all of which are in the G-protein-coupled receptor family.² Many dermal inflammatory disorders are related to the interaction between fibroblasts and histamine, yet the exact mechanism of this relation is not fully

explained.

Toll-like receptors (TLRs) are pattern recognition receptors that were first recognized as a functional component of the innate immune system. They interact with various endogenous and exogenous ligands and antigens. Also, TLRs influence acquired immune responses as well as various intracellular signaling processes.³ Until now, a total of 11 TLRs have been discovered, and research is ongoing on the ligands and some functional interactions of these receptors. Only a few studies on TLRs in fibroblasts have been presented in the literature, and those studies were mainly focused on certain groups of TLRs such as TLRs 2 and 4 and their co-expression pattern with keratinocyte.

Previous studies that focused on the role of TLRs 2 and 4 reported an enhanced expression of TLRs by histamine treatment, which resulted in stronger inflammatory reactions.⁴⁻⁶ Moreover, Proost et al.⁷ observed Th1 cytokine profile increment when TLRs 2, 3, 4, 5, and 9 were activated in human fibroblasts. These previous studies suggest a potential role of TLRs in fibroblasts under diverse inflammatory reactions.

TABLE 1. TLR primers for real-time RT-PCR in cultured human skin fibroblast

Gene	Forward sequence (5'-3') Reverse sequence (5'-3')	Size (bp)	PMID
TLR1	CAGAGTGAATGGTGCCATTATGAAC CCTTGGGCCATTCCAAATAAGTC	175	NM_003263
TLR2	GAAAGCTCCCAGCAGGAACATC GAATGAAGTCCCGCTTATGAAGACA	146	NM_003264
TLR3	GGACTTTGAGGCGGGTGT TGTTGAACTGCATGATGTACCTTGA	141	NM_003265
TLR4	TTGAGCAGGTCTAGGGTGATTGAAC ATGCGGGACACACACTTTCAAATA	143	NM_138554
TLR5	AGAGATGGCTGGTGCCTTGAA CATCAACTGGTACTGGGACAAGGA	114	NM_003268
TLR6	CCTGGCAAGAGCATTGTGGA TGCGTAATGGCACCACACTCACTCTG	107	NM_006068
TLR7	TCAAGCCTTAGATTGGCGATGTC CCAGAGATTGATTTGGGAATTTGTG	144	NM_016562
TLR8	CCTCATGCAGAGCATCAACCA ACATCCATGTTCTCATCCATTAGCC	129	NM_016610
TLR9	AGTCCTCGACCTGGCAGGAA GCGTTGGCGCTAAGGTTGA	168	NM_017442
TLR10	TTGCATTCCCACCAGGTATCATAA AGCTCGAAGGTTTGCCAGA	112	NM_001017
GAPDH	CATGACCACAGTCCATGCCATCACT TGAGGTCCACCACCTGTTGCTGTA	137	NM_002191

The purpose of the present study was to detect whether TLR expression in skin fibroblasts is detectable and then to see how TLR expression could be modulated by histamine, because histamine-associated chronic inflammatory reactions in the dermis have been seen in fibroblast-associated disorders such as scleroderma and keloid.

MATERIALS AND METHODS

1. Cell culture

Fibroblasts were collected from a 12-year-old boy during circumcision. The collected cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) with 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) and 10% fetal bovine serum (Hyclone, Logan UT, USA). An approximate amount of 1×10^5 cells was initially injected in a petri dish containing 10 ml of culture medium. The cells were cultured in a humidified incubator (MCO-18AIC, Sanyo, Sakata, Japan) with 5% CO₂ and at 37°C. Cells were subcultured when they reached 80% confluence. Subculture was done by the following procedures. The culture medium was removed from the dish and the cells were washed with phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA). PBS was removed and the plate was placed in the incubator with 2 ml of trypsin (Hyclone, Logan, UT, USA). After 2 minutes, trypsin was deactivated with 6 ml of horse serum (Hyclone, Logan, UT, USA) and all the contents were centrifuged at 800 rpm for 3 minutes. After removing the supernatants, 30 ml of culture medium was added and the samples were stirred

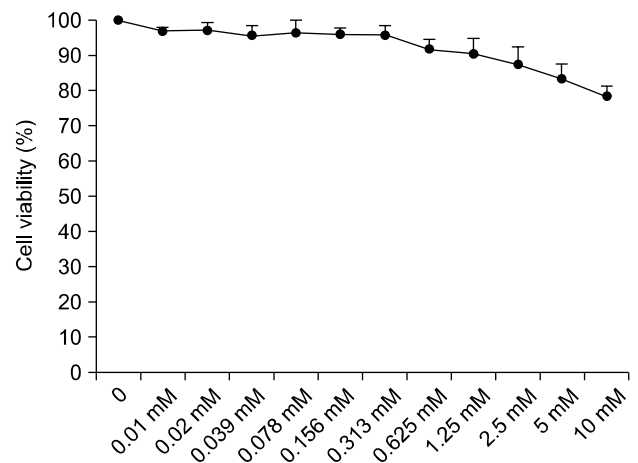


FIG. 1. MTT assay with various concentration of histamine was performed in fibroblast. No significant cytotoxicity was found below 0.313 mM.

well before being divided into three petri dishes. The cells were used in experiments when they reached 90% confluence at cell passages between 4 and 5.

2. Histamine treatment

Before treatment, cells were divided into a 6-well plate with approximately 5×10^5 cells per well. The MTT assay was carried out before treatment to check for cytotoxicity of histamine (Sigma Chemical Co., St. Louis, MO, USA). Histamine was treated for 1, 2, 3, and 6 hours at a concentration of 10 μ M. The cells were harvested with EDTA

(Hyclone, Logan, UT, USA) and trypsin to analyze TLR gene expression. A separate experiment was done to see the antagonism of antihistamine by using fibroblasts that were pre-treated with 10 μ M diphenhydramine (DPH, Sigma Chemical Co., St. Louis, MO, USA) for 1 hour. These cells were then further treated with 10 μ M histamine for 1, 2, and 6 hours.

3. Real-time PCR for TLRs

TriReagent (Molecular Research Center Inc., Cincinnati, OH, USA) was used to extract total RNA from experimental cells. Approximately 1 μ g of total RNA per group was treated with M-MLV (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions to synthesize cDNA. cDNA was quantified by using Qubit™

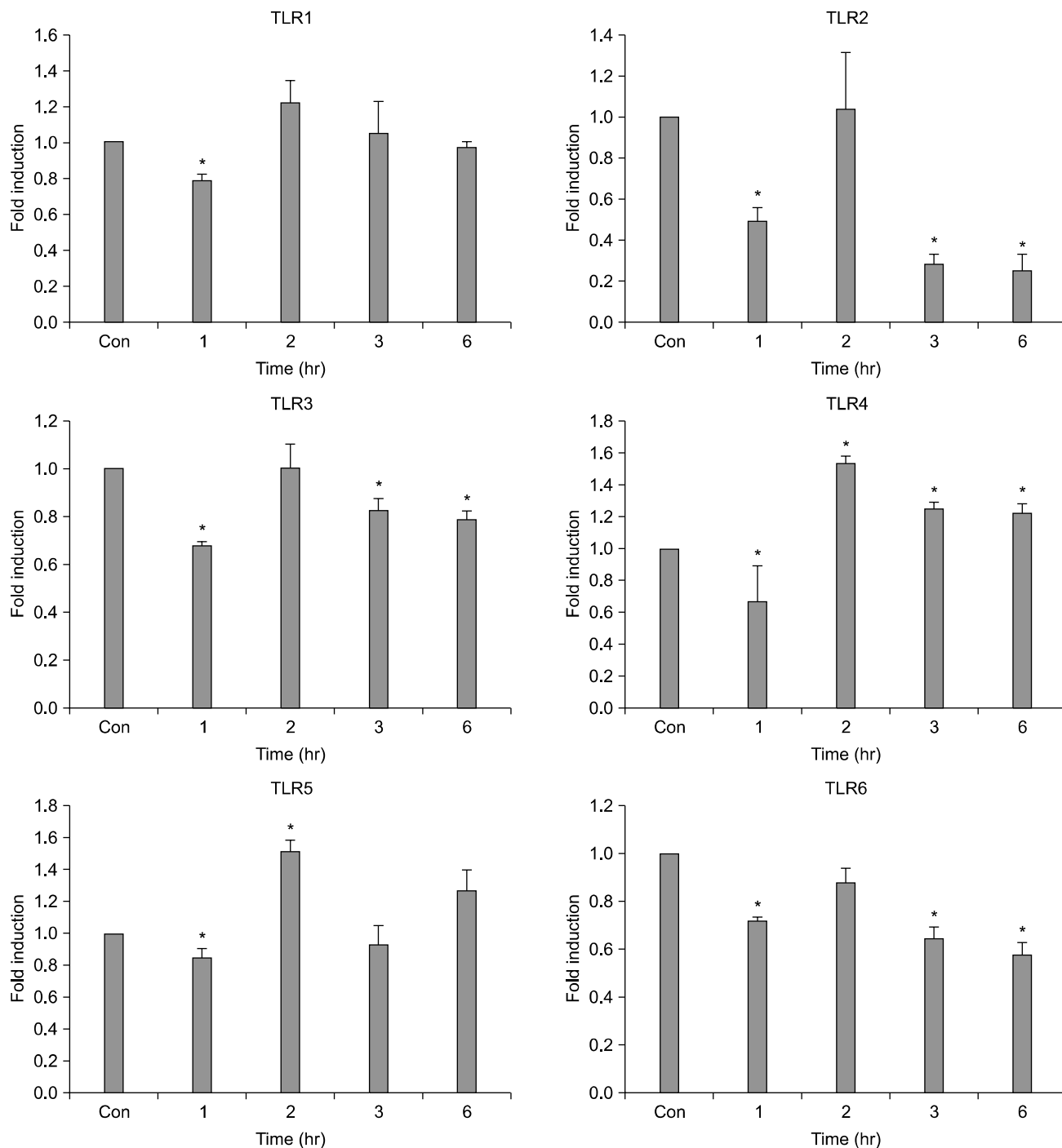


FIG. 2. Expression of toll like receptor (TLR) mRNA during the time course under histamine treatment. TLRs 4, 5 and 10 expressions were increased significantly after 2 hours of histamine treatment. TLR 2, 6, 7, 8 and 9 mRNA expressions were decreased comparing with control. TLR 7 and 8 showed continuous decrement regardless of treatment duration (Con: control. *p < 0.05, SEM as shown in error bar).

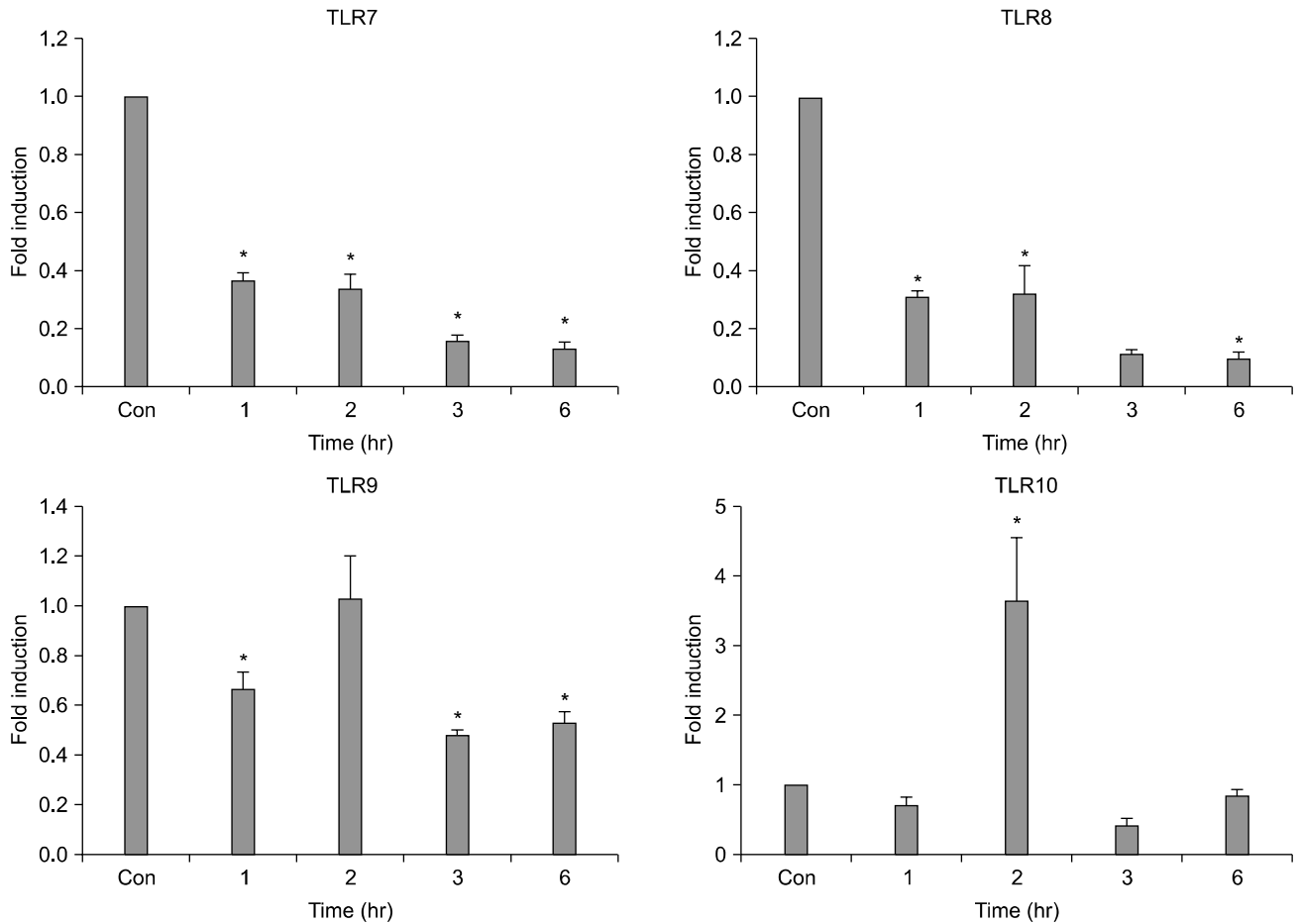


FIG. 2. Continued.

(Invitrogen, Carlsbad, CA, USA) and 15 pg of cDNA entered 50 cycles of real-time PCR (Takara Bio Inc., Shiga, Japan) with SYBR Green (Takara Bio Inc., Shiga, Japan) tags. TLR primers (Bioneer Co., Daejon, Korea) were specified by using basic BLAST (Basic logical alignment search tool) tools and the standard material was GAPDH (Bioneer Co., Daejon, Korea) (Table 1).

4. Statistical analysis

Data obtained from triplicate sets of experiments were analyzed under paired-t tests between control and experimental groups by using SPSS (SPSS Inc., Chicago, IL, USA). A p value derived from the average comparison less than 0.05 was regarded as being statistically significant.

RESULTS

1. Effect of histamine on cell viability

Cell viability tended to decrease as the histamine concentration was gradually increased. However, there was no significant cell toxicity under the concentration level of 0.313 mM (Fig. 1).

2. TLR mRNA expression in human dermal fibroblasts and effects of histamine treatment

TLRs 1 to 10 were all expressed in human dermal fibroblasts, and the level of expression did not differ significantly. The expression of each TLR was measured as a ratio of the standard expression of GAPDH via real-time PCR. The expression of all TLRs except TLRs 7 and 8 reached a peak value after 2 hours of histamine treatment and then showed a tendency to decrease. Among the increased TLRs, TLR 4 was the only receptor with a significant increase even after 6 hours of treatment, but TLRs 5 and 10 showed statistically significant increases only after 2 hours of treatment. The expression of TLRs 2, 3, 6, 7, 8, and 9 decreased significantly compared with the control after 6 hours of treatment. Among the TLRs showing decreased expression, TLRs 7 and 8 showed a continuously significant decreasing pattern throughout the treatment period (Fig. 2).

3. Changes in TLR mRNA expression with diphenhydramine

The mRNA expression of TLRs 3, 4, 5, and 8 of DPH-treated fibroblasts decreased instantly with statistical significance. The increments of TLRs 4, 5, and 10 were abro-

gated, but the changes in TLRs 2, 6, 7, 8, and 9 were insignificant compared with the results for treatment with the same concentration of histamine in the previous experiment. Interestingly, the expression of TLRs 3 and 10 was decreased compared with the control after alternative histamine treatment of 6 hours (Fig. 3).

DISCUSSION

Fibroblasts are the most abundant cells forming connective tissue and are known to interact with various proteins and mediators from surrounding tissues. Abnormal metabolic stimulation of the formation of extracellular ma-

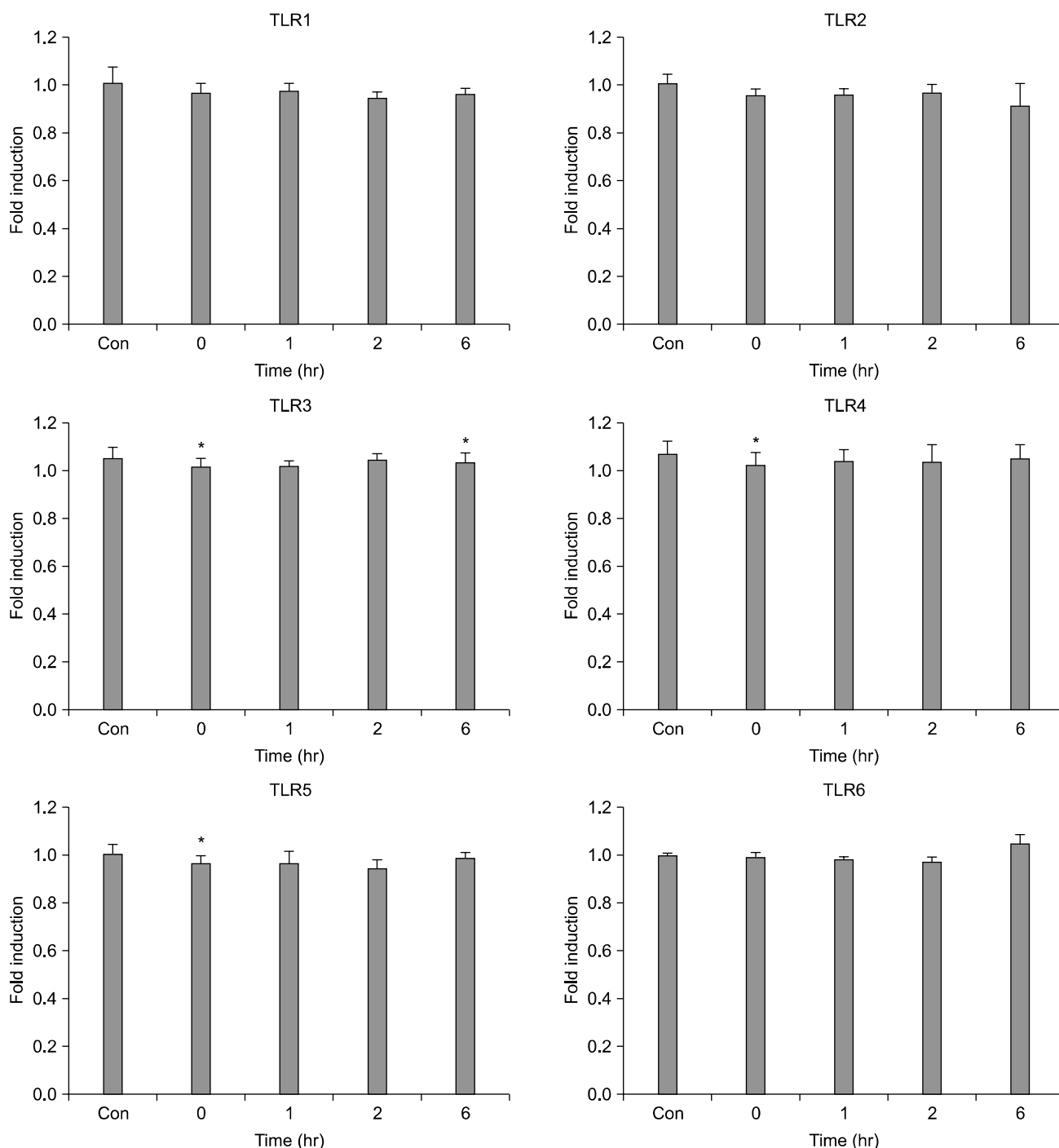


FIG. 3. TLR mRNA expression of diphenhydramine (DPH) pre-treated fibroblasts after histamine treatment. Fibroblast pre-treated with 10 μ M diphenhydramine expressed slightly decreased TLR mRNAs compared to control group. Among them TLR 3, 4, 5 showed statistically significant decrease expression (Con: control, 0: treated with diphenhydramine for 1 hour, 1: treated with diphenhydramine for 1 hour and with histamine for 1 hour afterwards, 2: treated with diphenhydramine for 1 hour and 2 hours with histamine, 6: treated with diphenhydramine for 1 hour and 6 hours with histamine. * $p < 0.05$, SEM as shown in error bar).

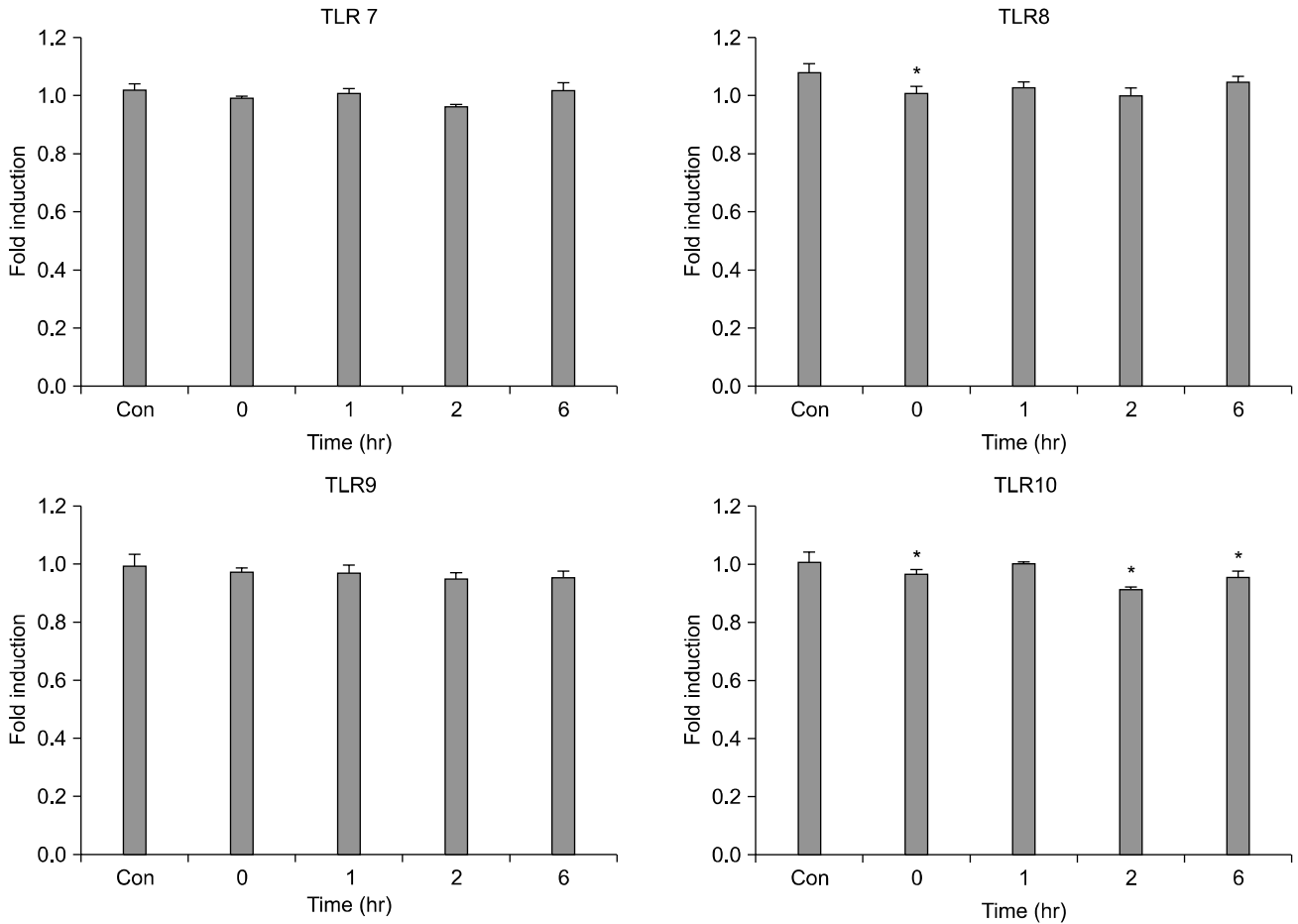


FIG. 3. Continued.

trix in the skin causes keloid or hypertrophic scars in which the over-activated chronic inflammatory process is involved.^{8,9} The signaling pathway of abnormal fibrosis caused by cellular proliferation and inflammation is now being studied from a different point of view, and recent reports have shown some proof that TLR activation can increase inflammatory cytokines in fibroblasts.⁷

It was shown in the present study by real-time PCR that the mRNAs of all 10 TLRs were well expressed in human dermal fibroblasts even though some results were rather inconsistent and the pattern was insufficient to verify the detail of activity of each receptor. However, the present data are enough to suggest that TLR research in fibroblasts could give us a clue to understanding the dermal inflammatory reaction, which seems to be closely related to TLR function. Histamine has been widely studied as an inflammatory mediator that acts in vascular dilation, gastric acid secretion, and neuronal signal transmitting.^{10,11} It is also known to control immune cells such as mononuclear cells, macrophages, T-cells, neutrophils, and endothelial cells related to inflammation.¹²⁻¹⁸ Besides the above known findings for histamine, a new functional class of its kind is also being explored. Recent study has shown an interesting role of histamine in enhancing the sensitivity and activity

of TLRs via H1 receptor activation. Talreja et al.⁴ treated human umbilical endothelial cells with histamine and lipopolysaccharide (LPS) in which increased TLR 2 and 4 expression and secretion of IL-6 were found compared with LPS treatment alone. Other experiments that were performed with exposure to peptidoglycan and lipoteichoic acid showed similar results. The effect of histamine was detected from 0.1 μ M and was strongest at a concentration of 10 μ M. This enhanced expression of TLRs 2 and 4 was abrogated by 10 μ M DPH but was not affected by H2 receptor antagonist, thus demonstrating the exclusive role of H1 receptor activation. The expression of TLR mRNA was most increased after 2 hours of treatment, and protein expression was highest after 16 hours.⁴ Hou et al.⁵ treated A549 and H292 cells with 1, 10, or 100 μ M histamine for 2, 12, and 24 hours. This study reported a histamine-dependent increase of TLR 3 mRNA expression that was antagonized by DPH treatment.⁵ Another study conducted by Kobayashi et al.⁶ reported an increase in TLR 2 receptor and cytokine secretion with 1 μ g/ml histamine treatment.

In the present study, we first focused on the expression of TLRs in fibroblasts from human skin. All TLRs seem to be well expressed. Next, we explored whether the TLR expression was influenced by histamine treatment and found

increases in the mRNA expression of TLRs 4, 5, and 10 in contrast with decreases in the expression of TLRs 2, 3, 6, 7, 8, and 9 after treatment with 10 μ M histamine. The increment of TLR 4 was similar to results from other studies, which suggests that TLR 4 responds to histamine similarly even in different cells. However, the mRNA expression of TLRs 7 and 8 was significantly decreased by histamine in this experiment. Known ligands of TLRs 7 and 8 are imidazoquinoline and virus-oriented ssRNA. Thus, we can speculate that this finding may be an explanation for the common clinical finding that patients with allergic disease are frequently more susceptible to low-infectivity viral infections than are normal patients. Indeed, there is a report concerning the increase of histamine-releasing factor along with allergic reaction mediators as a result of ssRNA virus treatment on low-airway endothelial cells.¹⁹ All these findings suggest the possibility of a negative feedback between TLRs 7 and 8 and tissue histamine concentrations, which may be a useful means of approach to prevent excessive allergic reactions.

Another interesting aspect of this study was the increasing pattern of TLR 4 mRNA expression with exposure time of histamine. This increased pattern was sustained for more than 6 hours of treatment, which was similar to TLRs 7 and 8 but in the opposite direction. According to Petricevic et al.²⁰ TLR 4 expression decreases in response to TLRs 7 and 8 ligand-activating treatment. Collectively, these results suggest a complex interaction between these ligands. It is well known that TLRs 4, 7, and 8 are essential in pathogenic antigen recognition in the response to bacterial and viral antigens, respectively, which have some common characteristics and differences. Their ligand-binding activation sites in the cell are different: TLR 4 is located on the cell membrane but TLRs 7 and 8 are located in the cytoplasm. TLR 4 ligand activation shares the MyD88 signaling pathway with TLRs 7 and 8 but it uses an additional TRIF pathway that is MyD88 independent. Because TLRs 4, 7, and 8 function as a gateway of pathogenic inflammation, excessive inflammatory reaction may happen in the case of synergistic infections. On the contrary, an inhibitory interaction between TLR 4 and TLRs 7 and 8 can reduce the severity of abnormal inflammatory activation, such as sepsis.

Thus, the present results suggest that an interesting interactive mechanism may exist in skin disorders in which TLRs 4, 7, and 8 are activated by histamine. However, it is also speculated that the histamine-induced TLR expression pattern and its kinetic interaction may be dependent on cell type or dominant inflammatory signals, because our data also show some inconsistent mRNA expression patterns. Thus, the post-translational process and protein expression during chronic inflammatory processes may vary if the histamine exposure pattern is modulated under specific conditions, as seen in previous reports.

It is well known that the chronic dermal inflammatory process in scleroderma and keloid results from the stimulation of fibroblasts and the overproduction of ex-

tracellular matrix such as collagen, in which histamine can act as an inflammatory signal. Our study indicated the complicated role of TLRs 4, 7, and 8 in dermal inflammatory conditions, and successive studies will re-evaluate these receptor-ligand interactions.

Another interesting finding in this experiment was the higher TLR 10 expression pattern in fibroblasts with histamine. Until now, the role of TLR 10 was not clear.

In conclusion, we found that human dermal fibroblasts express all TLRs 1 through 10, and among them, TLRs 4, 5, and 10 increase with histamine treatment but TLRs 2, 6, 7, 8, and 9 decrease. These effects were antagonized by DPH, which is known to inhibit the action of the H1 receptor. We believe that the functional interaction and signaling pathways of these TLR receptors in fibroblasts are worthy of being explored in various dermal inflammatory disorders.

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REFERENCES

- Hough LB. Genomics meets histamine receptors: new subtypes, new receptors. *Mol Pharmacol* 2001;59:415-9.
- Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci USA* 2002;99:12877-82.
- Kawai T, Akira S. TLR signaling. *Semin Immunol* 2007;19:24-32.
- Talreja J, Kabir MH, B Filla M, Stechschulte DJ, Dileepan KN. Histamine induces Toll-like receptor 2 and 4 expression in endothelial cells and enhances sensitivity to Gram-positive and Gram-negative bacterial cell wall components. *Immunology* 2004;113:224-33.
- Hou YF, Zhou YC, Zheng XX, Wang HY, Fu YL, Fang ZM, et al. Modulation of expression and function of Toll-like receptor 3 in A549 and H292 cells by histamine. *Mol Immunol* 2006;43:1982-92.
- Kobayashi M, Yoshiki R, Sakabe J, Kabashima K, Nakamura M, Tokura Y. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br J Dermatol* 2009;160:297-304.
- Proost P, Verpoest S, Van de Borne K, Schutysen E, Struyf S, Put W, et al. Synergistic induction of CXCL9 and CXCL11 by Toll-like receptor ligands and interferon-gamma in fibroblasts correlates with elevated levels of CXCR3 ligands in septic arthritis synovial fluids. *J Leukoc Biol* 2004;75:777-84.
- Kikuchi K, Kadono T, Takehara K. Effects of various growth factors and histamine on cultured keloid fibroblasts. *Dermatology* 1995;190:4-8.
- Falanga V, Soter NA, Altman RD, Kerdel FA. Elevated plasma histamine levels in systemic sclerosis (scleroderma). *Arch Dermatol* 1990;126:336-8.
- Barnes PJ. Histamine receptors in the lung. *Agents Actions Suppl* 1991;33:103-22.
- Hill SJ. Multiple histamine receptors: properties and functional

- characteristics. *Biochem Soc Trans* 1992;20:122-5.
12. Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J Immunol* 1998;161:2586-93.
 13. Clark RA, Gallin JI, Kaplan AP. The selective eosinophil chemotactic activity of histamine. *J Exp Med* 1975;142:1462-76.
 14. Raible DG, Schulman ES, DiMuzio J, Cardillo R, Post TJ. Mast cell mediators prostaglandin-D2 and histamine activate human eosinophils. *J Immunol* 1992;148:3536-42.
 15. Ogden BE, Hill HR. Histamine regulates lymphocyte mitogenic responses through activation of specific H1 and H2 histamine receptors. *Immunology* 1980;41:107-14.
 16. Beer DJ, Matloff SM, Rocklin RE. The influence of histamine on immune and inflammatory responses. *Adv Immunol* 1984;35:209-68.
 17. Li Y, Chi L, Stechschulte DJ, Dileepan KN. Histamine-induced production of interleukin-6 and interleukin-8 by human coronary artery endothelial cells is enhanced by endotoxin and tumor necrosis factor-alpha. *Microvasc Res* 2001;61:253-62.
 18. Ikawa Y, Shiba K, Ohki E, Mutoh N, Suzuki M, Sato H, et al. Comparative study of histamine H4 receptor expression in human dermal fibroblasts. *J Toxicol Sci* 2008;33:503-8.
 19. Contoli M, Caramori G, Mallia P, Johnston S, Papi A. Mechanisms of respiratory virus-induced asthma exacerbations. *Clin Exp Allergy* 2005;35:137-45.
 20. Petricevic B, Wessner B, Sachet M, Vrbanec D, Spittler A, Bergmann M. CL097, a TLR7/8 ligand, inhibits TLR-4-dependent activation of IRAK-M and BCL-3 expression. *Shock* 2009;32:484-90.