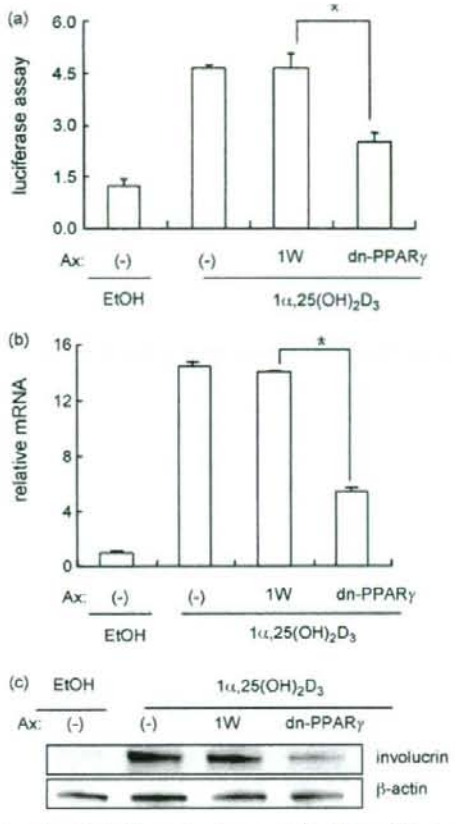

Fig. 2 dn-PPARγ interferes with the effect of $1\alpha,25(OH)_2D_3$ on involucrin expression. (a) Keratinocytes were transfected with pINV-Luc and pRL-TK for 24 h, and then infected with Ax1W or Axdn-PPARγ for an additional 24 h. The cells were exposed to $1\alpha,25(OH)_2D_3$ for 36 h. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System. The transfections were performed in triplicate. The relative luciferase activity was calculated by normalizing to the level of *Renilla* luciferase activity. (b) Keratinocytes were infected with Ax1W or Axdn-PPARγ for 24 h and then treated with $1\alpha,25(OH)_2D_3$. Total RNA was collected after 48 h, and involucrin mRNA was detected using real-time RT-PCR. (c) Keratinocytes were infected with Ax1W or Axdn-PPARγ for 24 h before the addition of $1\alpha,25(OH)_2D_3$. Cells were collected 48 h after treatment, and the level of involucrin protein was evaluated by Western blotting. The data are representative of at least three independent experiments.

mRNA (Fig. 2b) and protein (Fig. 2c) by more than 50%. Neither the control (1W) nor dn-PPARγ affected involucrin expression in vehicle cells (data not shown). These data indicate a positive role of PPARγ in involucrin expression during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation.

3.3. Regulation of $1\alpha,25(OH)_2D_3$-induced AP-1 transactivation by PPARγ

According to previous reports, the AP-1 site in the involucrin promoter is essential for the induction of involucrin by $1\alpha,25(OH)_2D_3$ [16]. We hypothesized that PPARγ affects involucrin expression by controlling AP-1 transactivation. Therefore, we performed EMSA to examine the DNA binding activity of AP-1 in nuclear extracts of keratinocytes. Our results confirmed those of previous reports [17,18], treatment with $1\alpha,25(OH)_2D_3$ for 36 h significantly upregulated AP-1 DNA binding activity (Fig. 3a). In addition, dn-PPARγ essentially blocked AP-1 transactivation in $1\alpha,25(OH)_2D_3$-treated human keratinocytes (Fig. 3a). In a competition experiment, pre-incubation with an unlabeled AP-1 probe completely abolished the binding reaction, indicating that the AP-1 probe was specific (Fig. 3b). Furthermore, the addition of anti-Fra1 or c-Fos antibody resulted in a supershifted band (Fig. 3c) demonstrating the presence of these two proteins in the AP-1 DNA binding complex [18]. We also detected increased levels of Fra1 and c-Fos proteins in $1\alpha,25(OH)_2D_3$-treated human keratinocytes (Fig. 3d), and this was decreased by the expression of dn-PPARγ (Fig. 3d). The blockage of AP-1 activity in Axdn-PPARγ-infected cells might depend on the inhibition of Fra1 and c-Fos expression by dn-PPARγ (Fig. 3d). Our data suggest that PPARγ regulates involucrin expression by controlling AP-1 transactivation in $1\alpha,25(OH)_2D_3$-treated human keratinocytes.

3.4. PPARγ regulates $1\alpha,25(OH)_2D_3$-induced involucrin expression probably by controlling the activation of p38 MAPK

During the late stages of keratinocyte differentiation, p38 MAP kinase (MAPK) is involved in upregulating involucrin expression [19]. In addition to the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) [18], $1\alpha,25(OH)_2D_3$ treatment resulted in a sustained increase in p38 phosphorylation (Fig. 4a). To examine whether p38 is involved in the induction of involucrin, cultures were incubated with SB203580, a specific inhibitor of p38 MAPK, before $1\alpha,25(OH)_2D_3$ treatment. The expression of involucrin in SB203580-treated vehicle cells was not different from that in untreated cells (data not shown), whereas SB203580 pretreatment of cells significantly impaired $1\alpha,25(OH)_2D_3$-induced expression of involucrin mRNA and protein (Fig. 4b and c). This suggests that p38 activity contributes to the $1\alpha,25(OH)_2D_3$-induced expression of involucrin.

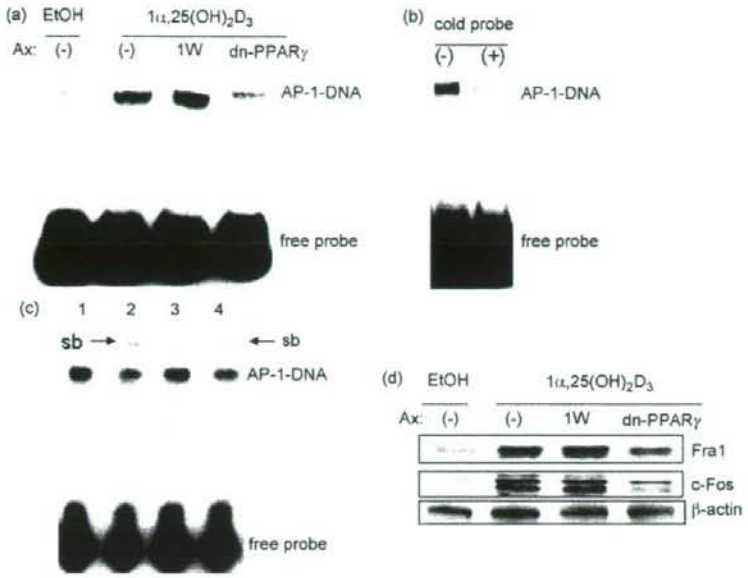

Fig. 3 Upregulation of AP-1 transactivation by $1\alpha,25(OH)_2D_3$ is inhibited by dn-PPARγ. (a) Keratinocytes were infected with Axdn-PPARγ or Ax1W and then treated with $1\alpha,25(OH)_2D_3$. Nuclear extracts were collected 36 h after treatment. A biotin-labeled AP-1 probe was incubated with the nuclear proteins, and EMSA was performed. (b) Lane competitor (−): only a biotin-labeled AP-1 probe was incubated with the nuclear extract from $1\alpha,25(OH)_2D_3$-treated cells; lane competitor (+): pre-incubation with the AP-1 unlabeled probe. (c) Nuclear extracts from $1\alpha,25(OH)_2D_3$-treated keratinocytes were mixed with biotin-labeled AP-1 probe in the presence of antibodies. Note the supershifted band (sb) after incubation with anti-Fra1 or anti-c-Fos antibody. Lane 1: no antibody added; lanes 2–4: added rabbit anti-Fra-1 antibody, normal rabbit IgG, and rabbit anti-c-Fos antibody, respectively. (d) Keratinocytes were infected with Axdn-PPARγ or Ax1W. Total cellular extracts were collected 36 h after the addition of $1\alpha,25(OH)_2D_3$, and the Fra1 and c-Fos protein levels were detected by immunoblotting. The data are representative of at least three independent experiments.

Next, we investigated whether PPARγ regulates the activation of p38. Infection with Axdn-PPARγ nearly blocked the $1\alpha,25(OH)_2D_3$-induced phosphorylation of p38, but the total level of p38 was unaffected (Fig. 4d). This suggests that p38 activity is involved in the regulation of involucrin expression by PPARγ during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation.

4. Discussion

In this study, we detected significant expression and activation of PPARγ during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation, which is consistent with the previous reports that PPARγ expression is increased significantly during *ex vivo* and *in vitro* epidermal differentiation [6,20]. We have demonstrated increased expression and transactivation of PPARγ, but not other PPARs, in keratinocyte suspension cultures [12]. The ability of PPARγ to promote cellular differentiation and to induce the expression of differentiation-associated genes has been reported in several epithelial cells [21,22], including keratinocytes [6]. We showed that blocking PPARγ signal interfered with involucrin induction in VD3-stimulated (this study) and suspension culture-triggered keratinocyte differentiation [12], suggesting PPARγ contributes to keratinocyte differentiation by regulating the expression of involucrin.

AP-1 activity is important for both basal and inducible involucrin transcription. $1\alpha,25(OH)_2D_3$ was shown to stimulate AP-1 transactivation via the phosphatidylinositol 3-kinase/Ras/MEK/ERK/JNK signal transduction pathway [17,18]. We also detected increased phosphorylation of ERK and JNK by $1\alpha,25(OH)_2D_3$ in this study, although this is not affected by dn-PPARγ (data not shown). The activated MAPKs influence AP-1 transcriptional activity by increasing the levels of specific Jun and Fos proteins and by altering the phosphorylation of AP-1 subunits [18]. We demonstrated the involvement of PPARγ in DNA binding by AP-1 in $1\alpha,25(OH)_2D_3$-stimulated keratinocytes, which is

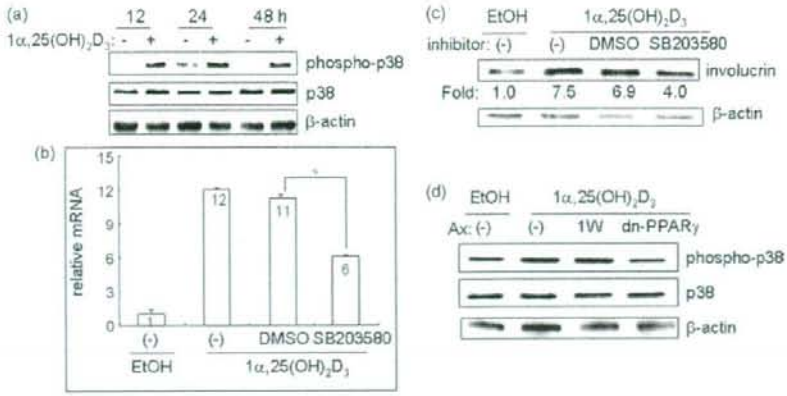

Fig. 4 p38 is involved in the regulation of involucrin expression by PPARγ. (a) Samples were collected as described in Fig. 1b, and the levels of p38 and phospho-p38 were detected. (b) Keratinocytes were incubated with SB203580 for 1 h before $1\alpha,25(OH)_2D_3$ treatment. Total RNA was collected after 48 h, and the level of involucrin mRNA was detected using real-time PCR. (c) Keratinocytes were treated as described in (b). Total protein was collected, and involucrin expression was measured by Western blotting. The intensity of protein band was quantified using NIH Image (the control signal was defined as one unit). (d) Keratinocytes were infected with Ax1W or Axdn-PPARγ before the addition of $1\alpha,25(OH)_2D_3$. The cells were collected after 24 h, and the levels of p38 and phospho-p38 were evaluated. The data are representative of at least three independent experiments.

probably dependent on the regulation of AP-1 proteins by PPARγ. A putative VDRE containing two half sites separated by three nucleotides (DR-3) is present in the proximal promoter of involucrin and acts specifically for $1\alpha,25(OH)_2D_3$ responsiveness [16]. In human keratinocytes, the increased binding activity of the DR-3 element by $1\alpha,25(OH)_2D_3$ [16] was not affected by dn-PPARγ (data not shown). Therefore, it is reasonable that AP-1 transactivation is PPARγ-dependent and responds to the induction of involucrin by $1\alpha,25(OH)_2D_3$.

Although the involvement of p38 in $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation is not supported by a previous report [18], we found that $1\alpha,25(OH)_2D_3$ stimulation resulted in persistent activation of p38, which contributes to involucrin expression. These conflicting results on the effects of $1\alpha,25(OH)_2D_3$ on p38 activity might be attributable to differences in culture conditions. For example, Johansen [18] cultured keratinocytes isolated from adult skin and stimulated them at 50–60% confluence; we separated primary normal human keratinocytes from neonatal skin and used subconfluent cells for experiments. PPARγ agonists are able to activate p38 [23]. Blocking PPARγ activation eliminated $1\alpha,25(OH)_2D_3$-induced p38 phosphorylation, suggesting that p38 is involved in the regulation of involucrin expression by PPARγ. The effect of p38 on involucrin expression could be associated with increased binding of AP-1 and C/EBP transcription factors to the involucrin promoter [19], and post-transcriptional control of involucrin gene [13,16].

The fact that the epidermis of PPARγ-deficient mice [7] shows no detectable defect indicates that PPARγ does not play a decisive role in controlling epidermal differentiation; other transcription factors might compensate for the lack of PPARγ. Nevertheless, our data suggest that the activation of PPARγ contributes to the effect of $1\alpha,25(OH)_2D_3$ on keratinocyte differentiation. This report describes early evidence that PPARγ regulates $1\alpha,25(OH)_2D_3$-induced involucrin expression by controlling AP-1 transactivation and p38 activity in normal human keratinocytes.

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The NF-κB, p38 MAPK and STAT1 pathways differentially regulate the dsRNA-mediated innate immune responses of epidermal keratinocytes

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Keywords: chemokines, cytokine, innate immunity, IRF3, SOCS1

Abstract

The epidermis is the primary boundary between the body and the environment, and it serves as the first line of defense against microbial pathogens. Production of chemokines and cytokines is an important step in the initiation of innate immune responses to viral infections. Epidermal keratinocytes produce IFN-α, -β and macrophage inflammatory protein (MIP)-1α in response to double-stranded RNA (dsRNA) or viral infections. We showed that human keratinocytes produced cytokines [tumor necrosis factor (TNF)-α, IL-1β and IL-15] and chemokines [MIP-1β, RANTES and liver and activation-regulated chemokine (LARC)] in response to dsRNA, with activation of the nuclear factor κB (NF-κB), p38 mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription 1 (STAT1) pathways. To study the roles of these pathways in their production, we transfected keratinocytes with adenoviral vectors (Ax) carrying a dominant-negative form of inhibitor κB α (IκBα) (IκBαM), a dominant-negative mutant form of STAT1 (STAT1F) or suppressors of cytokine signaling 1 (SOCS1). Transfection with AxIκBαM or addition of a p38 inhibitor (SB203580) significantly decreased the dsRNA-mediated production of TNF-α, IL-1β and MIP-1α, but not of IFN-β, IL-15, MIP-1β, RANTES or LARC. Transfection with AxSTAT1F or AxSOCS1 inhibited the dsRNA-mediated production of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC, but not IFN-β or IL-1β. In conclusion, the NF-κB, p38 MAPK and STAT1 pathways differentially regulate dsRNA-mediated innate immune responses in epidermal keratinocytes.

Introduction

The skin is the primary interface between the body and the environment and serves as the first line of defense against microbial pathogens. Epidermal keratinocytes, the main constituent of the epidermis, actively participate in innate immune responses by producing cytokines, chemokines (1–3) and anti-microbial peptides (4). In addition, human keratinocytes can be targets for viruses, such as herpes simplex virus (HSV) (5), human papillomavirus (6) and varicella-zoster virus (7). HSV- or varicella-zoster virus-infected keratinocytes are known to produce cytokines and chemokines (5, 8). Keratinocytes in virus-infected skin lesions produce macrophage inflammatory protein (MIP)-1α (7), suggesting that these cells play a role in the virus-mediated innate immune reaction of the skin. However, the regulatory mechanisms behind virus-mediated immune reaction of keratinocytes remain unclear.

Toll-like receptors (TLRs) play important roles in innate and adaptive immunity by recognizing microbial pathogens. The intracellular signals of the TLRs have been classified into myeloid differentiation primary response gene (88)-dependent and Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta-dependent pathways (9). TLR3 is a receptor for virus-associated double-stranded RNA (dsRNA) and activates nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs) and IFN regulatory factor 3 (IRF3) in an effort to control the viral infection (9, 10). In addition to TLR3, the RNA helicase retinoic acid-inducible gene (*RIG-1*) and melanoma differentiation-associated gene 5 (*MDA5*) have also been implicated in viral dsRNA recognition (11, 12). *In vitro* studies suggest that both *RIG-1* and *MDA5* detect RNA viruses and polyinosine–polycytidylic acid (polyI:C), a synthetic dsRNA analogue (11). Previously, we showed that the synthetic dsRNA, polyI:C, induced production of IFNs and MIP-1α in cultured human keratinocytes (8, 13). In airway epithelial cells, polyI:C induced the

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expression of various chemokines, including MIP-1α, MIP-1β, RANTES, 10 kDa IFN-γ-inducible protein (IP-10), IL-8 and liver and activation-regulated chemokine (LARC), as well as the expression of several cytokines, including IL-6, tumor necrosis factor (TNF)-α, IL-1β and IL-15 (14). However, production of these cytokines and chemokines in keratinocytes with dsRNA has not been studied. TNF-α and IL-1β regulate the early phase of inflammation in viral infections (15, 16), while IL-15 contributes to innate immunity by regulating the function of NK cells (17) and Langerhans cells (18). Local over-expression of IL-15 in the epidermis protects mice from cutaneous HSV infection by enhancing their anti-viral immune responses (19). Thus, it is important to study the regulatory mechanisms of the production of cytokines and chemokines in keratinocytes during viral infection to understand the defense mechanisms of the skin.

The production of most cytokines and chemokines is regulated primarily at the level of transcription, through activation of specific sets of transcription factors controlled by NF-κB, MAPKs and IRFs (13, 16). The signal transducers and activators of transcription (STAT) family plays an important role in cytokine production (20). Upon viral infection, type I IFN is rapidly induced and activates the transcription factor complex ISGF3, consisting of STAT1, STAT2 and IRF9. ISGF3 binds to *cis*-elements, termed IFN-stimulated response elements which usually reside within the promoters of IFN-inducible genes, such as *TLR3* (8) and *IRF7* (21). Some IFN-inducible genes can also be activated by IRF3 in virus-infected cells, where it forms a complex with CBP/p300 co-activators to bind to IRSE sites in the promoters of IFN-inducible genes, such as *ISG15* and *IP-10* (20, 21). The transcriptional regulation of these genes is dependent on the cellular context and stimulation (22). We reported previously that blockade of STAT1 suppressed the IFN- or polyI:C-induced expression of TLR3 and IRF7 in keratinocytes (13). However, details of the polyI:C-mediated production of other chemokines and cytokines in epidermal keratinocytes and the regulatory mechanisms involved remain unclear. Here, we report that dsRNA-induced production of chemokines and cytokines in epidermal keratinocytes was differentially regulated by NF-κB, p38 MAPK and STAT1 pathways.

Materials and methods

Reagents

PolyI:C (Amersham Biosciences Corp., Piscataway, NJ, USA) was dissolved in deionized distilled water at a concentration of 2 mg ml^{-1} and stored at −70°C. SB203580 (Calbiochem-Novabiochem International Co., San Diego, CA, USA) was dissolved in dimethyl sulfoxide at a concentration of 2 mM and stored at −20°C. Anti-IRF3 antibody, anti-β-actin, anti-RIG-I and anti-MDA5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb to TLR3 was obtained from Alexis Biochemicals (San Diego, CA, USA). The antibodies for inhibitor κB α (IκBα), phospho-IκBα, p38, phospho-p38, extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-STAT1, phospho-STAT2 and phospho-STAT3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture and polyI:C stimulation

Normal human keratinocytes were cultured in MCDB153 medium, supplemented with insulin (5 μg ml^{-1}), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg ml^{-1}) and Ca^{2+} (0.03 mM), as described previously (23). Cells that had been passaged four times were used in the experiments, and subconfluent keratinocyte cultures were treated with 100 ng ml^{-1} of polyI:C for a predetermined period.

This study was performed according to the principles set forth in the Declaration of Helsinki. All procedures involving human subjects received prior approval from the ethical committee of Ehime University School of Medicine. All subjects provided written informed consent to participation in the study.

Adenovirus construction and transfection

AxIκBαM, AxSOCS1 and AxSTAT1F were prepared as described previously (13). Cultured normal human keratinocytes were transfected with AxIκBαM, AxSOCS1 or AxSTAT1F and then stimulated as described previously (13). AxLacZ was used as a control.

Immunofluorescence microscopy

Keratinocytes on chamber slides were fixed for 5 min in methanol:acetone (1:1) and washed with PBS. The cells were then treated with anti-IRF3 antibody overnight at 4°C. After washing with PBS, the cells were incubated with fluorescein-labeled goat anti-rabbit IgG for 30 min at 37°C and then washed four times. The stained cells were visualized at a magnification of ×40 under a confocal microscope (LSM 510; Carl Zeiss, Jena, Germany).

Reverse Transcription–PCR

Total RNA from the cultured cells was isolated at the indicated time points using Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription (RT)–PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan), according to the manufacturer's protocol. The primer pairs used are listed in Table 1. The products were visualized on 2% agarose gels containing ethidium bromide and were then sequenced to confirm the accuracy of amplification.

ELISA

Culture supernatants were collected after treatment with polyI:C and stored at −70°C until use. ELISA kits for TNF-α and MIP-1α were purchased from Endogen (Auburn, MA, USA). ELISAs were performed according to the manufacturer's protocol. The optical density at 450 nm was measured with an Immuno Mini NJ-2300 microplate reader (Nalgene Nunc International K.K., Tokyo, Japan). All assays were performed in triplicate.

Protein preparation and western blotting

The cells were harvested by transferring them into an extraction buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM s-HCl (pH 7.4) and protease inhibitors. Equal amounts of protein were separated by

Table 1. The primer pairs used for RT-PCR

Primer name	Sequence: (5'-3')
GAPDH upper	5'-ACCACAGTCCATGCCATCAC-3'
GAPDH lower	5'-TCCACCACCCTGTTGCTGTA-3'
IFN-β upper	5'-CACGACAGCTCTTTCCATGA-3'
IFN-β lower	5'-AGCCAGTGCTCGATGAATCT-3'
TNF-α upper	5'-ACAAGCCTGTAGCCCATGTT-3'
TNF-α lower	5'-AAAGTAGACCTGCCCAGACT-3'
IL-1β upper	5'-AGACAAATTGCATGGTGAAGTCAGTT-3'
IL-1β lower	5'-ATGGCAGAAGTACCTAAGCTCGC-3'
IL-15 upper	5'-GGCTTTGAGTAATGAGAATTT-3'
IL-15 lower	5'-ATCAGTTGCAATCAAGAAGTG-3'
MIP-1α upper	5'-CACCATGGCTCTCTGCAAC-3'
MIP-1α lower	5'-TATTTCTGGACCCACTCCTC-3'
MIP-1β upper	5'-GGAATTCCCAGCCAGCTGTGGT-3'
MIP-1β lower	5'-CAAGCTTGAGCAGCTCAGTTCAGTTC-3'
RANTES upper	5'-GGAATTCGCCTCGCTGTCATCCTCATT-3'
RANTES lower	5'-CAAGCTTGCGGTTCTTTCGGGTGACAA-3'
LARC upper	5'-GGAATTCTGTACCAAGAGTTTGCTCCT-3'
LARC lower	5'-CAAGCTTCGGCTATGTCCAATTCCATT-3'

SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The analysis was performed using a Vistra ECF Kit (Amersham Biosciences K.K., Tokyo, Japan) and a FluorolmagerI (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Statistical analysis

At least three independent studies were performed and yielded similar results. The results of one representative experiment are shown in each of the figures. Quantitative data are expressed as means ± standard deviations. Statistical significance was determined using the paired Student's *t*-test. Differences were deemed statistically significant at $P < 0.05$. The levels of statistical significance are indicated in the figures as follows: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

Polyl:C enhanced mRNA expression of cytokines and chemokines in normal human keratinocytes

The presence of dsRNA during viral infection induces numerous inflammatory mediators in a variety of cell types (16, 22). As an initial step, we analyzed mRNA induction by polyI:C in cultured normal human keratinocytes. The levels of mRNA expression of inflammatory cytokines, including IFN-β, TNF-α, IL-1β and IL-15, and chemokines, including MIP-1α, MIP-1β, RANTES and LARC, were enhanced by polyI:C (Fig. 1a). The mRNA level of IL-18 was unchanged (data not shown).

The expression of the dsRNA detectors (24) TLR3, RIG-I and MDA5 in cultured human keratinocytes was also investigated using western blotting. As shown in Fig. 1(b), unstimulated cells (time, 0 h) expressed TLR3, but not RIG-I or MDA5. On stimulation with polyI:C, the level of TLR3 protein increased rapidly, and significant RIG-I and MDA5 expression was induced after 12 h of stimulation. The induction of RIG-I by polyI:C confirmed previous reports in human astrocytoma cells (25) and fibroblasts (26).

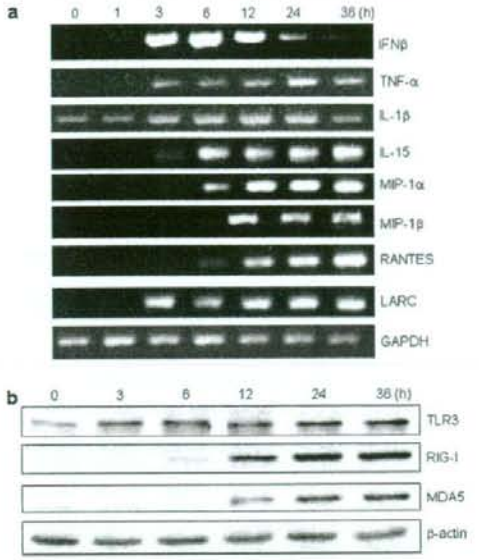

Fig. 1. PolyI:C enhanced mRNA expression of cytokines and chemokines in keratinocytes. (a) Keratinocytes were stimulated with polyI:C (100 ng ml^{-1}) for the indicated times, and RT-PCR was performed to detect the transcription of GAPDH, IFN-β, TNF-α, IL-1β, IL-15, MIP-1α, MIP-1β, RANTES and LARC. (b) Keratinocytes were stimulated with polyI:C for the indicated times, and the levels of TLR3, RIG-1 and MDA5 proteins were detected using western blotting.

NF-κB regulated polyI:C-induced TNF-α, IL-1β and MIP-1α production

As NF-κB regulates the production of many inflammatory cytokines (16), we examined its involvement in the polyI:C-induced production of cytokines and chemokines in keratinocytes. IκBα was rapidly phosphorylated by polyI:C (Fig. 2a), as reported previously (13). Next, the keratinocytes were transfected with an adenoviral vector (Ax) carrying a dominant-negative mutant form of IκBα (IκBαM) to block NF-κB signaling (13). Transfection with IκBαM significantly suppressed the mRNA induction of TNF-α, IL-1β and MIP-1α by polyI:C, while the levels of mRNA expression of other genes were unaffected (Fig. 2b). ELISAs for TNF-α and MIP-1α confirmed the inhibitory effect of IκBαM on their production (Fig. 2c). Thus, NF-κB signaling appeared to be involved in the induction of TNF-α, IL-1β and MIP-1α by polyI:C in keratinocytes.

p38 MAPK regulated polyI:C-induced TNF-α, IL-1β and MIP-1α production

The MAPK system is activated by infection with several viruses (16, 22). It has previously been shown in epithelial cells that dsRNA activated p38 MAPK, which is required for the induction of TNF-α and IL-1β (27). Considering the important role MAPK signaling plays in host defense, we examined the activation of three major MAPK subfamilies, p38, ERK1/2 and JNK, in keratinocytes upon polyI:C stimulation.

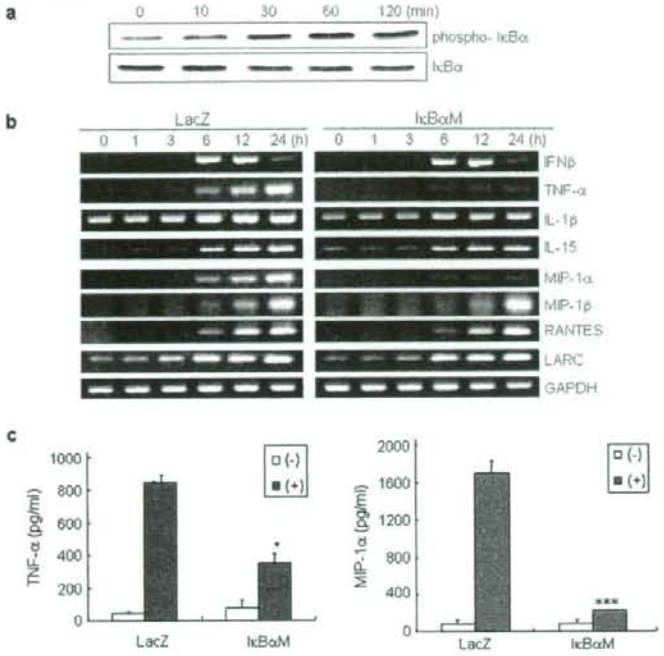

Fig. 2. NF-κB is involved in the polyI:C-induced expression of TNF-α, IL-1β and MIP-1α in keratinocytes. (a) Keratinocytes were treated with polyI:C for the indicated times, cell extracts were prepared and IκBα and phospho-IκBα expression were detected by western blotting. (b) Keratinocytes were infected with AxLacZ or AxIκBαM for 24 h, and the cultures were stimulated with polyI:C for the indicated times. RT-PCR was performed to determine the mRNA expression of various cytokines and chemokines. (c) Keratinocytes were infected with AxLacZ or AxIκBαM and then stimulated with polyI:C for 30 h. The supernatants were collected, and the expression of TNF-α and MIP-1α was analyzed by ELISA.

As shown in Fig. 2(a), after adding polyI:C, phospho-p38 was increased significantly, whereas the changes in phospho-ERK and phospho-JNK were not remarkable. To determine whether p38 is involved in polyI:C-induced cytokine and chemokine production in keratinocytes, p38 activity was inhibited using SB203580. Treatment with SB203580 resulted in a partial, but statistically significant, reduction in polyI:C-induced TNF-α, IL-1β and MIP-1α mRNA expression (Fig. 3B). This result was confirmed by ELISA (Fig. 3c). These observations suggest that polyI:C-induced production of TNF-α, IL-1β and MIP-1α involves p38 signaling.

Expression of suppressors of cytokine signaling 1 suppressed the activation of STATs and reduced polyI:C-induced TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC production

The JAK–STAT pathway is involved in immune regulation via IFNs (13), which are induced by viral infection and dsRNA. The suppressors of cytokine signaling (SOCS)/cytokine-inducible SH2-containing protein family, which is induced by STAT activation, negatively regulates the JAK–STAT pathway by inhibiting STAT activation (28). Previously, we showed that the STAT1–SOCS1 pathway regulates innate immunity in keratinocytes (13). In this study, we investigated whether the JAK–STAT pathway was involved in the production of cytokines and chemokines in polyI:C-treated keratinocytes. As in our previous report (13), we detected significant phosphorylation of STAT1, STAT2 and STAT3 upon polyI:C stimulation (Fig. 4a), which was blocked by SOCS1 (Fig. 4a), while the total protein levels of STATs were unchanged (data not shown). Furthermore, SOCS1 inhibited the mRNA induction of most of the genes, including the cytokines TNF-α and IL-15 and the chemokines MIP-1α, MIP-1β, RANTES and LARC (Fig. 4b). The data for TNF-α and MIP-1α were confirmed at the protein level by ELISA (Fig. 4c).

Expression of SOCS1 blocked the polyI:C-induced nuclear translocation of IRF3

It has been suggested that the action of SOCS is not confined to JAK/STAT signaling (29). We found that SOCS1 not only suppressed STAT activation (Fig. 4a) but also blocked the polyI:C-induced nuclear translocation of IRF3 (Fig. 4d), which is upstream of the JAK–STAT pathway during dsRNA signaling. These observations suggested that SOCS1 plays a central role in regulating the immune response in polyI:C-treated keratinocytes.

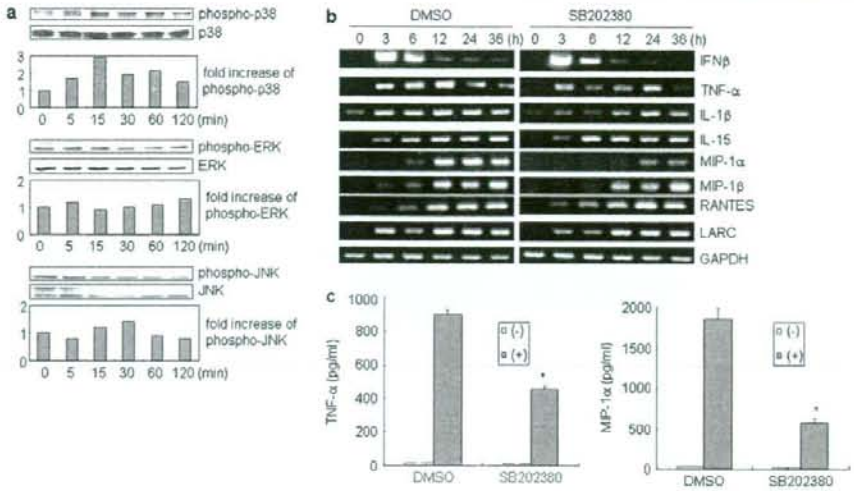

Fig. 3. Activation of the p38 pathway is required for polyI:C-induced TNF-α, IL-1β and MIP-1α expression in keratinocytes. (a) Keratinocytes were treated with polyI:C for the indicated times, cell extracts were prepared and the levels of p38, phospho-p38, ERK, phospho-ERK, JNK and phospho-JNK were determined by western blotting. The intensity of the protein band was quantified using NIH Image (Molecular Dynamics), and the relative increases in phospho-p38, phospho-ERK and phospho-JNK compared with the total protein are shown with the data. (b) Cultures were processed with dimethyl sulfoxide or SB202380 (20 μM) for 30 min prior to treatment with polyI:C for the indicated times. RNA was prepared, and RT-PCR was performed to detect the mRNA expression of several cytokines and chemokines. (c) Cultures were pre-treated with dimethyl sulfoxide or SB202380 for 30 min prior to stimulation with polyI:C for 30 h, and the supernatants were collected. TNF-α and MIP-1α production were examined by ELISA.

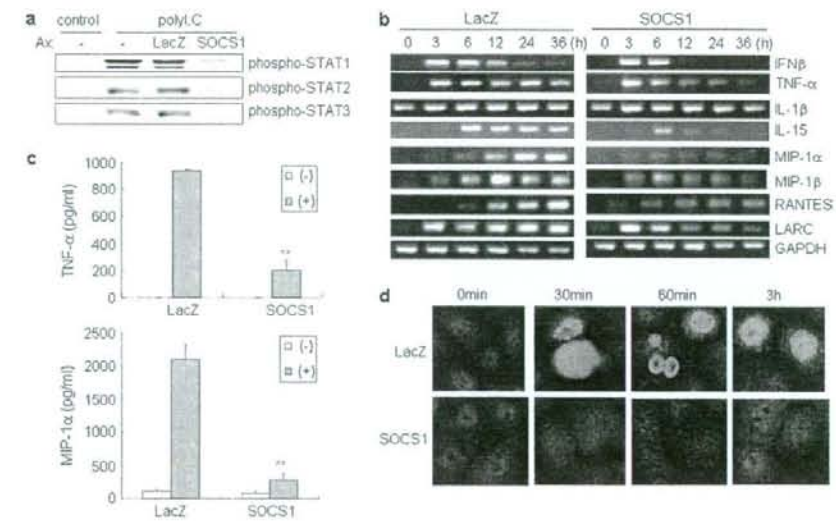

Fig. 4. Over-expression of SOCS1 suppressed the induction of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC and blocked the nuclear translocation of IRF3 in polyI:C-treated keratinocytes. (a) Keratinocyte cultures were transfected with AxLacZ or AxSOCS1 for 24 h, stimulated with polyI:C for 4 h and the extracts were subjected to western blotting to detect phospho-STAT1, phospho-STAT2 and phospho-STAT3. (b) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated time periods, and the transcription of several cytokines and chemokines was assayed by RT-PCR. (c) Keratinocyte cultures were transfected with AxLacZ or AxSOCS1 for 24 h. PolyI:C was added to the cultures, and the supernatants were collected after 30 h. ELISA was performed to evaluate the levels of TNF-α and MIP-1α. (d) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated times. The cells were then fixed, and immunofluorescence was used to detect the nuclear translocation of IRF3.

STAT1 regulated polyI:C-induced TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC production

A specific role of STAT1 activation in MIP-1α production in dsRNA signaling has been demonstrated (13). We examined whether STAT1 activation was essential for polyI:C-induced cytokine and chemokine expression. An Ax carrying a dominant-negative mutant form of STAT1 (STAT1F), which specifically blocks STAT1 activation with no effect on other STATs (13), was transfected into keratinocytes. These keratinocytes were then treated with polyI:C. STAT1F regulated the mRNA expression of polyI:C-induced cytokines and chemokines in a pattern similar to SOCS1 (Fig. 5a). Moreover, STAT1F decreased the production of TNF-α and MIP-1α (Fig. 5b). As STAT1F did not influence the dsRNA-induced activation of IRF3 (data not shown), the similarities between the effects of STAT1F and those of SOCS1 on polyI:C-triggered inflammation suggest that STAT1 contributed to the response to dsRNA in keratinocytes.

Discussion

We found that human keratinocytes produced TNF-α, IL-1β, IL-15, MIP-1β, RANTES and LARC following exposure to dsRNA, in addition to MIP-1α and IFN-β. Figure 6 summarizes the regulatory mechanisms of the polyI:C-induced production of cytokines and chemokines. Specific sets of transcription factors required for cytokine and chemokine production vary among cell types (16, 22). In airway epithelial cells, the p38 MAPK pathway alone regulates the dsRNA-induced production of TNF-α and IL-1β (27). In contrast, in keratinocytes, both p38 MAPK and NF-κB are required for the production of TNF-α and IL-1β. This may be explained by the observation that the p38 MAPK pathway is required for the efficient activation of NF-κB in response to viral infection (30) and other environmental stresses (31). In airway epithelial cells (14) and NIH 3T3 cells (32), both the IRF3 pathway and NF-κB signal are required for dsRNA-induced expression of RANTES. On the other hand, p38 MAPK regulates viral infection-induced RANTES in bronchial epithelial cells (33) and in microglia (30), but not in fibroblasts (32). In keratinocytes, STAT1, but not the NF-κB or p38 pathways, is required for RANTES production. Although all the NF-κB, p38 MAPK and STAT1 pathways regulate polyI:C-induced MIP-1α production in keratinocytes, NF-κB signaling is not essential for MIP-1α production in airway epithelial cells (14). Moreover, dsRNA-mediated LARC production is regulated by NF-κB activation in airway epithelial cells (14), but dsRNA-activated STAT1 signal is essential for LARC production in human keratinocytes. Although dsRNA elicits innate immune reactions in a variety of organs, the regulatory mechanisms to produce cytokines and chemokines differ among them. We also noted that the *IFN-β*, *TNF-α* and *LARC* genes were induced much earlier compared with other cytokines or chemokines, in response to polyI:C stimulation. In addition, inhibition of the STAT1 signal significantly suppressed the late phase, but not the early phase, of TNF-α and LARC production. This kinetic regulation of TNF-α and LARC demonstrates that different transcription factors contribute to the early and late phases of expression of these genes. In particular, the early phase of TNF-α and LARC induction might occur in response to direct dsRNA receptor-mediated TNF-α and LARC transcription, which might be activated by NF-κB and p38, although neither blocking the NF-κB signal nor inhibiting p38 resulted in a remarkable

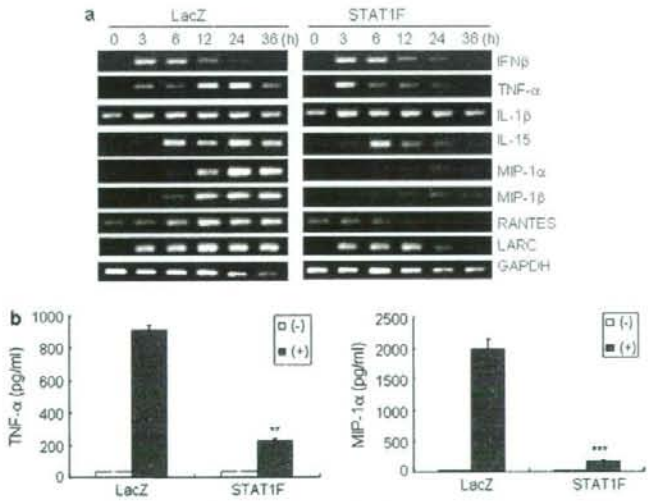

Fig. 5. STAT1F inhibited the induction of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC by polyI:C in keratinocytes. (a) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated time periods, and the transcription of several cytokines and chemokines was measured by RT-PCR. (b) Keratinocytes were infected with AxLacZ or AxSTAT1F and then stimulated with polyI:C for 30 h. The supernatants were collected, and the levels of TNF-α and MIP-1α were analyzed by ELISA.

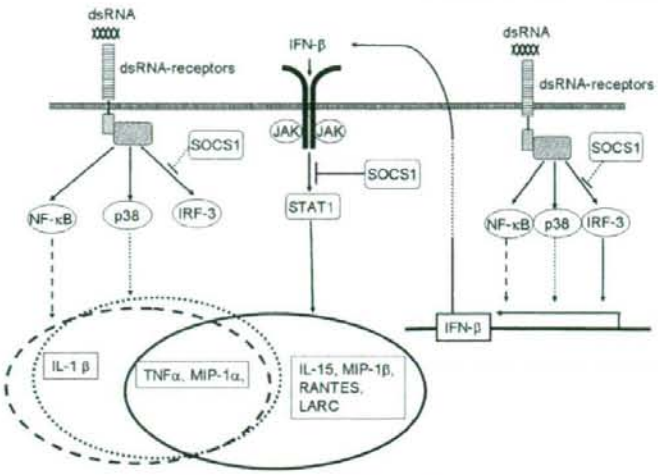

Fig. 6. Regulation of dsRNA-mediated cytokines and chemokines by different signaling pathways in keratinocytes. After infection with virus or treatment with dsRNA, NF-κB, p38 MAPK and IRF3 are activated, resulting in IFN-β production. The *de novo*-expressed IFN-β stimulates activation of the JAK-STAT pathway. The activation of NF-κB, p38 MAPK and JAK-STAT pathways differentially regulates dsRNA-mediated production of cytokines and chemokines.

decrease in LARC production. As opposed to early and direct TNF-α and LARC induction, the late phase of the production of these genes is indirect and is most probably mediated by dsRNA-induced IFN-β, which activates STAT1 and amplifies the dsRNA signaling (13).

TLR3, *RIG-I* and *MDA5* have been implicated in the recognition of dsRNA and the subsequent induction of anti-viral responses (24). In the endosome, the viral dsRNA and its mimic polyI:C are recognized by TLR3, whereas RIG-I and MDA5 have been identified as cytosomal dsRNA detectors. Moreover, RIG-I and MDA5 also activate NF-κB and IRF3 and stimulate the subsequent production of type I IFNs and pro-inflammatory cytokines when stably expressed in cells (24, 34). Despite this knowledge, the exact contributions of *TLR3*, *RIG-I* and *MDA5* to dsRNA-mediated signal transduction and cytokine production have yet to be clarified. In human astrocytoma cells, polyI:C-induced RANTES upregulation is significantly inhibited by siRNA for *RIG-I* (25), and over-expression of RIG-I in gingival fibroblasts enhances the production of IL-1β and IL-8 induced by polyI:C (26). However, the knockdown of *RIG-I* and *MDA5* through siRNA transfection failed to inhibit polyI:C-mediated RANTES, IP-10 and IL-8 production in airway epithelial cells (35). In this study, we demonstrated that cultured normal human keratinocytes express substantial TLR3, but not MDA5 and RIG-I, suggesting that the primary reactions triggered by polyI:C are most probably mediated by TLR3, while RIG-I and MDA5, both of which are significantly induced by polyI:C, might contribute to the amplification of dsRNA signaling in keratinocytes. Further research is required to elucidate the respective functions of *TLR3*, *RIG-I* and *MDA5* in dsRNA signaling and anti-viral responses in human keratinocytes.

Our finding that SOCS1 blocked polyI:C-induced IRF3 nuclear translocation was unexpected. The mechanism of IRF3 activation by polyI:C is probably dependent on the kinases I kappa B kinase ε and TRAF-associated NF-κB activator (TANK)-binding kinase 1 (36). Although Kinjyo *et al.* (29) have suggested that SOCS1 suppressed TRAF6-dependent IKK activation when over-expressed, no studies have described the effect of SOCS in the region directly upstream of IRF3. Further research is required to clarify the molecular mechanism by which SOCS1 inhibited IRF3 activation.

Induction of type I IFNs is one of the earliest events in viral infections, in fact preceding the generation of a specific immune response (37). The promoter of the human IFN-β gene is complex, with several partially overlapping positive and negative regulatory elements (38). Three families of transcription factors, IRF3 (39), NF-κB and AP-1 (38, 40), all of which are activated in response to dsRNA or viral infection, have been shown to participate in the induction of IFNs. Activation of the IFN-β promoter requires the coordinated action of several transcription factors (41); however, not all these transcription factors may be necessary for dsRNA-mediated IFN-β induction if one of them is present in excess (42). In the current study, we found that SOCS1 over-expression blocked the activation of IRF3, but that it had no effect on IFN-β expression; furthermore, both the introduction of AxIκBαM and treatment with a p38 MAPK inhibitor failed to block IFN-β induction. It has been shown that individual *cis*-elements, in the absence of the others, can drive dsRNA-induced transcription of transfected reporter genes, indicating that each element is capable of communicating with the basal transcription machinery and with the relevant co-activators (38), and this may apply to the complex promoter of the *IFN-β* gene in polyI:C-treated keratinocytes. Compared with the regulation of the other cytokines and chemokines, the contribution of the many overlapping signaling mechanisms in *IFN-β* transcription suggests that *IFN-β* is a key element in viral infection.

Overall, our findings provide insight into the precise roles of NF-κB, p38 and STAT1 in the virus-provoked innate immune responses of epidermal keratinocytes.

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Abbreviations

Ax	adenovirus vector
dsRNA	double-stranded RNA
ERK	extracellular signal-regulated kinase
HSV	herpes simplex virus
IκB	inhibitor κB
IκBαM	dominant-negative mutant form of IκBα
IRF	IFN regulatory factor
JNK	c-Jun N-terminal kinase
LARC	liver and activation-regulated chemokine
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated gene 5
MIP	macrophage inflammatory protein
NF-κB	nuclear factor κB
polyI:C	polyinosine-polycytidylic acid
RIG-1	RNA helicase retinoic acid-inducible gene
RT	reverse transcription
SOCS	suppressors of cytokine signaling
STAT	signal transducers and activators of transcription
STAT1F	dominant-negative mutant form of STAT1
TLR	Toll-like receptor
TNF	tumor necrosis factor

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Cytokine-immobilized microparticle-coated plates for culturing hematopoietic progenitor cells

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ABSTRACT

The purpose of this study was to provide a culture method for an effective expansion of human CD 34 positive hematopoietic progenitor cells (CD 34 (+) HCs) utilizing low molecular weight heparin/protamine microparticles (LH/P MPs) which can be stably coated onto plastic surfaces and cytokines. CD 34 (+) HCs optimally proliferated on LH/P MP-coated plates in the presence of stem cell factor (SCF; 5 ng/ml), thrombopoietin (Tpo; 10 ng/ml), and Flt-3 ligand (Flt-3; 10 ng/ml) in hematopoietic progenitor growth medium (HPGM). After 6 days, the total cells expanded 16.5-fold. Those cytokines were shown to be partially immobilized on the LH/P MP-coated plates, and the immobilized cytokines were gradually released into the medium with half releasing time of 3–4 days. Since flow cytometry analyses revealed that 90% of initial cells and 44.5% of expanded cells were CD 34 positive, CD 34 (+) HCs were estimated to have increased 8.0-fold after 6 days, and to have increased to over 31.9-fold after 12 days. In contrast, cultured CD 34 (+) HCs on non-coated tissue culture plates increased only 2.9-fold in the identical medium after 6 days, and only 5.2-fold after 12 days.

1. Introduction

Mammalian hematopoiesis originates in bone marrow where a rare population of quiescent stem cells gives rise to expanding populations of committed progenitors that then replenish all of the blood cell lineages throughout the lifetime of the organism. The regulated proliferation, conservation, and differentiation of hematopoietic progenitor cells are believed to occur in intimate contact with the bone marrow microenvironment. This results in the preservation of the hematopoietic progenitor cell pool while permitting controlled cell proliferation and differentiation [1,2]. The progenitor cells are localized in stem cell niches and local area networks in the microenvironment, where they interact with the components of their niche including stromal cells, extracellular matrix proteins, heparan sulfate proteoglycans, and immobilized cytokines. Regional variations in these components within the hematopoietic microenvironment may create niches that are specific for cells at a given stage of differentiation [3–5]. However, the identity and structural characteristics of macromolecules that mediate the formation of such niches are not well known. In addition, a routine method for *ex vivo* amplification of human hematopoietic progenitor cells has not yet been established [6].

Hematopoietic progenitor cells proliferate and mature in semi-solid media when stimulated by exogenous hematopoietic cell growth factors (HCGFs) such as stem cell factor (SCF), thrombopoietin (Tpo), Flt-3 ligand (Flt-3), interleukin (IL)-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [7,8]. They also proliferate in association with bone marrow-derived stromal cells [9,10] although biologically active amounts of HCGFs cannot be detected in stromal culture supernatants [10]. It is possible that HCGFs are synthesized by the stromal cells but remain bound to the stromal cells and/or their extracellular matrix. In fact, it was demonstrated that both natural and recombinant HCGFs, such as IL-3 and GM-CSF, could be adsorbed by heparan sulfate, which is the major sulfated glycosaminoglycan of bone marrow stroma [9,10]. On the other hand, it has been reported that serum-free medium requires large amounts of SCF, Tpo, and Flt-3 to proliferate CD 34 (+) HCs [11,12]. Although such medium is commercially available (hematopoietic progenitor growth medium (HPGM), Lonza Japan Corp. Tokyo, Japan), it is prohibitively expensive. In the present study, we demonstrated that recombinant HCGFs such as SCF, Tpo, and Flt-3 were immobilized onto LH/P MP-coated plates, and the immobilized cytokines were gradually released into the medium. Furthermore, these cytokines, once bound, can be presented in the biologically active form to hematopoietic

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progenitor cells [9,10]. Furthermore, only one-fourth of the concentration of the cytokines recommended by the manufacturer was required for maximal expansion of CD 34 (+) HCs on the LH/P MP-coated plates. These findings may have important implications of the use of heparinoid as an artificial matrix for *ex vivo* expansion of hematopoietic progenitor cells.

Because heparin and low-molecular weight heparin (LH) are known to interact with a variety of functional proteins such as growth factors, cytokines, extracellular matrix components, and adhesion molecules [13,14], heparin could be useful as a therapeutic agent for various pathological conditions in which such functional proteins are involved [15]. But high dose heparin cannot be used because it creates an excessive risk of bleeding [16]. In contrast, LH has pharmacological and practical advantages compared to heparin [17]. The lower protein binding of LH produces a low, stable, and predictable anticoagulant response, thereby obviating the need for laboratory monitoring to adjust the dosage [17]. In addition, one or two subcutaneous injections per day are sufficient to maintain therapeutic concentrations, because of the longer plasma half-life of LH [17].

On the other hand, protamine, a purified mixture of proteins obtained from fish sperm, neutralizes heparin and LH by forming a stable complex that lacks anticoagulant activity [18]. Protamine is also in clinical use as an antidote of heparin to reverse heparin's anticoagulant activity following cardiopulmonary bypass and in cases of heparin-induced bleeding [19]. We previously reported on low molecular weight heparin/protamine microparticles (LH/P MPs), which are about 1–0.5 μm in diameter, which thereby allow FGF-2-containing LH/P MPs to be easily injected [20]. FGF-2-containing LH/P MPs show a substantial effect in inducing vascularization and fibrous tissue formations due to the gradual release of FGF-2 molecules. In this study, we found that LH/P MPs can be stably coated onto plastic surfaces. The purpose of the present study was to evaluate LH/P MP-coated plates as a stem cell niche for the immobilization and the gradual release of hematopoietic cytokines such as SCF, Tpo, and Flt-3 that stimulate the proliferation of human CD 34 positive hematopoietic progenitor cells (CD 34 (+) HCs). Here we report that CD 34 (+) HCs can be expanded 8-fold on LH/P MP-coated plates within 6 days in the presence of lower concentrations of SCF, Tpo, and Flt-3.

2. Methods

2.1. Preparation of low molecular weight heparin/protamine microparticles (LH/P MPs) and LH/P MPs-coated plates

The preparation of LH/P MPs was previously described [20]. Briefly, 0.3 ml of protamine solution (Protamine sulfate; MW: average about 3500; 10 mg/ml; Mochida Pharmaceutical Co., Tokyo, Japan) was added drop by drop to 0.7 ml of LH solution (dalteparin sodium; MW: average about 5000; 6.4 mg/ml; Kissei Pharmaceutical Co., Tokyo, Japan) with slight vortexing for approximately 2 min. In order to maximize the production of the microparticles, protamine and dalteparin solutions were mixed in a 3:7 ratio (vol:vol) in this study. The produced milky solution of LH/P MPs (1 ml) was then subjected to washing twice with phosphate-buffered saline (PBS) to remove unreactants by centrifugation (6000 rpm, 10 min), and the pellet of microparticles finally filled up to 1 ml with PBS. More than 7 mg of dry LH/P MPs was obtained from 1 ml of the LH/P MPs milky solution.

Twelve-well tissue culture plates (3.6 cm^2 of surface area; Sumitomo Bakelite Corp., Tokyo, Japan) were coated overnight at 4 °C with 0.3 ml of 0.7 mg/ml LH/P MPs in PBS solution. The LH/P MPs milky solutions were removed from the wells by pipetting, and the plates were gently washed with PBS. Although LH/P MPs were loosely bound to the plastic surface, the particles were easily rinsed away from the plastic surface by washing. Alternatively, when a 12-well plate with bound LH/P MPs was air-dried for 1 h on a clean bench, the LH/P MPs were stably coated to the plastic surface. It was estimated that about 0.2 mg of LH/P MPs was immobilized to each well of 12-well tissue culture plates. When fluorescence-labeled LH/P MPs were produced using Texas Red-X Protein Labeling Kit (Invitrogen Japan K. K., Tokyo, Japan) and were coated to the plastic plates, the coated LH/P MPs became thin paste and stably covered whole plastic surface (data not shown).

2.2. ELISA for evaluating the binding of cytokines to LH/P MP-coated plates

An ELISA using human recombinant SCF, Tpo, and Flt-3 (Acris Corp. Hiddenhausen, Germany) and LH/P MP-coated plates was performed to evaluate the adsorption of cytokines to the LH/P MP-coated plates. Hematopoietic progenitor growth medium (HPGM; Lonza Japan Corp. Tokyo, Japan) including antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) (200 μl, including the indicated concentrations of SCF, Tpo, and Flt-3) was added to the LH/P MP-coated 48-well tissue culture plates (0.65 cm^2 of surface area; Sumitomo Bakelite Corp., Tokyo, Japan), and bound to the plates for 2 h at 37 °C. The plates were then washed thoroughly with PBS/BSA (0.1%) (bovine serum albumin; Wako Pure Chemical Industries, Ltd. Osaka, Japan) three times. Diluted (1:200 with PBS/BSA) anti-SCF, anti-Tpo, or anti-Flt-3 (R&D Systems Inc. Minneapolis, MN, USA) was added to the plates and incubated for 30 min at room temperature. The plates were again washed thoroughly with PBS/BSA and 200 μg/ml of anti-IgG horseradish peroxidase conjugate (diluted 1:1000 with PBS/BSA) (Bio-Rad Lab., Hercules, CA, USA) was added to the plates and incubated for 30 min at room temperature. Each well was again washed thoroughly with PBS/BSA, and the color was developed by adding 300 μl/ml of horseradish peroxidase substrate solution (Bio-Rad). The plates were mixed for 30 min and 50 μl/ml sulfuric acid (1 M) was finally added to each well to stop the reaction. The plates were read at 450 nm using Mini Plate Reader (Nunc InterMed, Tokyo, Japan).

For experiment on immobilization of cytokines (SCF, Tpo, and Flt-3) onto LH/P MP-coated plates (Fig. 1), HPGM including antibiotics containing each concentration of cytokines (1/2×, 1/4×, 1/8×, and 1/16×) was added into the first LH/P MP-coated plates and incubated at 37 °C for 2 h. The concentrations (1×) of cytokines were 20 ng/ml of SCF, 40 ng/ml of Tpo, and 40 ng/ml of Flt-3. HPGM containing each concentration of cytokines was then added into the second LH/P MP-coated plates and incubated at 37 °C for 2 h. The amounts of immobilized cytokines on LH/P MP-coated plates were quantified using the ELISA as described above.

For experiment on release profiles of cytokines from cytokine-immobilized LH/P MP-coated plates (Fig. 2), amounts of cytokines (SCF, Tpo, and Flt-3) initially immobilized into the cytokine-immobilized LH/P MP-coated plates (day 0) were evaluated using the ELISA as described above. The cytokine-immobilized LH/P MP-coated plates were incubated with HPGM without cytokines and the medium was changed everyday. The amounts of immobilized cytokines on LH/P MP-coated plates were quantified on the indicated days using the ELISA as described above.

For experiment on competitive inhibition of various glycosaminoglycans to the bindings of cytokines to LH/P MP-coated plates (Fig. 3), various concentrations of chondroitin sulfate A (CSA; Seikagaku Corp., Tokyo, Japan) and chondroitin sulfate C (CSC; Seikagaku Corp.), heparin, and LH added into 200 μl of HPGM including the concentration (1/4×) of SCF (5 ng/ml), Tpo (10 ng/ml), and Flt-3 (10 ng/ml)) were applied to the LH/P MP-coated 48-well tissue culture plates, and bound to the plates for 2 h at 37 °C. The plates were then washed thoroughly with PBS/BSA (0.1%)/indicated concentrations of either CSA, CSC, heparin, or LH three times. The amounts of immobilized cytokines on LH/P MP-coated plates were then quantified on the indicated days using the ELISA as described above.

2.3. Culture of human CD 34 positive hematopoietic progenitor cells from bone marrow (CD 34 (+) HCs)

CD 34 (+) HCs were purchased from Takara Bio. Corp., Tokyo, Japan. The cells were plated at an initial density of 30,000 cells/well on either 12-well tissue culture plates (Sumitomo Bakelite Corp.) or LH/P MP-

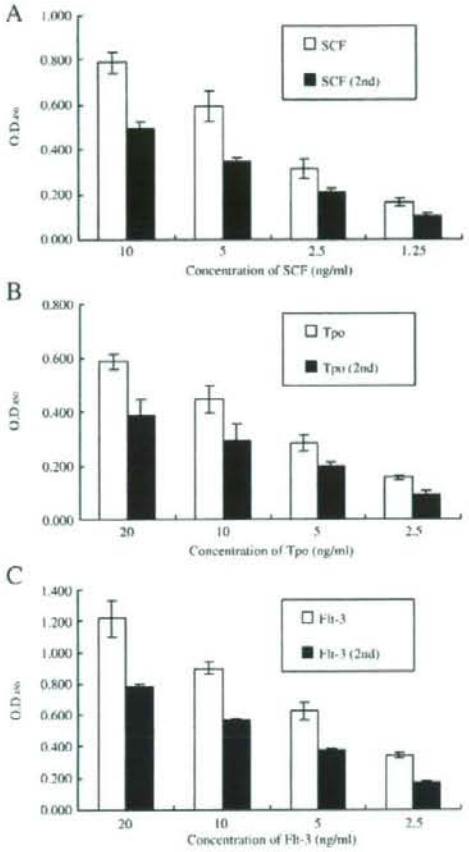

Fig. 1. Immobilization of cytokines (SCF, Tpo, and Flt-3) onto LH/P MP-coated plates. HPGM containing each concentration of cytokines (1/2×, 1/4×, 1/8×, and 1/16×) was added into the first LH/P MP-coated plates and incubated at 37 °C for 2 h (white bars). HPGM containing each concentration of cytokines was then added into the second LH/P MP-coated plates and incubated at 37 °C for 2 h (black bars). The amounts of immobilized cytokines (SCF (A), Tpo (B), and Flt-3 (C)) on LH/P MP-coated plates were quantified using an ELISA as described in Methods. Data represent mean ± SD of six determinations.

coated plates in 2.5 ml of hematopoietic progenitor growth medium (HPGM; Lonza Japan Corp.) including antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin), and the indicated concentrations of recombinant human stem cell factor (SCF; Acris Corp. Hiddenhausen, Germany), thrombopoietin (Tpo; Acris Corp.), Flt-3 ligand (Flt-3; Acris Corp.), and the cells were cultured at 37 °C in an atmosphere of 5% CO_2 in air and 100% relative humidity for the indicated periods. Although a minority of CD 34 (+) HCs loosely adhered to the LH/P MP-coated plates, almost all cells were easily suspended in the medium by several shakes of the plates and the number of cells was counted using a hemocytometer (Sigma Aldrich Japan). The culture medium was changed on days 6 and 10 for 12-days culture.

2.4. Flow cytometry analyses

Cultured CD 34 (+) HCs (about 1.0 × 105 cells) were suspended in 1 ml of stain buffer (FBS (10 ml) + PBS (40 ml) + NaN_3 (0.5 g)) and centrifuged (3000 rpm) for 2 min twice to wash the cells. Five μl of PE labeled anti-human CD 34 (BD Biosciences Pharmingen, San Diego, CA. USA) was added to the pellet and was incubated in an ice bath at 4 °C for 15 min.

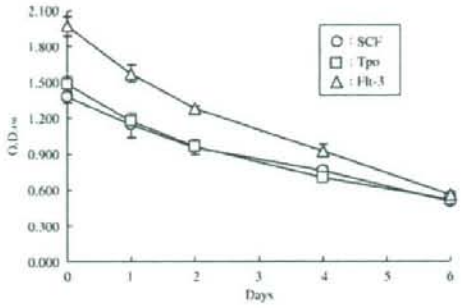

Fig. 2. Release profiles of cytokines from cytokine-immobilized LH/P MP-coated plates. Amounts of SCF (○), Tpo (□), and Flt-3 (△) initially immobilized into the cytokine-immobilized LH/P MP-coated plates (day 0) were evaluated using an ELISA as described in Methods. The remaining cytokines were quantified after incubating for the indicated number of days. Data represent mean ± SD of six determinations.

The cells were washed twice with 1 ml of stain buffer, and then 500 μl of IsoFlow (Beckman Coulter, Fullerton, CA. USA) was added to the pellet. The samples were analyzed using EPICSRXL (Beckman Coulter).

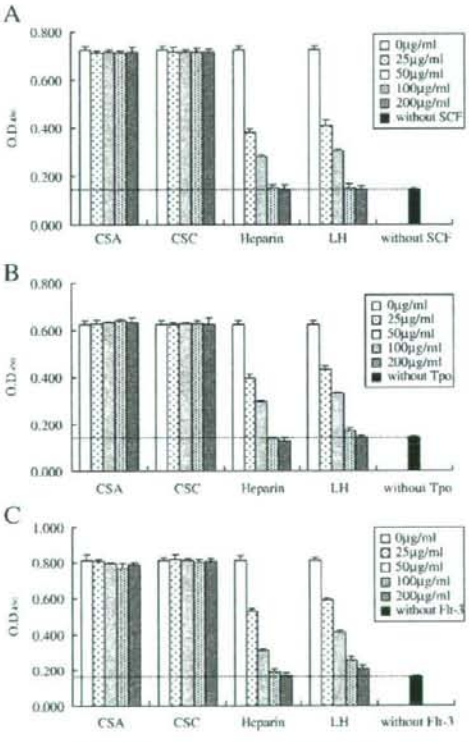

Fig. 3. Competitive inhibition of various glycosaminoglycans to the bindings of cytokines to LH/P MP-coated plates. Various concentrations of CSA, CSC, heparin, and LH added into HPGM including SCF (A), Tpo (B), and Flt-3 (C) were applied to the LH/P MP-coated 48-well tissue culture plates, and bound to the plates for 2 h at 37 °C. The plates were then washed thoroughly with PBS/BSA (0.1%)/either CSA, CSC, heparin, or LH three times. The amounts of immobilized cytokines on LH/P MP-coated plates were then quantified on the indicated days using the ELISA as described in Methods. The broken lines in the figure show the levels of cell growth cultured without SCA (A), Tpo (B), and Flt-3 (C) and any glycosaminoglycan.

3. Results

3.1. Adsorption of cytokines (SCF, Tpo, and Flt-3) to LH/P MP-coated plates

Various cytokines are known to specifically bind to heparin. Although it has not yet been demonstrated that SCF, Tpo, and Flt-3 are heparin-binding proteins, each of these were retained on the LH/P MP-coated plates in a concentration-dependent manner, indicating that all SCF, Tpo, and Flt-3 are heparin-binding cytokines (Fig. 1). In this study we used 48-well culture plates using 0.2 ml of medium to save various expensive reagents such as cytokines and their antibodies for the ELISA. Two hundred μl of various concentrations of SCF, Tpo, and Flt-3 in HPGM was added to the LH/P MP-coated 48-well culture plates and incubated at 37 °C for 2 h. Subsequently, the culture medium containing the indicated concentrations of cytokines was transferred to other LH/P MP-coated 48-well culture plates and incubated at 37 °C for 2 h. When the immobilized cytokines in both the LH/P MP-coated 48-well culture plates were measured by ELISA, the cytokine levels of the latter plates decreased to about 50–70% of the former plates. Furthermore, we observed that the amount of cytokines bound to LH/P MP-coated plates was determined by only the concentrations of cytokine when used more than 0.2 ml of the medium in 48-well plates. These results indicated that roughly 0.4 ng of SCF, 0.7 ng of Tpo, and 0.7 ng of Flt-3 were immobilized onto the LH/P MP-coated 48-well culture plates using lower concentrations (1/4×) of the cytokines in HPGM (Fig. 1).

When the cytokines immobilized on the LH/P MP-coated plates were incubated with HPGM at 37 °C with gentle shaking, approximately 17%, 20%, and 20% of the immobilized SCF, Tpo, and Flt-3, respectively, were found to have been released from the LH/P MP-coated plates within one day, followed by further gradual release after that (Fig. 2). Subsequently, approximately 36%, 35%, and 28% of immobilized SCF, Tpo, and Flt-3, respectively, remained on the LH/P MP-coated plates after incubating for 6 days. Thus, the immobilized cytokines were gradually released into the medium over 6 days.

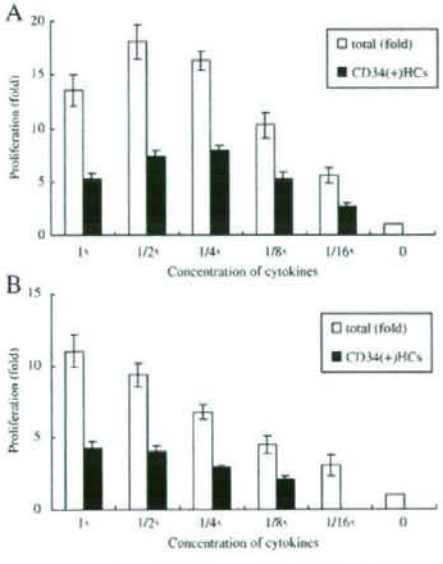

Fig. 4. Optimal concentrations of cytokines. Optimal concentrations of SCF/Tpo/Flt-3 were determined for both LH/P MP-coated (A) and non-coated tissue culture plates (B) with 6 days-incubation. The concentration of SCF (20 ng/ml), Tpo (40 ng/ml), and Flt-3 (40 ng/ml) in HPGM is represented as 1×. White bars represent total HCs and black bars represent CD 34 (+) HCs which were calculated using data from flow cytometry analyses listed in Table 1. Data represent mean ± SD of six determinations.

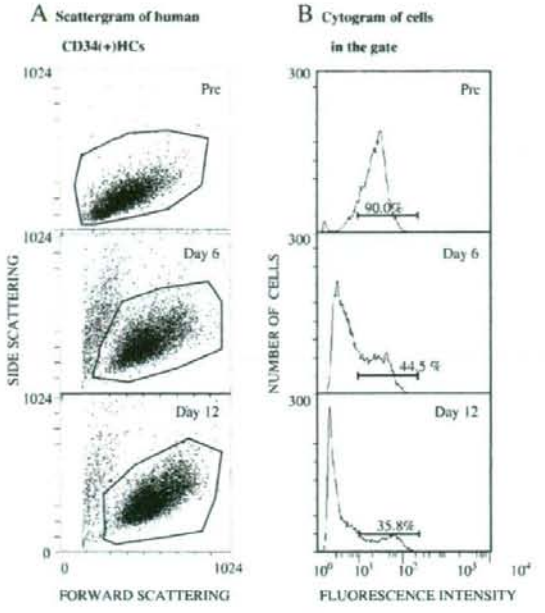

Fig. 5. Scattergram of expanded human CD 34 (+) HCs (A) and cytogram of the cells in the gate (B). The positive ratio of CD 34 (+) HCs in pre-cultured cells (upper panels) and the cultured cells on the LH/P MP-coated plates with lower concentrations (1/4×) of SCF (5 ng/ml), Tpo (10 ng/ml), and Flt-3 (10 ng/ml) in HPGM for 6 days (middle panels) and 12 days (lower panels) were evaluated using flow cytometry data as described in Methods. The data in Tables 1 and 2 were measured using identical flow cytometry analyses.

3.2. Expansion of CD 34 (+) HCs

In this study we used 12-well culture plates using 2.5 ml of the medium to culture the CD34 (+) hematopoietic progenitor cells since more than 100,000 cells were required to evaluate flow cytometry for the marker (CD 34) as well as cell number. When CD 34 (+) HCs were plated on 12-well tissue culture plates, the cells did not adhere to the plates. However, the cells could be grown in suspension culture (without LH/P MP-coating) with high concentrations (1×) of SCF (20 ng/ml), Tpo (40 ng/ml), and Flt-3 (40 ng/ml) in HPGM. The total cell number increased 11-fold after 6 days of culturing (Fig. 4). Since flow cytometry analysis (Fig. 5) revealed that 90% of initial cells (Fig. 6, Table 2) and 34.6% of expanded cells were CD 34 positive (Table 1), CD 34 (+) HCs were estimated to have expanded 4.3-fold after 6 days of culturing (Fig. 4). The effect of total cell expansion on the tissue culture plates (without LH/P MP-coating) decreased with cytokine concentrations, and high concentrations (1×) (SCF (20 ng/ml), Tpo (40 ng/ml), Flt-3 (40 ng/ml)) were required for maximal effect. However, the ratio of CD 34 (+) HCs to the total cells increased with lower concentrations of the cytokines on the tissue culture plates (without LH/P MP-coating) after 6 days as measured by flow cytometry (Table 1).

On the other hand, the total cells cultured on the LH/P MP-coated plates with lower concentrations (1/4×) of SCF (5 ng/ml), Tpo (10 ng/ml), and Flt-3 (10 ng/ml) in HPGM were expanded to 16.3-fold. And CD 34 (+) HCs were estimated to have expanded 8.0-fold (Fig. 4). Although the maximal effect for total cell expansion on the LH/P MP-coated plates was achieved at cytokine concentrations of 1/2× (SCF (10 ng/ml), Tpo (20 ng/ml), and Flt-3 (20 ng/ml)) (Fig. 4), the ratio of CD 34 (+) HCs to the total cells increased with lower concentrations of the cytokines on the LH/P MP-coated plates after 6 days as measured by flow cytometry (Table 1). Considering the total cell growth and the ratio of CD 34 (+) HCs to the total cells, we chose the lower concentrations (1/4×) of SCF (5 ng/ml),

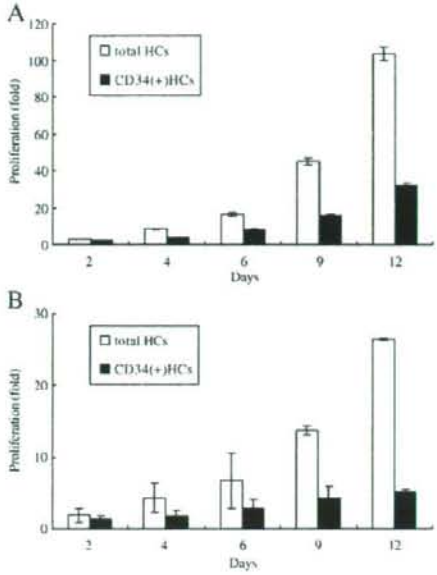

Fig. 6. Expansion of total HCs and CD 34 (+) HCs. Human CD 34 (+) HCs were plated onto LH/P MP-coated (A) and non-coated (B) plates and incubated with the lower concentration (1/4×) of SCF (5 ng/ml), Tpo (10 ng/ml), and Flt-3 (10 ng/ml) in HPGM for the indicated time periods. Total HCs (white bars) were counted using a hemocytometer and CD 34 (+) HCs were calculated using flow cytometry data to figure the ratio of CD 34 (+) HCs to total HCs (Table 2). Data represent mean ± SD of six determinations.

Tpo (10 ng/ml), and Flt-3 (10 ng/ml) in HPGM for optimal culture conditions for CD 34 (+) HCs on LH/P MP-coated plates.

The expansion of CD 34 (+) HCs cultured on the LH/P MP-coated plates with the lower concentrations of SCF, Tpo, and Flt-3 in HPGM were continued for 12 days. Total cells expanded 103-fold after 12 days and CD 34 (+) HCs expanded 31.9-fold (Fig. 6). Furthermore, the ratio of

Table 1
Effect of concentrations of cytokines (SCF, Tpo, and Flt-3) on maintaining CD 34 (+) HCs

	Ratio of CD 34 (+) HCs to expanded total cells (%)					
Concentrations	1×	1/2×	1/4×	1/8×	1/16×	0
On LH/P MP-coated	35.3	37.2	44.5	45.0	48.0	N.D.
On non-coated	34.6	38.8	42.8	45.6	N.D.	N.D.

N.D.: Not determined.
High concentration (1×) indicates 20 ng/ml SCF, 40 ng/ml Tpo, and 40 ng/ml Flt-3. Flow cytometry analyses were carried out after 6 days of culturing. Data represent mean of duplicated determinations.

Table 2
Time course on ratio of CD 34 (+) HCs to the total cells cultured with lower concentrations of the cytokines

	Ratio of CD 34 (+) HCs to expanded total cells	
Culture time	On LH/P MP-coated (%)	On non-coated (%)
Day 0	90.0	90.0
Day 2	66.3	71.2
Day 4	53.4	52.1
Day 6	44.5	42.8
Day 9	43.2	40.6
Day 12	35.8	30.8
Day 15	30.8	19.5

CD34 (+) HCs were cultured with lower concentrations (5 ng/ml SCF, 10 ng/ml Tpo, and 10 ng/ml Flt-3) of cytokines in HPGM for the indicated time periods. Data represent mean of duplicated determinations.

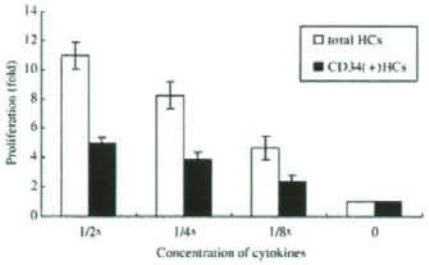

Fig. 7. Expansion of total HCs and CD 34 (+) HCs on cytokines-immobilized LH/P MP-coated plates. Human CD34-positive hematopoietic cells were plated onto cytokines-immobilized LH/P MP-coated plates and incubated at 37 °C for 2 h with HPGM containing various concentrations of cytokines (1/2×, 1/4×, and 1/8×). The cells were also incubated with HPGM without cytokines for 6 days. Total HCs (white bars) were counted using a hemocytometer and CD 34 (+) HCs were calculated using flow cytometry data to figure the ratio of CD 34 (+) HCs to total HCs. Data represent mean ± SD of six determinations.

CD 34 (+) HCs to the total cells as measured by flow cytometry decreased over time: 90% at day 0, 66.3% at day 2, 53.4% at day 4, 44.5% at day 6, 43.2% at day 9, 35.8% at day 12, and 30.8% at day 15 (Table 2). In contrast, when the expansion of CD 34 (+) HCs cultured on tissue culture plates (without LH/P MP-coating) with lower concentrations (1/4×) of the cytokines in HPGM was continued for 12 days, the total cells expanded 26.5-fold and CD 34 (+) HCs expanded only 5.2-fold (Fig. 6). Furthermore, the ratio of CD 34 (+) HCs to the total cells from as measured by flow cytometry decreased over time (Table 2). Furthermore, the pre-immobilized cytokines appeared to be bioactive for proliferation of CD 34 (+) HCs, since the cells grew well on the cytokine-immobilized LH/P MP-coated plates in HPGM without cytokines (Fig. 7).

4. Discussion

In this study, LH/P MP-coated plates serving as hematopoietic stem cell niches could stimulate the growth of CD 34 (+) HCs in the presence of lower concentrations of cytokines (5 ng/ml SCF, 10 ng/ml Tpo, and 10 ng/ml Flt-3) in hematopoietic progenitor growth medium (HPGM). Our observation was that CD 34 (+) HCs grew in suspension without cell adhesion to LH/P MP-coated plates, resulting in 8-fold expansion after 6 days and 31.9-fold after 12 days. Commercially available such a medium (HPGM) is prohibitively expensive, since the manufacturer for the medium is recommending high concentrations of cytokines (20 ng/ml SCF, 40 ng/ml Tpo, and 40 ng/ml Flt-3). CD 34 (+) HCs were estimated to have expanded only 4.3-fold after 6 days of culturing in the HPGM including the high concentration of cytokines without LH/P MP-coating (Fig. 4). Furthermore, flow cytometry analysis (Fig. 5) revealed that the rate of CD 34 (+) in the expanding cells was decreased from 90% to 44.5% during 6 days-culturing (Table 1). Thus, the LH/P MP-coated plate in the presence of lower cytokine concentrations is a useful and low cost biomaterial for rapid expansion of CD 34 (+) HCs. However, we have to continue to do this work to effectively maintain the purity of CD34 (+) cells during the culturing and to clarify the expanded cell population with various surface antigens and with different morphology/function in the stem cells, since the rates of CD 34 (+) in the expanding cells were decreased from 90% to 44.5% during 6 days-culturing.

Hematopoietic progenitor cells are localized in stem cell niches and local area networks in the microenvironment of bone marrow, where they interact with components of their niche including stromal cell surfaces, extracellular matrix proteins, heparan sulfate proteoglycans, and cytokines [9,10]. Heparan sulfate proteoglycans are found in the extracellular matrix produced by the stromal cells. They are prime candidates for selectively retaining hematopoietic progenitor cell stimulatory factors in the niche (stromal layers), and they may regulate

hematopoiesis [9]. In the presented study, the pre-immobilized cytokines (SCF, Tpo, and Flt-3) may provide a matrix like stem cell niches to be bioactive for proliferation of CD 34 (+) HCs, since the cells grew well on the cytokine-immobilized LH/P MP-coated plates in HPGM without those cytokines (Fig. 7).

It is recognized in polymer chemistry that a positively and a negatively charged polymer interact ionically [21,22]. Basic protamine molecules complexed with acidic molecules (low molecular weight heparin; LH) form microparticles through ionic interactions. The amounts of LH and protein (protamine) on the LH/P MP-coated plates used for culturing CD 34 (+) HCs were not changed during at least 7 days (data not shown). However, it seems likely that polypeptides such as SCF, Tpo, and Flt-3, once bound to the LH/P MPs, are gradually released from the LH/P MP-coated plates (decreasing by half within 5 days). Even if LH/P MPs are not biodegradable *in vitro*, incorporating various cytokines such as SCF, Tpo and Flt-3 on the coating will provide an excellent cytokine-containing matrix for hematopoietic progenitor cells [7–10]. No report so far has demonstrated that SCF, Tpo, and Flt-3 are heparin-binding proteins, but our present results demonstrated that all three interacted with LH/P MPs, and that the immobilized cytokines were gradually released into the culture medium over 6 days. Furthermore, it has been shown that heparin addition enhances the expansion of cord blood hematopoietic progenitor cells in three-dimensional coculture with stromal cells [23].

Dalteparin is LH with an anti-coagulant activity much lower than that of native heparin, allowing it to be administered subcutaneously [17,18]. Therefore, dalteparin was used to prepare LH/P MPs in these studies. No bleeding complications were observed in animals injected with LH/P MPs in the previous study [20]. In addition, since the two components for LH/P MPs, LH and protamine, are already in clinical use, their clinical safety is ensured.

Hematopoietic progenitor cells in mice are often defined in cell transplantation experiments by their ability to engraft and to maintain hematopoiesis in lethally irradiated recipients [24]. *Ex vivo* amplified hematopoietic progenitor cells by this method using the cytokines-immobilized LH/P MP-coated plates appear to be usable for cell transplantation. In addition we are now working on effective expansion of mesenchymal stem cells using lower serum (1–2%) and growth factor (FGF-2)-immobilized LH/P MP-coated plates. Furthermore, this cell culture system using LH/P MP-coated plates can be applied for expansions of mouse ES cells with cytokines (LIF etc.) without feeder cells. Thus, the cell culture system using LH/P MP-coated plates may be useful for a large-scale autologous cell culture in cell-based therapies.

5. Conclusion

The purpose of this study was to evaluate LH/P MPs as a stem cell niche for immobilization and gradual release of hematopoietic cytokines such as SCF, Tpo, and Flt-3 that stimulate the growth of CD 34 (+) HCs. Our present results demonstrate that all three cytokines interact with LH/P MPs and were gradually released into the culture medium over 6 days. Here we report that CD 34 (+) HCs can be expanded 8-fold and 31.9-fold on LH/P MP-coated plates after 6 days and 12 days, respectively, in the presence of lower concentrations of SCF (5 ng/ml), Tpo (10 ng/ml), and Flt-3 (10 ng/ml) on LH/P MP-coated plates. It is thus proposed that LH/P MP-coating in the presence of these cytokines may be a promising new biomaterial for the rapid expansion of CD 34 (+) HCs.

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Effect of controlled release of fibroblast growth factor-2 from chitosan/fucoidan micro complex-hydrogel on *in vitro* and *in vivo* vascularization

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Abstract: We produced a chitosan/fucoidan micro complex-hydrogel as a carrier for controlled release of heparin binding growth factors such as fibroblast growth factor (FGF)-2. Material consisting of a soluble chitosan (CH-LA) mixed with fucoidan yielded a water-insoluble and injectable hydrogel with filamentous particles. In this study, we examined the ability of the chitosan/fucoidan complex-hydrogel to immobilize FGF-2 and to protect its activity, as well as the controlled release of FGF-2 molecules. The chitosan/fucoidan complex-hydrogel has high affinity for FGF-2 ($K_d = 5.4 \times 10^{-9} M$). The interaction of FGF-2 with chitosan/fucoidan complex-hydrogel substantially prolonged the biological half-life time of FGF-2. It also protected FGF-2 from inactivation, for example by heat and proteolysis, and enhance FGF-2 activity. When FGF-2-containing complex-hydrogel was subcutaneously injected into the back of mice, significant neovascularization and fibrous tissue formation were induced near the site of injection at 1 week, and the complex-hydrogel was biodegraded and disappeared by 4 weeks. These findings indicate that controlled release of biologically active FGF-2 molecules is caused by both slow diffusion and biodegradation of the complex-hydrogel, and that subsequent induction of vascularization occurs. FGF-2-containing chitosan/fucoidan micro complex-hydrogel is thus useful and convenient for treatment of ischemic disease. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 85A: 619–627, 2008

Key words: chitosan/fucoidan micro complex-hydrogel; fibroblast growth factor-2; stabilization; complex formation; vascularization

INTRODUCTION

Preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia, a concept termed therapeutic angiogenesis.[1] Although many studies using growth factors have been carried out in the field of tissue regeneration and vascularization, use of them has not always been successful *in vivo*.[2] Reasons for this include the high diffusibility and very short half-lives of growth factors *in vivo*. Thus, enhancement of their biological activity *in vivo* is needed for application of growth factors to tissue regeneration and vascularization.

Fibroblast growth factor (FGF)-2 has been characterized well[1] and is currently available as a pharmaceutical agent. It is stored at various sites in the body interacting with heparinoids (heparin and heparin-like molecules),[3] and plays an important role in regeneration processes.[4,5]

FGF-2 binds specifically to heparin and heparan sulfate with high affinity, and its mitogenic activity and biological stability are effected by heparin and heparan sulfate.[6] Heparin and heparan sulfate also protect FGF-2 from inactivation by acid, heat, and degradation by proteases.[6,7] Other studies have shown that heparin and heparan sulfate serve as cofactors to promote binding of FGF-2 to receptors,

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