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Short Communication

Transcriptional downregulation of DC-SIGN in human herpesvirus 6-infected dendritic cells

Hironari Niiya, Taichi Azuma, Lei Jin, Naoyuki Uchida, Atsushi Inoue, Hitoshi Hasegawa, Shigeru Fujita, Mikiko Tohyama, Koji Hashimoto and Masaki Yasukawa

First Department of Internal Medicine and Department of Dermatology, Ehime University School of Medicine, Shigenobu, Ehime 791-0295, Japan

Correspondence
Masaki Yasukawa
yasukawa@m.ehime-u.ac.jp

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DC-SIGN expressed on dendritic cells (DCs) efficiently binds and transmits various pathogens, including human immunodeficiency virus, to lymphoid tissues and permissive cells. Consequently, alteration of DC-SIGN expression may affect susceptibility and resistance to pathogens. The present study shows that infection with human herpesvirus 6 (HHV-6) induces downregulation of DC-SIGN expression on immature DCs. Expression levels of DC-SIGN mRNA and intracellular protein appeared to decrease following infection with HHV-6, indicating that downregulation of surface DC-SIGN occurs at the transcriptional level. Downregulation of DC-SIGN was not induced by inoculation of UV-inactivated HHV-6 or culture supernatant of HHV-6-infected DCs, indicating that replication of HHV-6 in DCs is required for downregulation of DC-SIGN. The present study demonstrates for the first time that expression of DC-SIGN is altered at the transcriptional level by virus infection.

Human herpesvirus 6 (HHV-6) exhibits tropism mainly for leukocytes, including T lymphocytes, monocytes/macrophages and dendritic cells (DCs) (Dockrell, 2003). Among them, DCs are considered to be the professional antigen-presenting cells, possessing the ability to activate the immune response by capturing antigens in peripheral tissues and migrating to secondary lymphoid organs, where they sensitize naive T lymphocytes to the antigens. It has been reported that various viruses, including HHV-6, induce phenotypic and functional alterations of DCs (Salio *et al.*, 1999; Ho *et al.*, 2001; Kakimoto *et al.*, 2002).

DC-SIGN, also called CD209, has recently been identified as a cellular adhesion molecule on DCs by the generation of antibodies that inhibit binding to intracellular adhesion molecule-3 (ICAM-3) (Geijtenbeek *et al.*, 2000a). DC-SIGN is a type II transmembrane protein that belongs to the C-type lectin family. The interaction of DC-SIGN with ICAM-3 mediates the early transient clustering of DCs and T lymphocytes necessary for T lymphocyte activation. Another important function of DC-SIGN is interaction with ICAM-2. The interaction of DC-SIGN with ICAM-2 mediates rolling along endothelial linings and transmigration of DCs into the periphery (Geijtenbeek *et al.*, 2000b).

DC-SIGN has also been identified as the human immunodeficiency virus 1 (HIV-1) *cis*- and *trans*-receptor on DCs (Geijtenbeek *et al.*, 2000c). DC-SIGN binds HIV-1 and transmits it very efficiently to neighbouring permissive target cells. It has been reported that DC-SIGN also interacts with various micro-organisms, including Ebola virus (Alvarez *et al.*, 2002), hepatitis C virus (Pohlmann *et al.*, 2003), human cytomegalovirus (Halary *et al.*, 2002), dengue virus (Tassaneetrithep *et al.*, 2003), *Leishmania* (Colmenares *et al.*, 2002), *Mycobacterium tuberculosis* (Geijtenbeek *et al.*, 2003), *Candida albicans* (Cambi *et al.*, 2003) and *Schistosoma mansoni* (van Die *et al.*, 2003). These recent findings strongly suggest that alteration of DC-SIGN expression may affect the immune response as well as susceptibility to and resistance against various pathogens. In the present study, we examined the effect of HHV-6 on the expression of DC-SIGN and the importance of this molecule in HHV-6 infection of DCs.

The U1102 strain of HHV-6A and the Z29 strain of HHV-6B were used in the present study. Immature DCs were generated from peripheral blood monocytes by culturing in the presence of granulocyte–macrophage colony-stimulating factor and IL4, as described previously (Romani *et al.*, 1996). Immature DCs were inoculated with HHV-6 at an approximate m.o.i. of 1 $TCID_{50}$.

Replication of HHV-6 was examined quantitatively by real-time PCR, as described previously (Locatelli *et al.*, 2000). Sequences of the primers were 5′-TTTGCAGTCATCACG-ATCGG-3′ and 5′-AGAGCGACAAATTGGAGGTTTC-3′. The sequence of the fluorogenic probe was 5′-AGCCACAG-CAGCCATCTACATCTGTCAA-3′.

The expression of cell-surface molecules on DCs was examined by flow cytometric analysis using the following monoclonal antibodies (mAbs): anti-CD80, anti-CD83, anti-CD86 and anti-DC-SIGN (clone AZND1) (all from Immunotech). Hybridomas producing anti-DC-SIGN mAbs 3G2 and 6D1 were established in our laboratory by immunizing mice with human *DC-SIGN* gene-transfected K562 cells (K562-DC-SIGN), which were established as follows. The whole coding region of the *DC-SIGN* gene was obtained by RT-PCR as described previously (Baribaud *et al.*, 2001). The cDNA for *DC-SIGN* was transfected stably into an erythroleukaemia cell line, K562, by use of the pCXN2 vector (Niwa *et al.*, 1991). The mAbs 3G2 and 6D1 specifically recognize K562-DC-SIGN but not parent K562 cells. Expression of intracellular DC-SIGN was examined as follows. Mock-infected and HHV-6-infected DCs were cultured in the presence of Brefeldin A overnight and the cells were then fixed with 3·0% formaldehyde, permeabilized with 0·05% saponin and incubated with FITC-conjugated anti-DC-SIGN mAb AZND1. After being washed, the cells were analysed with a FACSCalibur (Becton Dickinson).

Expression of mRNA for *DC-SIGN* was examined by semi-quantitative RT-PCR and Northern blot analysis. Sequences of the primers for PCR were 5′-CTGCAACTCCTCTCC-TTCAC-3′ and 5′-TCGTTCCAGCCATTGCCACT-3′.

The role of DC-SIGN in *cis*- and *trans*-infection of HHV-6 was examined with K562-DC-SIGN cells and anti-DC-SIGN mAbs. Immature DCs were incubated with anti-DC-SIGN mAbs for 1 h and inoculated with HHV-6. After incubation for 1 h, DCs were washed extensively and cultured for 5 days. HHV-6 copy numbers in DCs treated with and without anti-DC-SIGN mAbs were determined by real-time RT-PCR as described above. To examine *trans*-infection of HHV-6, K562-DC-SIGN and parent K562 cells were inoculated with HHV-6. After incubation for 1 h, the cells were washed extensively and frozen and thawed. These cell lysates were added to cord blood lymphocytes that had been stimulated with phytohaemagglutinin (PHA) and the lymphocytes were cultured for 5 days. HHV-6 copy numbers were determined as described above.

Fig. 1(a) shows phenotypic changes of surface molecules on immature DCs induced by HHV-6B infection. As we reported previously (Kakimoto *et al.*, 2002), the surface expression levels of CD80, CD83 and CD86 increased following infection with HHV-6B. Expression of surface DC-SIGN appeared to decrease significantly after infection with HHV-6B. Downregulation of DC-SIGN appeared to require the replication of HHV-6, since UV light-inactivated HHV-6 showed no effect on DC-SIGN expression (data not shown). The kinetic study showed that downregulation of DC-SIGN on HHV-6-infected DCs was first detectable 3 days after HHV-6 infection. Similar results were also obtained in HHV-6A-inoculated immature DCs (data not shown). Flow cytometric analysis revealed that the expression level of intracellular DC-SIGN in DCs also decreased following infection with HHV-6 (Fig. 1b). As shown in Fig. 1(c), the downregulation of DC-SIGN in HHV-6-infected DCs was confirmed by Western blot analysis using whole-cell lysates.

To investigate the level at which the expression of DC-SIGN was downregulated in HHV-6-infected DCs,

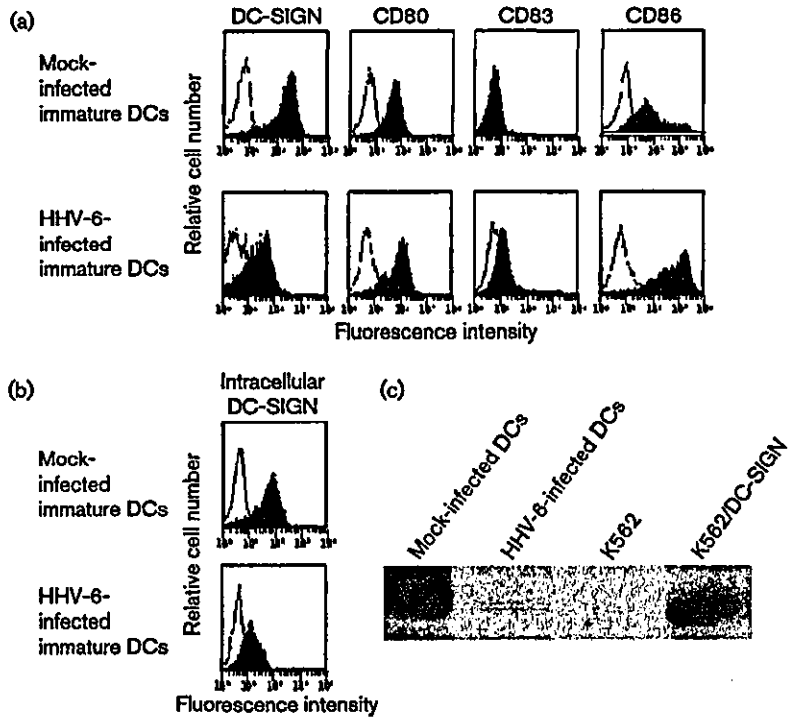

Fig. 1. Downregulation of surface and intracellular DC-SIGN by HHV-6 infection. (a) Flow cytometric analysis of cell-surface molecules on mock-infected and-HHV-6-infected immature DCs. Staining with negative-control Abs is shown as open histograms. (b) Flow cytometric analysis of intracellular DC-SIGN in mock-infected and HHV-6-infected DCs. (c) Western blot analysis of DC-SIGN protein in mock-infected DCs, HHV-6-infected DCs, K562 cells and K562-DC-SIGN cells. Whole-cell extracts were subjected to Western blotting using anti-DC-SIGN mAb.

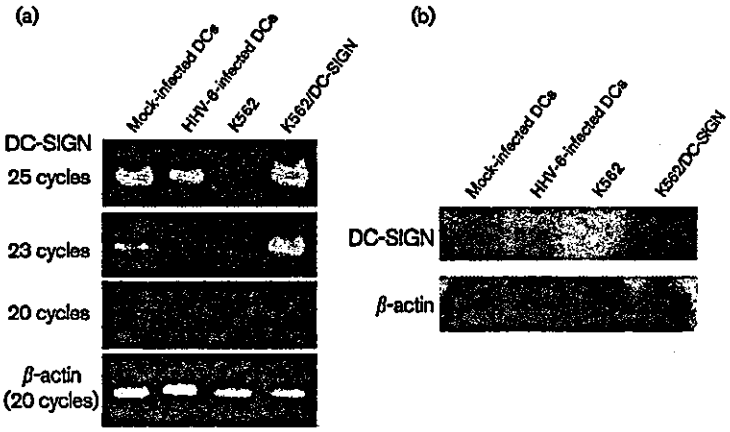

Fig. 2. Transcriptional downregulation of DC-SIGN by HHV-6 infection. Semi-quantitative RT-PCR of mRNA (a) and Northern blot analysis of RNA (b) for *DC-SIGN* in mock-infected DCs, HHV-6-infected DCs, K562 cells and K562-DC-SIGN cells.

semi-quantitative RT-PCR and Northern blot analysis of mRNA for *DC-SIGN* was performed. As shown in Fig. 2(a, b), the expression level of mRNA for *DC-SIGN* decreased significantly following infection with HHV-6, indicating that downregulation of DC-SIGN by HHV-6 infection is induced at the transcriptional level.

DC-SIGN has been reported to act as a receptor for *trans*-infection by various pathogens. In the present study, we investigated the possibility that HHV-6 infects *in cis* or *in trans* via binding with DC-SIGN. Although CD46, a cellular receptor for HHV-6 (Santoro *et al.*, 1999), is expressed on K562 cells, K562-DC-SIGN cells, as well as parent K562 cells, appeared to be resistant to HHV-6 infection. Furthermore, none of the three anti-DC-SIGN mAbs, AZND1, 3G2 and 6D1, which react to different epitopes on DC-SIGN, was able to inhibit HHV-6 infection of immature DCs, which are permissive to HHV-6 infection. In addition, K562-DC-SIGN cells did not transfer HHV-6 to PHA-stimulated cord blood lymphocytes, which are highly sensitive to HHV-6 infection and replication (detailed data not shown). These data indicated that DC-SIGN is not necessary for binding of HHV-6 on the cell surface.

The present study demonstrates for the first time that viral infection directly alters expression of DC-SIGN, which is an important molecule for *trans*-infection by various pathogens, including HIV. It has recently been reported that the expression of DC-SIGN is affected by cytokines: DC-SIGN expression is IL4 dependent and negatively regulated by IFN-α, IFN-γ and transforming growth factor β (Relloso *et al.*, 2002). It has also been reported that HHV-6 infection induces and inhibits production of various kinds of cytokine (Flamand *et al.*, 1991; Mayne *et al.*, 2001; Smith *et al.*, 2003). Taken together, it seems possible that cytokines produced by DCs following infection with HHV-6 might inhibit DC-SIGN expression. However, this possibility seems unlikely, because the repeated addition of culture supernatant from HHV-6-infected DCs did not affect the expression level of DC-SIGN (data not shown). In addition, expression of DC-SIGN was not altered by inoculation of inactivated HHV-6, strongly suggesting that replication of HHV-6 in DCs is essential to induce transcriptional downregulation of DC-SIGN.

Downregulation of DC-SIGN induced by HHV-6 infection may provide new insights into the role of HHV-6 in the pathogenesis of various infectious diseases, especially HIV infection. We reported previously that HHV-6 infection induces downregulation of CXCR-4, resulting in resistance of $CD4^+$ T lymphocytes to X4 HIV infection (Yasukawa *et al.*, 1999; Hasegawa *et al.*, 2001). It was reported that HHV-6 suppressed R5- but not X4-tropic HIV-1 replication in lymphoid tissue (Grivel *et al.*, 2001). Downregulation of DC-SIGN induced by HHV-6 infection may render DCs resistant to HIV infection. On the other hand, HHV-6 infection was reported to induce upregulation of CD4 (Lusso *et al.*, 1991), which is the main receptor for HIV. In addition, HHV-6 was shown to induce *trans*-activation of the HIV long terminal repeat and cell death (Ensoli *et al.*, 1989). Therefore, the interaction between HHV-6 and HIV appears to be complex. Further *in vivo* studies using HHV-6-permissive animals are necessary to clarify this important issue.

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Keratinocyte G2/M Growth Arrest by 1,25-Dihydroxyvitamin D3 Is Caused by Cdc2 Phosphorylation Through Wee1 and Myt1 Regulation

Xiuju Dai, Kenshi Yamasaki, Lujun Yang, Koji Sayama, Yuji Shirakata, Sho Tokumara, Yoko Yahata, Mikiko Tohyama, and Koji Hashimoto
Department of Dermatology, Ehime University School of Medicine, Ehime, Japan

1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) has an antiproliferative effect on keratinocyte growth, and its derivatives are used for the treatment of psoriasis. It was reported previously that 1,25[OH]$_2$VD3 induced cell cycle arrest not only at the G0/G1 phase but also at the G2/M phase. However, the mechanism of 1,25[OH]$_2$VD3-induced G2/M phase arrest in keratinocytes has not been fully understood. The addition of 10^{-8} to 10^{-6} M 1,25[OH]$_2$VD3 to cultured normal human keratinocytes enhanced the expression of Myt1 mRNA preceding Wee1 mRNA; 10^{-6} M 1,25[OH]$_2$VD3 unregulated Myt1 mRNA from 6 h to 24 h and Wee1 mRNA from 12 to 48 h. Interestingly, the levels of phosphorylated Cdc2 were increased between 6 h and 48 h after 1,25[OH]$_2$VD3 treatment, although the expression levels of Cdc2 mRNA and its protein production were reduced. 1,25[OH]$_2$VD3 also decreased the expression of cyclin B1, which forms a complex with Cdc2. These data indicated that the increase of Myt1 and Wee1 induced the phosphorylation of Cdc2 leading to G2/M arrest. In conclusion, the induction of Cdc2 phosphorylation due to the increase of Wee1 and Myt1 as well as the reduction of Cdc2 and cyclin B1 are involved in 1,25[OH]$_2$VD3-induced G2/M arrest of keratinocytes.

Key words: 1,25-dihydroxyvitamin D3/Keratinocyte/Wee1/Myt1/Cdc2/cell cycle
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The compound 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3), which is the hormonal form of vitamin D3, regulates cell growth in a variety of cells (Walters, 1992). The skin is one of the target organs for 1,25[OH]$_2$VD3, and the vitamin D receptor (VDR) is present in the cells of both the epidermis and the dermis (Stumpf *et al*, 1979, 1984; Pillai *et al*, 1988; Milde *et al*, 1991). The effects of 1,25[OH]$_2$VD3 on keratinocytes have been investigated extensively, and well characterized *in vitro* and *in vivo*. In the *in vitro* studies, 1,25[OH]$_2$VD3 demonstrated a potent inhibitory effect on proliferation, and promoted the differentiation of both murine and human keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986; Matsumoto *et al*, 1990; Kobayashi *et al*, 1993; Gniadecki, 1996; Kobayashi *et al*, 1998; Segaert *et al*, 2000). The critical effects on the epidermis are also manifested *in vivo* (Holick *et al*, 1987; Dubertret *et al*, 1992; el-Azhary *et al*, 1993; Langner *et al*, 1993).

Several reports have demonstrated that 1,25[OH]$_2$VD3 suppresses keratinocyte growth through cell cycle regulation. The most distinguished effect of 1,25[OH]$_2$VD3 on cell cycle regulation is G1 block. G1 block is associated with changes of various kinds of cell cycle regulatory molecules: increased levels of p21 (Segaert *et al*, 1997; Zhuang and Burnstein, 1998; Moffatt *et al*, 2001), p27 (Segaert *et al*, 1997; Wang *et al*, 1997), and transforming growth factor-βGF-β (Segaert *et al*, 1997), decreased Cdk2 activity (Wang *et al*, 1997; Zhuang and Burnstein, 1998), reduced levels of cyclin E (Zhang *et al*, 1996), repressed E2F transactivation (Zhuang and Burnstein, 1998), and retinoblastoma hypophosphorylation (Kobayashi *et al*, 1993; Segaert *et al*, 1997). Interestingly, 1,25[OH]$_2$VD3 induces the accumulation of cells not only in the G1 compartment but also in the G2/M compartment. These features have been noted in HL60 cell cultures (Godyn *et al*, 1994; Zhang *et al*, 1996; Harrison *et al*, 1999), in breast cancer cell lines (Eisman *et al*, 1989a, b), and also in human keratinocytes (Kobayashi *et al*, 1993). In contrast to the numerous studies on the mechanism of G1 block in 1,25[OH]$_2$VD3-treated keratinocytes, the underlying mechanism of G2 block induced by 1,25[OH]$_2$VD3 in keratinocytes has not been reported.

Orderly progression through the cell cycle is mediated by the activation of a highly conserved family of protein kinases, namely, cyclin-dependent kinases (Cdks) (Pines, 1995; Morgan, 1997). Activation of a particular Cdk requires binding to a specific regulatory subunit, which is termed a cyclin. A pivotal regulatory step for G2/M transition in eucaryotes is the activation of the cell division cycle (Cdc)2/cyclin B complex (initially called the maturation or mitosis promoting factor, MPF) (Coleman and Dunphy, 1994; Morgan, 1997). Cdc2/cyclin B is maintained in an inactive

Abbreviations: ATP, adenosine triphosphate; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; 1,25[OH]$_2$VD3, 1,25-dihydroxyvitamin D3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPA, ribonuclease protection assay; RXR, retinoid X receptor; Thr, threonine; Tyr, tyrosine; VDR, vitamin D receptor; VDRE, vitamin D response elements

form during the S and G2 phases by inhibitory phosphorylation of the Cdc2 residues threonine 14 (Thr14) and tyrosine 15 (Tyr15), which are positioned within the adenosine triphosphate (ATP)-binding cleft (Gould and Nurse, 1989; Krek and Nigg, 1991; Jin *et al*, 1996). The inhibitory phosphorylation of Cdc2 is modulated by the Wee1 family protein kinases, i.e., human Wee1 and Myt1. The Wee1 gene product is a tyrosine-specific protein kinase, which locates in nucleus and phosphorylates Cdc2 exclusively at Tyr15 (McGowan and Russell, 1993, 1995). The Myt1 kinase, which is a homologue of the product of the *mik1* gene, is a dual-specificity protein kinase that phosphorylates Cdc2 at both the Thr14 and Tyr15 residues (Booher *et al*, 1997; Fattaey and Booher, 1997; Gould and Nurse, 1989; Krek and Nigg, 1991; Mueller *et al*, 1995). Myt1 kinase is a membrane-associated protein that localizes to the endoplasmic reticulum and Golgi complex (Liu *et al*, 1997); except for inhibitory phosphorylation of Cdc2, Myt1 seems to influence normal shuttling of the Cdc2/cyclin B complex into the nucleus (Liu *et al*, 1999). Phosphorylation of Tyr15 and Thr14 suppresses catalytic activity of Cdc2 by disrupting the orientation of the ATP molecule to the ATP-binding cleft of Cdc2 (Atherton-Fessler *et al*, 1993; De Bondt *et al*, 1993). Cdc25 family members, which are dual-specificity phosphatases, have been identified as positive regulators of Cdc2. They counteract Wee1/Myt1 activities (Russell and Nurse, 1986; Sebastian *et al*, 1993). Dephosphorylation of the Thr14 and Tyr15 residues of Cdc2 by Cdc25B (in the cytoplasm) and Cdc25C (in the nucleus) in the late G2 phase activates the cyclin B/Cdc2 complex directly, and the accumulated, active Cdc2/cyclin B1 then triggers the initiation of mitosis (Dunphy and Kumagai, 1991; Strausfeld *et al*, 1991; Coleman and Dunphy, 1994; Karlsson *et al*, 1999). Therefore, the active status of the Cdc2/cyclin B complex is controlled by several G2/M-specific cell cycle regulatory kinases and phosphatases that reversibly phosphorylate Cdc2.

To elucidate the mechanism of G2 block induced in human keratinocytes by 1,25[OH]$_2$VD3, we investigated the effects of 1,25[OH]$_2$VD3 on the regulatory molecules of G2/M transit. We demonstrated here that 1,25[OH]$_2$VD3 increased Wee1 and Myt1, and the phosphorylated Cdc2. This provides a potential mechanism for the accumulation of VD3-treated keratinocytes in the G2/M compartment.

Results

cDNA microarray analysis of the effect of 1,25[OH]$_2$VD3 on Wee1, cyclin B1, Cdc25B, and Cdc25C in human keratinocytes We initially studied the expression of cell cycle-related genes in 1,25[OH]$_2$VD3-treated keratinocytes using the cDNA microarray technique. Six hours of stimulation with 1,25[OH]$_2$VD3 modified the expression patterns of four genes that are thought to be important regulators of G2/M transition (Fig 1). The expression of the Wee1 mRNA, whose protein product phosphorylates Cdc2 at Tyr15 and suppresses Cdc2 activity, was induced after 1,25[OH]$_2$VD3 treatment. In contrast, the expression levels of cyclin B1, Cdc25B, and Cdc25C were suppressed to various degrees by 1,25[OH]$_2$VD3. These data suggest the possibility that 1,25[OH]$_2$VD3 induces G2/M cell cycle arrest in keratinocytes by affecting Cdc2/cyclin B1.

Effect of 1,25[OH]$_2$VD3 on Wee1 and Myt1 in human keratinocytes The cDNA microarray analysis showed that 1,25[OH]$_2$VD3 upregulated the expression of the Wee1 gene. To ascertain the effect of 1,25[OH]$_2$VD3 on Wee1, we examined the level of Wee1 mRNA, and Wee1 protein in 1,25[OH]$_2$VD3-treated human keratinocytes. Wee1 mRNA expression was upregulated gradually in a time-dependent manner from 12 h up to 48 h by 10^{-6} M of 1,25[OH]$_2$VD3 (Fig 2*A*). The optimum induction of Wee1 mRNA was 6-fold at 48 h compared with the control (vehicle). Wee1 protein began to increase at 12 h and reached the maximum, 3-fold at 48 h (Fig 2*B*). This time course of Wee1 protein induction is consistent with that of Wee1 mRNA. Furthermore, 1,25[OH]$_2$VD3 increased Wee1 mRNA expression in a concentration-dependent manner, 1.6-fold at 10^{-8} M, 2.4-fold increase at 10^{-7}M, and 4.0-fold 10^{-6} M (Fig 2*C*). Wee1 protein was increased almost similarly by the addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} (Fig 2*D*). These data demonstrate that 1,25[OH]$_2$VD3 increases both Wee1 mRNA and Wee1 protein similarly in human keratinocytes.

Since Myt1 is a Wee1 family kinase that has been shown to phosphorylate Cdc2 both at Tyr15 and Thr14 (Mueller *et al*, 1995; Booher *et al*, 1997; Fattaey and Booher, 1997), we examined the effect of 1,25[OH]$_2$VD3 on Myt1 expression. 1,25[OH]$_2$VD3 enhanced the expression of Myt1 mRNA (Fig 3*A*). But the time course of Myt1 mRNA induction by 1,25[OH]$_2$VD3 was distinguished from that of Wee1 mRNA. Myt1 mRNA increased markedly from 6 to 12 h, and decreased at 24 h. Then, Myt1 mRNA expression returned to the baseline level at 36 h. Myt1 protein increased and decreased in the same time course as Myt1 mRNA (Fig 3*B*). 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M increased Myt1 mRNA, 2.3-, 3-, and 1.5-fold, respectively, compared with the control (vehicle) (Fig 3*C*). The optimum induction of Myt1 protein was also observed at 10^{-7} M of 1,25[OH]$_2$VD3 (Fig 3*D*). Taken together, 1,25[OH]$_2$VD3 induces Myt1 kinase preceding Wee1 kinase in human keratinocytes. Myt1 is localized in the cytoplasm and Wee1 is localized in the nucleus. This difference of localization may affect the time lag of Myt1 and Wee1 induction by 1,25[OH]$_2$VD3 in human keratinocytes.

Effect of 1,25[OH]$_2$VD3 on Cdc2 and phosphorylation of Cdc2 in human keratinocytes Next, we assessed the

Figure 1
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) regulates the expression of regulators of G2/M transit. Normal human keratinocytes were cultured for 6 h in MCDB medium that contained 1,25[OH]$_2$VD3 (10^{-6} M) or vehicle, and RNA samples from these cells were analyzed using the cDNA microarray. Red, green, and yellow signify increased, decreased, and unchanged gene expression, respectively, in response to 1,25[OH]$_2$VD3. *A*, Wee1; *B*, cyclin B1; *C*, Cdc25B; *D*, Cdc25C.

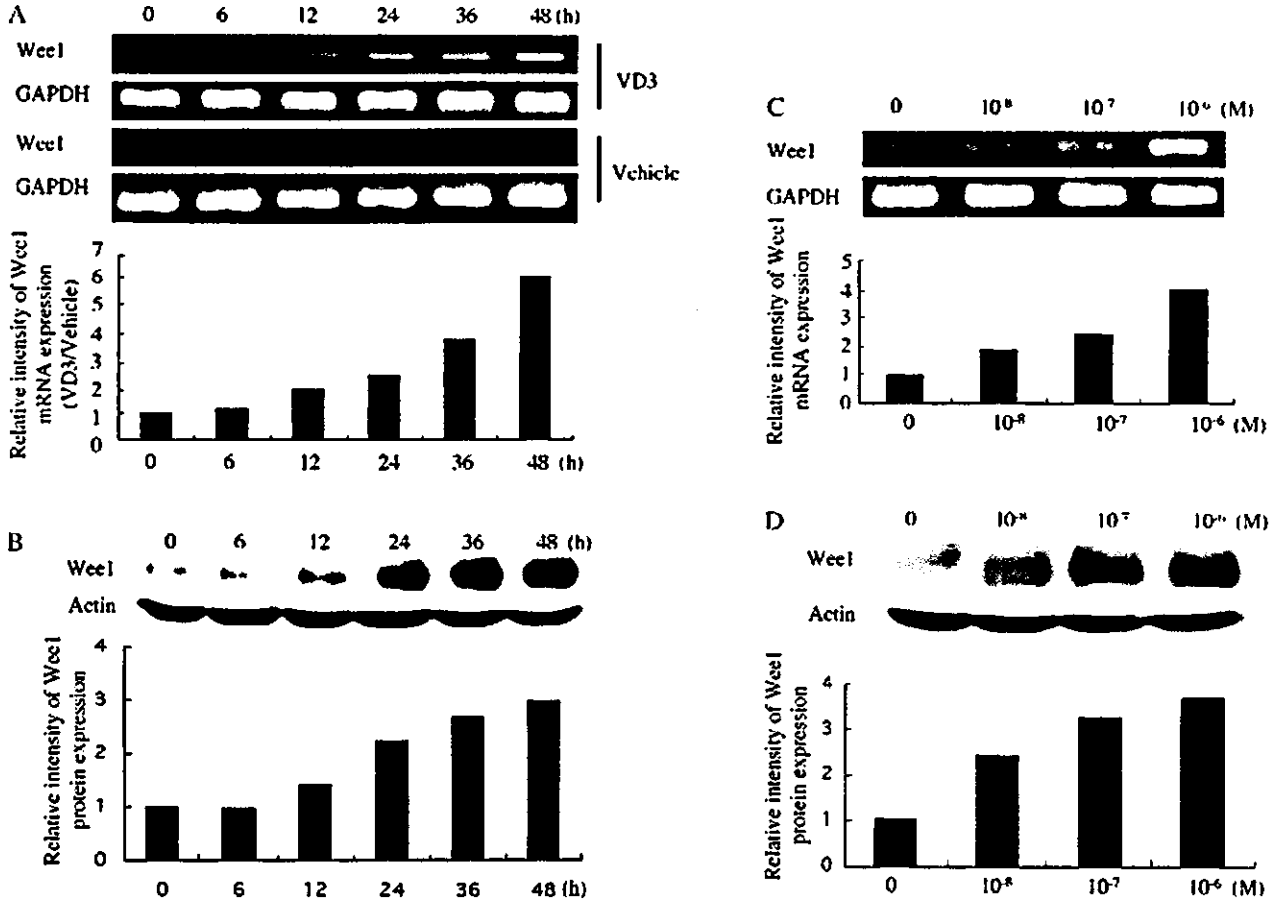

Figure 2
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) increases the expression of Wee1 mRNA and protein. (*A*) Keratinocytes were incubated for the indicated time periods in MCDB medium that contained either 1,25[OH]$_2$VD3 (10^{-6} M) or the vehicle, and total RNA samples were collected. RT-PCR was performed using specific primers for human Wee1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Wee1 mRNA expression was firstly estimated using GAPDH as the internal reference, and then the relative levels of Wee1 mRNA for each time point in 1,25[OH]$_2$VD3-treated cells was estimated by using that of vehicle-treated cells at a corresponding time point. Lastly, the relative Wee1 mRNA expression was normalized against that at 0 h as 1 U, and plotted in the graph. (*B*) Keratinocytes were cultured and stimulated as described in (*A*), and protein was extracted at the indicated time. Western blotting was performed, and the levels of Wee1 protein expression were estimated using actin as the internal reference and normalized against the value of 0 h as 1 U. (*C*) Keratinocytes were cultured in different concentrations of 1,25[OH]$_2$VD3 or vehicle for 24 h. Wee1 mRNA expression was estimated using GAPDH as the internal reference, and normalized against the relative value for the vehicle-treated sample (0 M) as 1 U. (*D*) Keratinocytes were cultured and stimulated as described in (*C*), and protein was extracted after 24 h of stimulation. Western blotting was performed and the levels of Wee1 protein expression were estimated using actin as the internal reference and normalized against the value for the vehicle-treated sample (0 M) as 1 U.

effect of 1,25[OH]$_2$VD3 on Cdc2 and phosphorylated Cdc2, since Wee1 and Myt1 participate in the inhibitory phosphorylation of Cdc2 (McGowan and Russell, 1993; McGowan and Russell, 1995; Mueller *et al*, 1995; Booher *et al*, 1997; Fattaey and Booher, 1997).

The addition of 10^{-6} M 1,25[OH]$_2$VD3 suppressed the expression of the Cdc2 mRNA in a time-dependent manner (Fig 4*A*). Cdc2 mRNA began to decrease at 12 h, and was barely detectable at 36 h. Western blotting demonstrated that 10^{-6} M of 1,25[OH]$_2$VD3 reduced Cdc2 protein at 12 h, and was barely detectable at 36 and 48 h like Cdc2 mRNA expression (Fig 4*B*).

Interestingly, phosphorylated Cdc2 was upregulated by 1,25[OH]$_2$VD3, although the overall level of Cdc2 protein expression was decreased (Fig 4*B*). The level of phosphorylated Cdc2 increased from 6 h and reached a plateau at 24–48 h. Therefore, the relative ratio of phosphorylated Cdc2/total Cdc2 increased in a time-dependent manner, about 10-fold at 48 h. Cdc2 protein was markedly reduced in a concentration-dependent manner by addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M (Fig 4*C*). In contrast, phosphorylated Cdc2 was increased almost equally by 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M (Fig 4*C*).

Taken together, these data indicate that treatment with 1,25[OH]$_2$VD3 suppresses Cdc2 production and inhibits its activity in human keratinocytes. This seems likely to result in G2/M growth arrest of 1,25[OH]$_2$VD3-treated keratinocytes eventually.

Effect of 1,25[OH]$_2$VD3 on cyclin B1 in human keratinocytes The cyclin B1 subunit is essential for Cdc2 activity, and the level of cyclin B1 affects the activity of Cdc2 and progression from the G2 to the M phase. So, we examined the effect of 1,25[OH]$_2$VD3 on cyclin B1 mRNA and protein. The addition of 10^{-6} M 1,25[OH]$_2$VD3 induced the decrease of both cyclin B1 mRNA and cyclin B1 protein in a time-

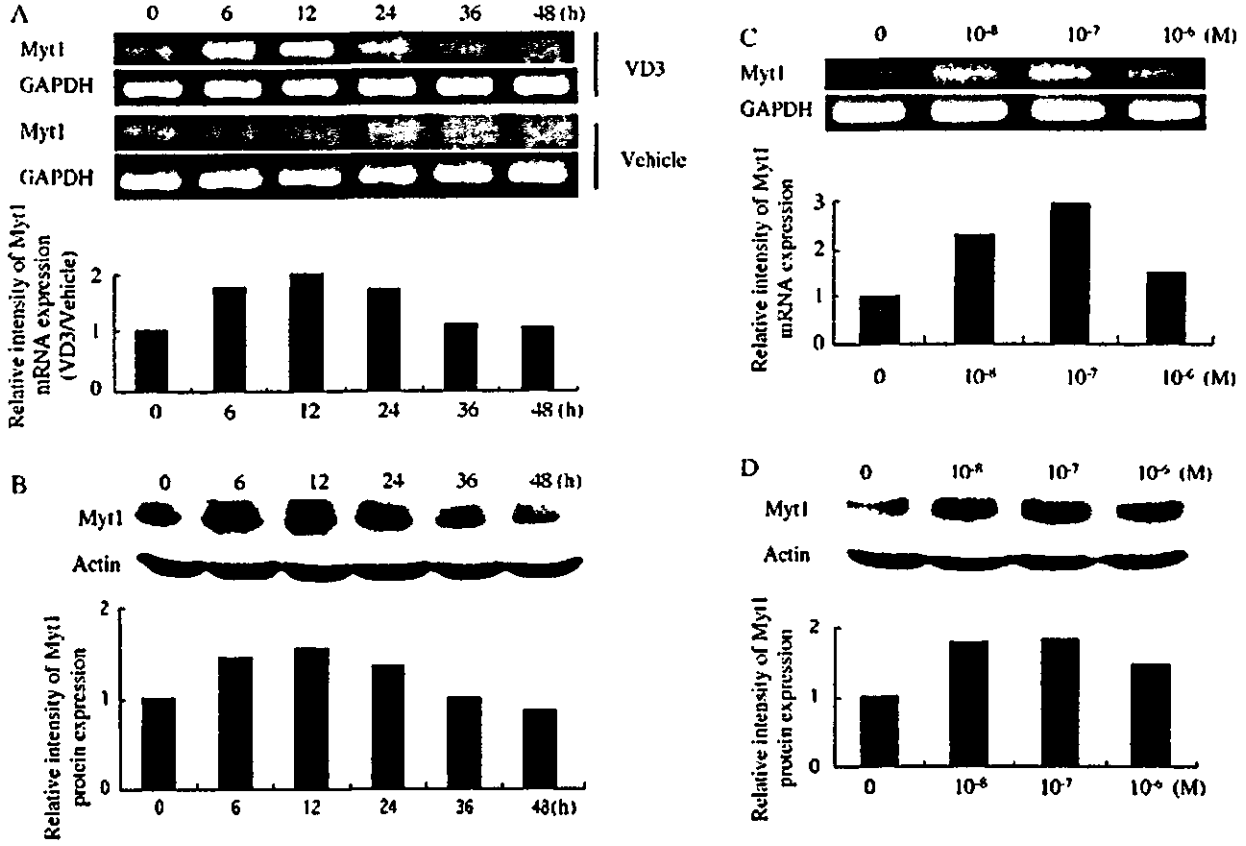

Figure 3
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) increases the expression of Myt1 mRNA and Myt1 protein. (*A*) RT-PCR was performed using specific primers for human Myt1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the relative intensity of Myt1 mRNA expression was estimated as described in Fig 2*A*. (*B*) Western blotting was performed as described in Fig 2*B*, and the levels of Myt1 protein expression were estimated and normalized as described in Fig 2*B*. (*C*) Keratinocytes were cultured in different concentrations of 1,25[OH]$_2$VD3 or vehicle for 12 h. The Myt1 mRNA expression was estimated and normalized as described in Fig 2*C*. (*D*) Keratinocytes were cultured and stimulated as described in (*C*), and proteins were extracted after 12 h of stimulation. Western blotting was performed, and the levels of Myt1 protein expression were estimated and normalized as described in Fig 2*D*.

dependent manner (Figs 5*A*, *B*). In addition, cyclin B1 mRNA and protein were reduced in a concentration-dependent manner by addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M(Figs 5*C*, *D*), although cyclin B1 mRNA decreased more remarkably than cyclin B1 protein. This suppression of cyclin B1 may also contribute to G2/M growth arrest of 1,25[OH]$_2$VD3-treated keratinocytes.

Discussion

We used a cDNA microarray to conduct a systematic analysis of 1,25[OH]$_2$VD3-dependent cell cycle regulation, and found that 1,25[OH]$_2$VD3 strongly induced the Wee1 family in keratinocytes. Accompanying Wee1 and Myt1 induction, 1,25[OH]$_2$VD3 induced inhibitory phosphorylation of Cdc2. This paper reports that Wee1 and Myt1 are target molecules of 1,25[OH]$_2$VD3, and that Wee1 and Myt1 might be key molecules in 1,25[OH]$_2$VD3-induced G2/M arrest in keratinocytes. 1,25[OH]$_2$VD3 is reported to induce G2/M arrest in other cells (Godyn *et al*, 1994; Harrison *et al*, 1999). Wee1 and Myt1 might be involved in the 1,25[OH]$_2$VD3-induced G2/M arrest of these cells.

Wee1 inhibits mitosis (McGowan and Russell, 1995). Despite extensive studies of the functions of the Wee1 family, there are few reports on their mechanisms. Interestingly, we found a time lag between Myt1 (6–12 h post-stimulation) and Wee1 (12–48 h post-stimulation) induction, which resulted in continuous phosphorylation of Cdc2 (6–48 h post-stimulation). Wee1 phosphorylates Cdc2 in the nucleus, whereas Myt1 phosphorylates Cdc2 in the cytoplasm. The significance of this localization and the mechanism producing the time lag between Myt1 and Wee1 induction is unclear. We speculate that Myt1 kinase phosphorylates and inhibits Cdc2 activation and nuclear translocation of the Cdc2/cyclin B complex during the early period of 1,25[OH]$_2$VD3 treatment. Moreover, Cdc2 phosphorylation is retained as high in the late period of 1,25[OH]$_2$VD3 treatment, whereas the level of Wee1 increases steadily. Therefore, 1,25[OH]$_2$VD3 might keep Cdc2 inactive with the sequential increase in the two kinases: Myt1 and Wee1 (Fig 6).

1,25[OH]$_2$VD3 is a physiologically active ligand for VDR. VDR forms stable receptor complexes preferentially as heterodimers with the retinoid X receptor (RXR). The VDR-RXR dimer binds vitamin D response elements (VDRE), which are located in the promoters of 1,25[OH]$_2$VD3-

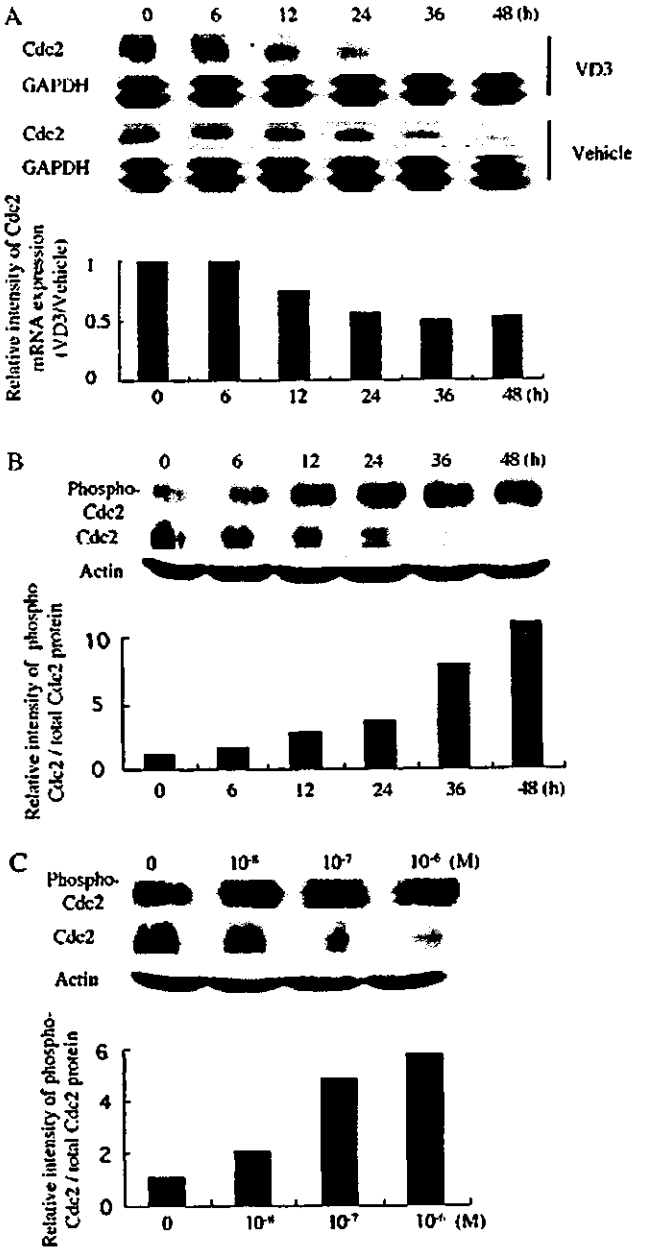

Figure 4
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) downregulates the expression of Cdc2 mRNA and Cdc2 protein, and increases the level of phospho-Cdc2 protein. (*A*) Total RNA samples were collected as described in Fig 2*A*. Cdc2 mRNA was detected by ribonuclease protection assay, and the relative levels of Cdc2 mRNA expression were estimated as described in Fig 2*A* and plotted on the graph. (*B*) Western blotting was performed as described Fig 2*B*. The levels of Cdc2 and phospho-Cdc2 protein expression were, respectively, estimated against actin, the relative level of phospho-Cdc2 at each point was estimated using total Cdc2 expression as the reference, and then normalized against the relative value at 0 h as 1 U. (*C*) Keratinocytes were cultured and stimulated as described Fig 2*C*, and protein was extracted after 24 h of stimulation. Western blotting was performed, and Cdc2 and phospho-Cdc2 protein level at each point was estimated using actin as an internal reference. The relative levels of phospho-Cdc2 against total Cdc2 protein at each time point were normalized against that relative value at 0 M as 1 U.

regulated genes. The Wee1 promoter does not contain a confirmed VDRE, so the VDR-VDRE pathway might not regulate Wee1 directly. A recent study found one activator protein 1 (AP-1)-binding motif in the Wee1 promoter region, and c-Fos transactivates the Wee1 kinase gene directly (Kawasaki *et al*, 2001). In addition, prolonged c-Fos expression elicits abnormally increased expression of the Wee1 gene, which induces and maintains inactive phosphorylation of Cdc2 kinase. Another study reported that 1,25(OH)$_2$VD3 stimulates AP-1 DNA-binding activity in keratinocytes (Johansen *et al*, 2003), and we confirmed that 1,25(OH)$_2$VD3 increased c-Fos mRNA expression and AP-1 transcription beginning 6 h after stimulation in a time-dependent manner (unpublished data). The increased c-Fos and activated AP-1 transcription seem to be dependent on the binding of 1,25[OH]$_2$VD3 to its surface membrane receptor, annexin II (Johansen *et al*, 2003). Combined with these data, this AP-1 activation induced by 1,25(OH)$_2$VD3 might contribute to 1,25(OH)$_2$VD3-dependent Wee1 expression and inactive phosphorylation of Cdc2 kinase, and this signal pathway might be independent of VDR.

Previous reports have indicated that Cdc2 phosphorylation is not the only mechanism that regulates G2 arrest, but that Cdc2 and cyclin B1 levels are also related to the G2/M transition (Kao *et al*, 1997; McVean *et al*, 2002). We found that 1,25[OH]$_2$VD3 decreased Cdc2 and cyclin B1 expression in human keratinocytes. Combined with the fact that 1,25[OH]$_2$VD3 increases inhibitory phosphorylation of Cdc2, 1,25[OH]$_2$VD3 suppresses Cdc2 activation efficiently, since almost all the Cdc2 is inhibited by phosphorylation by 1,25[OH]$_2$VD3, as shown in Figs 4*B, C*. Cdc2 expression appears to be regulated at the mRNA level, and to begin 12 h after treatment. We postulate that 1,25[OH]$_2$VD3 suppresses Cdc2 transcription. The basal promoter region of human Cdc2 contains the E2F, E-box, and Sp1 motifs. Of these, the E2F motif is important for activating the Cdc2 promoter (Shimizu *et al*, 1995). In the pRb/E2F pathway, pRb is phosphorylated (inactive) and hypophosphorylated (active) during the cell cycle, and hypophosphorylated pRb appears to repress gene transcription via its interaction with E2F (Harbour and Dean, 2000). 1,25(OH)$_2$VD3 induces hypophosphorylation of pRb in keratinocytes within 6 h of treatment (Kobayashi *et al*, 1993). Therefore, 1,25(OH)$_2$VD3 might suppress Cdc2 transcription via pRb activation and consequent E2F silencing in keratinocytes. In fact, 1,25(OH)$_2$VD3 blocks the transcription of E2F-regulated genes, such as cyclins A and E, via the G1 CDK-pRb-E2F pathway in MCF-7 cells (Jensen *et al*, 2001). Treatment of keratinocytes with TGF-β1 also results in the formation of a DNA-binding complex between pRb and E2F, which contributes to suppressing the E2F-regulated Cdc2 gene and inhibiting cell cycle progression (Herzinger *et al*, 1995). Cyclin B1 and Cdc25 are also E2F-target genes. This suggests an interesting link in 1,25(OH)$_2$VD3-treated keratinocytes between the G1 CDk-pRb-E2F pathway and the mitotic kinase Cdc2. Since the CDk inhibitor p21 is a 1,25(OH)$_2$VD3-regulated gene and 1,25(OH)$_2$VD3 increases p21 expression in a VDRE-dependent manner (Liu *et al*, 1996), 1,25(OH)$_2$VD3 probably decreases Cdc2 expression via a VDR-dependent pathway. c-Myc deregulation by 1,25(OH)$_2$VD3 in keratinocytes (Matsumoto *et al*, 1990)

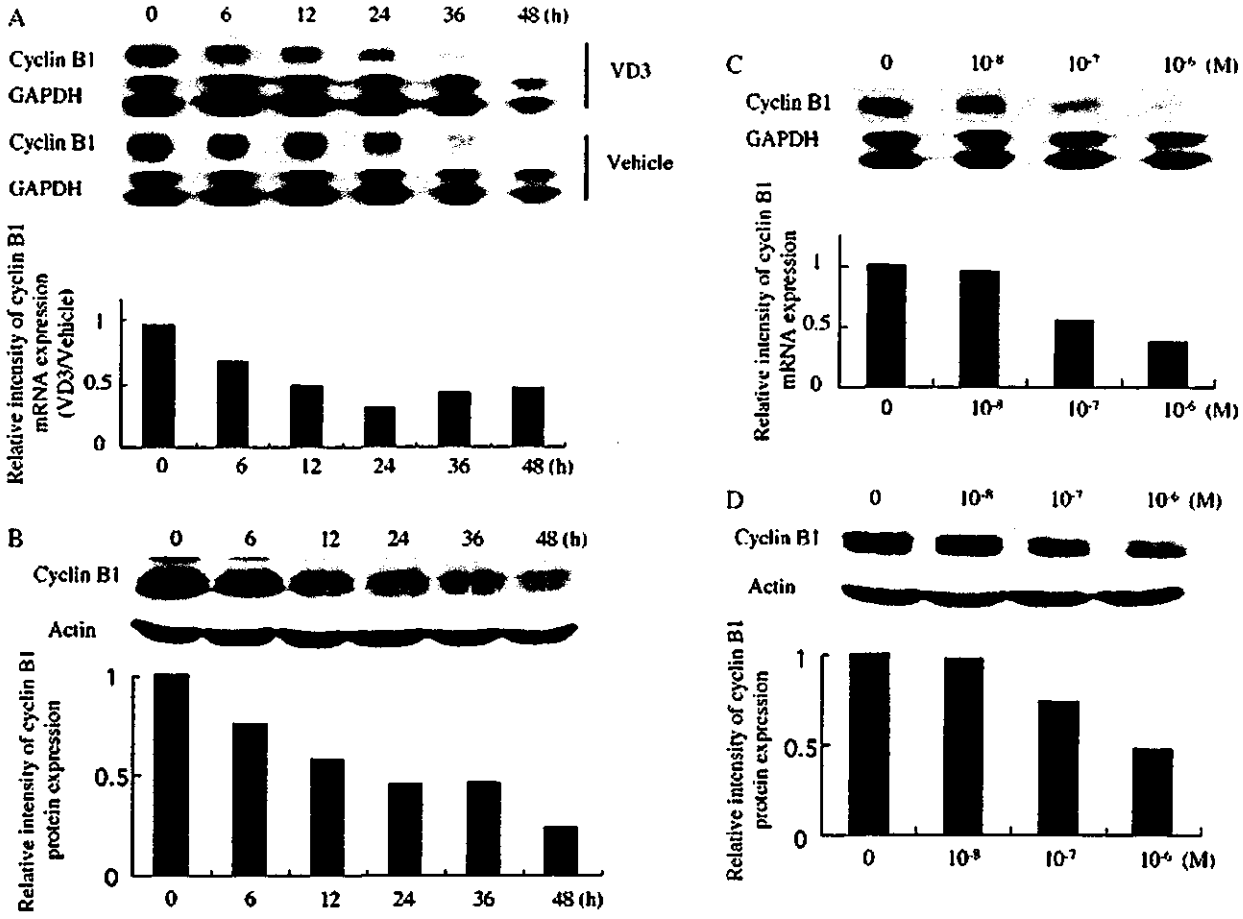

Figure 5
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3 VD3) downregulates the expression of cyclin B1 mRNA and protein. (*A*) Ribonuclease protection assay was performed as described in Fig 4*A*. The relative levels of cyclin B1 mRNA expression were estimated and normalized as described in Fig 2*A*. (*B*) Western blotting was performed as described in Fig 2*B*, and the levels of cyclin B1 protein expression were estimated and normalized as described in Fig 2*B*. (*C*) Keratinocytes were cultured and stimulated as described in Fig 2*C*. Cyclin B1 mRNA expression was estimated and normalized as described in Fig 2*C*. (*D*) Keratinocytes were cultured and stimulated as described in Fig 2*B*, and protein was extracted after 24 h of stimulation. Western blotting was performed, and the levels of cyclin B1 protein expression were estimated and normalized as described in Fig 2*D*.

could also contribute partially to the decreased Cdc2 and cyclin B1 expression. c-Myc activates the transcription of Cdc2 and cyclin B1 by occupying the E-box (North *et al*, 1999; Menssen and Hermeking, 2002). Activation of JNK and p38 reduces cyclin B1 mRNA expression (Garner *et al*, 2002) and 1,25(OH)$_2$VD3 increases the activities of JNK and SAPK kinase in normal keratinocytes (our unpublished data). The reductions in Cdc2 and cyclin B1 expression might contribute to the G2 arrest of human keratinocytes via two pathways: (1) a reduction in the amount of Cdc2/cyclin B1 complex and (2) a decrease in cyclin B1-dependent Cdc2 activation.

In this report, we presented data obtained from samples treated with a dose of 1,25(OH)$_2$VD3 (10^{-6} M), which is slightly higher than the dose of vitamin D3 ointment that is used to treat psoriasis (25 ~ 50 μg per g or about 10^{-7} M). Note that 10^{-7} M 1,25(OH)$_2$VD3 elicited a remarkable effect in our work in concentration-dependent experiments. We also studied the temporal effect of 10^{-7} M 1,25(OH)$_2$VD3 and found that its influence was apparent, but moderate, compared with that of 10^{-6} M (data not shown). We present the data for 10^{-6} M 1,25(OH)$_2$VD3 because Kobayashi *et al*, (1993) reported that G2/M accumulation was obvious in 10^{-6} M 1,25(OH)$_2$VD3-treated keratinocytes and was induced weakly by 10^{-7} M 1,25(OH)$_2$VD3. Therefore, these changes might occur under physiological conditions and might contribute to the improvement of psoriatic skin treated with vitamin D3 analogue ointments.

The 1,25(OH)$_2$VD3 signaling pathways, via either VDR or non-genomic signaling via a possible 1,25(OH)$_2$VD3 membrane receptor, are poorly understood. Our results suggest that 1,25[OH]$_2$VD3 induces G2/M arrest in normal human keratinocytes by increasing the levels of Wee1 and Myt1, which results in the inhibitory phosphorylation of Cdc2 (Fig 6). Furthermore, the decreased levels of Cdc2 and cyclin B1 contribute to G2/M arrest. This is the first report of the interaction of 1,25[OH]$_2$VD3, Wee1, and Myt1, and this paper presents new molecular mechanisms for 1,25[OH]$_2$VD3-induced biological function.

Materials and Methods

Cell culture Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (5 μg per mL),

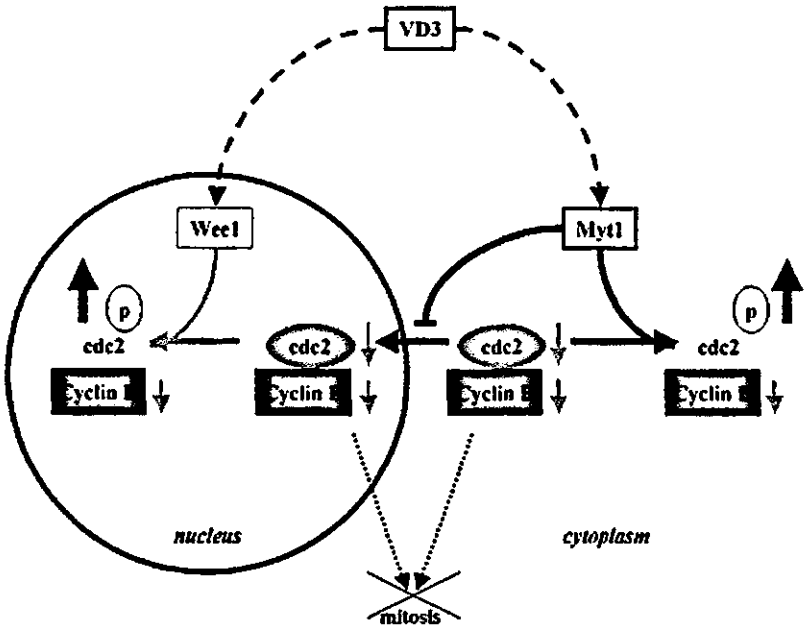

Figure 6
Schema of 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3)-induced G2/M arrest of keratinocytes. This summarizes the possible mechanisms of 1,25[OH]$_2$VD3-induced G2/M arrest of keratinocytes. 1,25[OH]$_2$VD3 induces Wee1 and Myt1 in keratinocytes, and these kinases phosphorylate Cdc2 and inactivate the Cdc2 protein in keratinocytes, resulting in G2/M arrest. The suppressed Cdc2 and cyclinB expression by 1,25[OH]$_2$VD3 may also contribute to G2/M blockade.

hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg per mL), and Ca^{2+} (0.03 mM) as previously described (Yamasaki *et al*, 2003a). Second or third passage cells were used in all of the experiments. All the procedures that involved human subjects received prior approval from the Ethical Committee of Ehime University School of Medicine, and all subjects provided written informed consent.

Reagents 1,25[OH]$_2$VD3 was a generous gift from Teijin Pharmaceutical Co. Ltd (Tokyo, Japan). The rabbit anti-human-Wee1, goat anti-human-Myt1, and goat anti-human-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Mouse anti-human-cyclin B1 was obtained from PharMingen (San Diego, California). The rabbit anti-human-Cdc2 and rabbit anti-human-phospho-Cdc2 (Tyr15) antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts).

1,25[OH$_2$VD3 treatment of human keratinocytes Subconfluent human keratinocyte cultures were incubated with various concentrations (10^{-8}, 10^{-7}, and 10^{-6} M) of 1,25[OH]$_2$VD3, or vehicle (ethanol) alone. After 24 h, the cells were harvested and total RNA and protein were extracted. For the time-course experiment, we stimulated cells with 10^{-6} M 1,25[OH]$_2$VD3 and extracted total RNA and protein at 0, 6, 12, 24, 36, and 48 h.

cDNA microarray analysis The cDNA array analysis was performed with the Atlas cDNA expression array system, which includes human cell cycle-related genes (Clontech Laboratories Inc., Palo Alto, California), according to the manufacturer's instructions. Briefly, keratinocytes were treated with 10^{-6} M 1,25[OH]$_2$VD3 or vehicle for 6 h, and total RNA was isolated with the Atlas Pure System (Clontech). One microgram of total RNA was labeled with the cDNA Synthesis Primer Mix and [α-^{32}P]dATP. Each radioactively labeled probe mix was then hybridized with a separate array membrane. After a high-stringency wash, the hybridization pattern was analyzed by autoradiography. The relative mRNA expression levels of stimulated keratinocytes were assessed by comparison with those of the control cells. Red indicated a signal that was enhanced by 1,25[OH]$_2$VD3, and green indicated a signal that was suppressed by 1,25[OH]$_2$VD3. Yellow indicated unchanged expression of mRNA.

RT-PCR analysis Total RNA from cultured human keratinocytes was prepared using Isogen (Nippon Gene, Toyama, Japan), and treated with 50 U per mL of DNase I (Clontech) at 37°C for 30 min to remove any genomic DNA contamination. The following specific primers were used for the PCR: human Wee1, 5′-GGACAGTGTCGTCGTAGAAAG-3′ and 5′-GGCAGCATTTGGGATTGAGGT-3′; human Myt1, 5′-AAGCTGGGTGACTTCGGACT-3′ and 5′-ACAGAACGCAGCTCGGAAGA-3′; and human GAPDH (glyceraldehyde 3-phosphate dehydrogenase), 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. The RT-PCR was performed using RT-PCR High Plus (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was added to a 50 μL reaction mixture that contained 10 μL of 5 × reaction buffer, 6 μL of 2.5 mM dNTPs, 5 μL of 25 mM $Mn(OAc)_2$, 19 μL of RNase-free H_2O, 2 μL of 10 U per μL RNase inhibitor, 2 μL of 2.5 U per μL of rTth DNA polymerase, and 2 μL of 10 pmol per μL of each primer. The cDNA was reverse transcribed from total RNA for 30 min at 60°C and heated to 94°C for 2 min. Amplification was performed using the DNA Thermal Cycler (Astec, Fukuoka, Japan) for 23–25 cycles. A cycle profile consisted of 1 min at 94°C for denaturation, and 1.5 min at 53°C–60°C for annealing and primer extension. A 5 μL sample of the reaction mixture was electrophoresed on a 2.0% agarose gel that contained ethidium bromide. The PCR products were also sequenced to confirm proper amplification. We performed at least three independent studies and confirmed similar results. One representative experiment is shown in the figures. The intensity of each band was quantified using the NIH *Image* software. The relative levels of Wee1 and Myt1 mRNA expression were estimated using GAPDH mRNA expression of each time point as the internal reference, and normalized against the respective relative level of the control (the point at 0 h was considered as a control for the time course, whereas the point at 0 M of 1,25[OH]$_2$VD3 was considered as a control for the concentration-dependent experiments) as 1 U.

Ribonuclease protection assay (RPA) Single-stranded antisense riboprobes were prepared by *in vitro* transcription of human cDNA fragments using the RiboQuant In Vitro Transcription Kit (Pharmingen) in the presence of [α-^{32}P]UTP. The hCC-1 and hCYC-1 probe sets (Pharmingen) were used as the templates for *in vitro* transcription. Samples of total RNA (10 μg each) were hybridized with the ^{32}P-labeled riboprobe and digested with RNase using the RiboQuant RPA Kit (Pharmingen) according to the manufacturer's

instructions. The hybridization products were separated on a gel and exposed to a film as previously described (Yamasaki *et al*, 2003b). We performed at least three independent studies and confirmed similar results. One representative experiment is shown in figures. Band density was analyzed using the NIH *Image* program. GAPDH bands are doublet, which may be due to that the end of GAPDH mRNA is highly susceptible to the RNase digestion even though it is double stranded. So, the sum of the two bands' intensity was considered as GAPDH mRNA expression. The relative levels of Cdc2 and cyclin B1 mRNA were estimated using GAPDH as the internal reference, and normalized against the respective relative values of the control signal as 1 U. The relative values of Cdc2 and cyclin B1 mRNA were plotted on graphs.

Western blot analysis The cells were harvested by scraping in extraction buffer that contained 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes. The analysis was performed using the Vistra ECF kit (Amersham Biosciences K.K., Tokyo, Japan) and FluorImager (Molecular Dynamics Inc., Sunnyvale, California) as previously described (Yamasaki *et al*, 2003b). We performed at least three independent studies and confirmed similar results. One representative experiment is shown in figures. The intensity of each band was quantified with ImageQuant (Molecular Dynamics Inc.), the relative protein expressions were estimated using actin as the internal reference, and normalized to the control as 1 U. The relative values of proteins are plotted on the graphs.

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Address correspondence to: Kenshi Yamasaki, Department of Dermatology, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan. Email: yamasaki@m.ehime-u.ac.jp

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SOCS3/CIS3 negative regulation of STAT3 in HGF-induced keratinocyte migration

Sho Tokumaru[a], Koji Sayama[a,*], Kenshi Yamasaki[a], Yuji Shirakata[a], Yasushi Hanakawa[a], Yoko Yahata[a], Xiuju Dai[a], Mikiko Tohyama[a], Lujun Yang[a], Akihiko Yoshimura[b], Koji Hashimoto[a]

[a] *Department of Dermatology, Ehime University School of Medicine, Ehime, Japan*
[b] *Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyusyu University, Fukuoka, Japan*

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Abstract

Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes. Because HGF has strong effects on the motility of keratinocytes and is produced by fibroblasts, HGF is thought to regulate keratinocyte migration during wound healing. However, the intracellular signaling mechanism of HGF-induced keratinocyte migration is poorly understood. In this report, we clarify the roles of STAT3 and SOCS/CIS family in HGF-induced keratinocyte migration. HGF activated STAT3 and strongly induced keratinocyte migration. Transfection with the dominant-negative mutant of STAT3 almost completely abolished HGF-induced keratinocyte migration and STAT3 phosphorylation. Next, we studied the mechanisms that regulate STAT3 phosphorylation. HGF enhanced the expression of SOCS3/CIS3 by sixfold within 1 h, but had minimum effect on SOCS1/JAB expression. Transfection with SOCS3/CIS3 almost completely abolished HGF-induced STAT3 phosphorylation and keratinocyte migration, indicating that SOCS3/CIS3 acts as a negative regulator of HGF-induced keratinocyte migration. In conclusion, SOCS3/CIS3 regulates HGF-induced keratinocyte migration by inhibiting STAT3 phosphorylation.

Keywords: Keratinocytes; HGF; Migration; STAT3; STAT1; SOCS3/CIS3; SOCS1/JAB; Wound healing; Adenovirus vector

The migration of epidermal keratinocytes is an important step in skin wound healing. Several growth factors regulate keratinocyte migration [1]. Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes [2,3]. HGF also has mitogenic, motogenic, morphogenic, and tumor-inhibitory activities for a variety of cells, including epithelial, endothelial, and some types of stromal cells [4–6]. Because HGF has potent effects on the motility of keratinocytes and because dermal fibroblasts produce HGF in the skin [7], HGF has been suggested to play a regulatory role in keratinocyte migration during wound healing [8,9]. Recently, an application of HGF has been demonstrated as a potential therapeutic approach for the treatment of cutaneous ulcer [10–13]. However, the intracellular signaling mechanism of HGF-induced migration is poorly understood.

c-Met is an HGF receptor that is autophosphorylated upon binding of HGF. Phosphorylated c-Met recruits a number of substrates with Src homology (SH)2 domains, such as phosphatidylinositol 3-kinase [14], Grb-2 (ASH)/Sos complex [15], Ras GTPase activating protein, $pp60^{src}$, and phospholipase C [15]. Grb-2 has also been implicated in the recruitment of the large adaptor protein Grb-2-associated binding protein-1 (Gab1) to the Met signaling complex [16–18]. These signaling molecules lead to mitogenic activity via the Ras–Raf1–MEK–MAPK pathway. In addition to these

* Corresponding author. Fax: +81 960 5352.
E-mail address: sayama@m.ehime-u.ac.jp (K. Sayama).

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pathways, two signal transducers and activators of transcription (STAT) proteins, STAT1 and STAT3, are signaling cascade proteins located downstream from c-Met [19].

The STAT signaling pathways are negatively regulated by proteins of the suppressor of cytokine signaling (SOCS)/cytokine-inducible SH2-containing protein (CIS) family to avoid oversignaling [20]. The SOCS/CIS family is induced by cytokine stimulation and binds to tyrosine-phosphorylated sites of the cytokine receptor or to Jak through an SH2 domain, resulting in the inhibition of tyrosine kinase phosphorylation. CIS was the first member of the SOCS/CIS family to be identified; it binds to the tyrosine-phosphorylated site of the erythropoietin receptor and inhibits the STAT5 signal downstream [21]. Additional members of the SOCS/CIS family have been identified independently [22,23]. SOCS1/JAB binds mainly to Jak2 [24] and regulates the IFN-γ/STAT1 [25] and IL-6/STAT3 signaling pathways [23]. SOCS2/CIS2 regulates insulin-like growth factor and the insulin-like growth factor receptor signaling pathways [26]. SOCS3/CIS3 regulates the IL-6/STAT3 and IFN-γ/STAT1 signaling pathways.

Because STATs are involved in the HGF-c-Met signaling pathway, we hypothesized that the SOCS/CIS family regulates HGF-induced keratinocyte migration by inhibiting STAT pathways. To prove this, we first studied whether STAT3 was involved in HGF-induced keratinocyte migration. Next, we tested whether the SOCS/CIS family affects HGF-induced keratinocyte migration through the inhibition of STAT3.

Materials and methods

Reagents and antibodies. Recombinant HGF was kindly provided by Dr. Kunio Matsumoto (Osaka University, Osaka, Japan). Antibodies were purchased as follows: mouse monoclonal STAT3 (Transduction Laboratories) and phospho-STAT3 (New England Biolabs).

Keratinocyte culture. Human skin samples were obtained after plastic surgery under a protocol approved by the Institutional Review Board of Ehime University School of Medicine. Primary normal human keratinocytes were isolated from the normal human skin. Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (1 μg/ml), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (BHE; 50 μg/ml), and Ca^{2+}(0.1 mM). This supplement was as described elsewhere [27].

Migration assay. Keratinocytes were cultured at 1×10^5 cells per 35-mm type I-collagen-coated culture plate in culture medium without BHE for 12 h. After stimulation, keratinocyte migration was observed using time-lapse video microscopy (IX-IBC, CK30; Olympus, Tokyo, Japan) in a controlled chamber at 37 °C and 5% CO_2.

Keratinocyte migration was assayed quantitatively with a Boyden chamber, as previously described [28]. Designated amounts of HGF were added to the bottom wells of a 48-well Boyden chamber (Neuro Probe, Cabin John, MD), and an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe) was placed on the wells. The membrane was precoated with type I collagen (10 μg/ml in PBS, Nitta Gelatin, Osaka, Japan) at room temperature for 1 h and then extensively washed with PBS. Subconfluent keratinocytes were harvested with trypsin–EDTA (0.05% trypsin and 0.5 mM EDTA) and resuspended in the culture medium without BHE at 1×10^5 cells/ml. Fifty microliters of the keratinocyte suspension (5000 cells/well) was added to the upper wells, and the chamber was incubated overnight at 37 °C in a humidified atmosphere of air with 5% CO_2. Cells that adhered to the upper surface of the filter membrane were removed by scraping with a rubber blade. Cells that moved through the filter and stayed on the lower surface of the membrane were considered to be migrated cells. The membrane was fixed with 10% buffered formalin overnight and then stained with Gill's hematoxylin overnight. The membrane was then mounted between two glass slides with 90% glycerol and the number of migrated cells was determined by counting under a microscope.

Western blotting. Subconfluent keratinocytes were starved for 2 h in BHE-free medium and stimulated with HGF as indicated. Cells were harvested on ice in lysis buffer containing 5 mM EDTA, 100 μM sodium orthovanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 5 μM 3,4-dichloroisocoumarin, 1 μg/ml aprotinin, and 1% Triton X-100 in PBS. Twenty micrograms of protein was separated on 10% SDS–PAGE and then transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk in PBS overnight at 4 °C. The blocked membranes were incubated for 6 h with the first antibody as indicated. After three washes with PBS containing 0.05% Tween 20, the membranes were treated with ABC reagents (VECTOR Laboratories, Burlingame, CA) for 20 min at room temperature, washed three times with PBS containing 0.05% Tween 20, treated with ECL detection reagents (Amersham–Pharmacia Biotech, Piscataway, NJ) for 1 min at room temperature, and then exposed to films (Kodak, Rochester, NY).

Quantitative PCR analysis. Total RNA from cultured human keratinocytes was prepared using Isogen (Nippon Gene, Toyama, Japan) and was treated with 50 U/ml DNase I (Clontech) at 37 °C for 30 min to remove any genomic DNA contamination.

To quantify the mRNA expression in vivo, we performed quantitative RT-PCR using the ABI Prism 7700 sequencer detection system (Perkin–Elmer Applied Biosystems, Foster City, CA). RT-PCR mixtures were prepared according to the manufacturer's instructions for the TaqMan One-Step RT-PCR Master Mix Reagent kit (Perkin–Elmer Applied Biosystems). Briefly, 50 ng of total RNA was added to each 50-μl reaction mixture containing 1 μl master mix, 1× multiScribe and RNase inhibitor mix, 200 nM of each primer, and 100 nM hybridization probe for specific detection of target cDNA. For SOCS1/JAB detection, the sense primer 5′-TTTTTCGCCCTTAGC GTGAA-3′, the antisense primer 5′-GCCATCCAGGTGAAAGCG-3′, and the probe 5′-CCTCGGGACCCACGAGCATCC-3′ were added. For SOCS3/CIS3 detection, the sense primer 5′-TTCAGCA TCTCTGTCGGAAGAC-3′, the antisense primer 5′-GCATCGTAC TGGTCCAGGAACT-3′, and the probe 5′-AACGGCCACCTGG ACTCCTATGAGAAA-3′ were added. The probe was labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′-end. For GAPDH detection, 1 μl of Pre-Developed TaqMan Assay Reagent (Perkin–Elmer Applied Biosystems) was added. The thermal conditions were 48 °C for 30 min for reverse transcription and 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 15 s for denaturing and 55 °C for 1.5 min for annealing and extension. The PCR products were sequenced to confirm the proper amplification. To compare mRNA expression, the results were estimated as relative values using GAPDH as an internal reference. The relative quantified expression was calculated using the following formula: relative expression $= 2[-(\sum CT_s/N_s - \sum CT_{GAPDH_s}/N_s) - (\sum CT_r/N_r - \sum CT_{GAPDH_r}/N_r)]$, where CT_s denotes the cycle threshold for the candidate gene in the sample, N_s is the number of samples, CT_{GAPDH_s} is the cycle threshold for GAPDH in the same sample, CT_r is the cycle threshold for the candidate gene in the overall reference sample, N_r is the number of reference samples, and CT_{GAPDH_r} is the cycle threshold for GAPDH in the same reference sample. In each group, there were $n = 3$ samples.

Adenovirus vectors (Ax). STAT3 has a phosphorylation site at tyrosine705. In the dominant negative mutants of STAT3 (STAT3F), the phosphorylatable tyrosine residue is substituted with phenylalanine. Axs encoding STAT3F (AxCA STAT3F), SOCS1/JAB (AxCAJAB), and SOCS3/CIS3 (AxCACIS3) were generated as previously described [29] using the COS–TPC method [30]. Ax encoding lacZ (Ax LacZ) was a gift from Dr. Izumi Saito (University of Tokyo). Virus stocks were prepared using a standard procedure [30]. Concentrated, purified virus stocks were prepared using a CsCl gradient, and the virus titer was checked using a plaque formation assay. We infected normal human keratinocytes with Ax at a multiplicity of infection (MOI) of five.

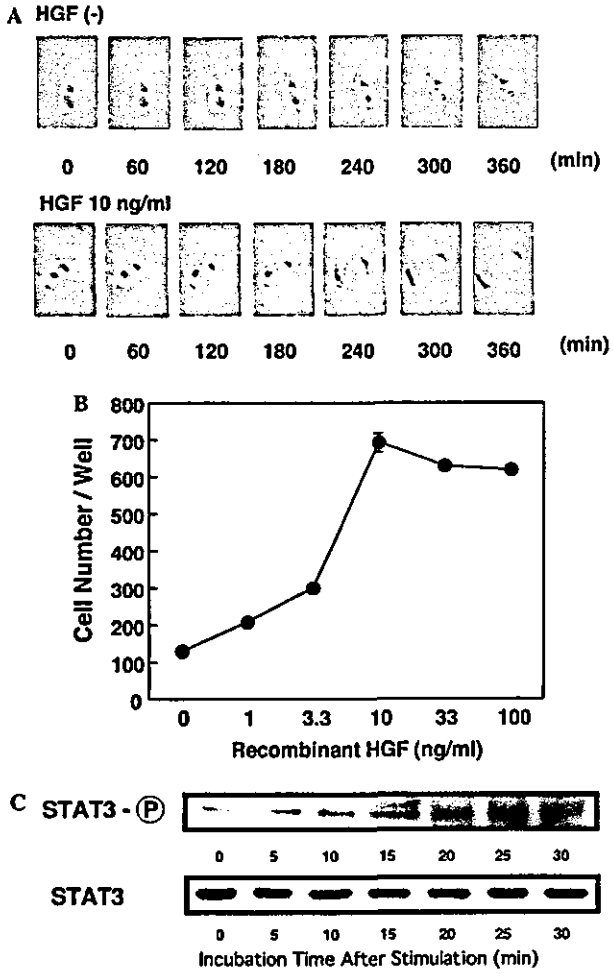

Fig. 1. Keratinocyte migration and phosphorylation of STAT3 by HGF. (A) HGF-induced keratinocyte migration. After adding 10 ng/ml HGF, keratinocyte migration was observed under time-lapse video microscopy every 60 min. (B) Quantification of keratinocyte migration induced by HGF. The indicated amount of HGF was added to the bottom wells of a 48-well Boyden chamber, and then an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane was placed on the wells. Keratinocytes were added to the upper wells at 5000 cells/well. After overnight incubation, the membrane was stained with Gill's hematoxylin. The number of cells that had migrated through the filter was determined by counting under a microscope. Each point shows means ± SD of quadruplicate measurements. (C) Phosphorylation of STAT3 by HGF. Subconfluent keratinocytes were starved for 2 h in BHE-free medium and stimulated with 10 ng/ml HGF. Cells were harvested and the phosphorylation of STAT3 was analyzed by Western blotting.

Results

HGF induces keratinocyte migration and phosphorylates STAT3

We first observed whether HGF induces the migration of normal human keratinocytes by using time-lapse video microscopy (Fig. 1A). Keratinocytes started to migrate within 180 min after HGF stimulation. Without HGF, no migration occurred. Then, the migration was analyzed quantitatively using the Boyden chamber assay (Fig. 1B). HGF induced keratinocyte migration sixfold that of control. The optimum concentration of HGF was 10 ng/ml. The phosphorylation of STAT3 was analyzed by Western blotting (Fig. 1C). HGF phosphorylated STAT3 at 25 min.

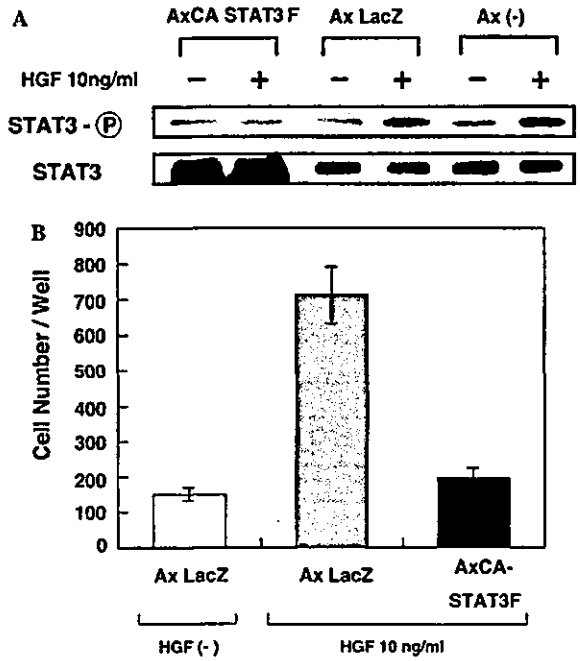

Fig. 2. Inhibition of HGF-induced phosphorylation of STAT3 and keratinocyte migration by STAT3F. (A) Inhibition of STAT3 phosphorylation. Ax LacZ and AxCA STAT3F were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were stimulated with 10 ng/ml HGF or vehicle alone for 25 min. Then the cells were harvested and analyzed by Western blotting. (B) Inhibition of keratinocyte migration. Ax LacZ and AxCA STAT3F were transfected into normal human keratinocytes at an MOI of five. After 24 h, the keratinocytes were harvested and transferred to a Boyden chamber and HGF (10 ng/ml) was added to the lower chamber. The migration was analyzed as in Fig. 1B. Each point shows the mean ± SD of quadruplicate measurements.

Phosphorylation of STAT3 is essential for HGF-induced keratinocyte migration

We next constructed dominant negative mutants of STAT3 (STAT3F) to study the role of STAT3 in HGF-induced keratinocyte migration. The expression of STAT3F using Ax (AxCA STAT3F) almost completely blocked HGF-induced STAT3 phosphorylation, while the transfection of LacZ had no effect on the STAT3 phosphorylation (Fig. 2A).

Using AxCA STAT3F, we analyzed the functions of STAT3 in HGF-induced keratinocyte migration. After the transfection of keratinocytes with AxCA STAT3F, the HGF-induced migration of the keratinocytes was quantitatively analyzed with the Boyden chamber assay (Fig. 2B). The expression of STAT3F almost completely blocked HGF-induced keratinocyte migration, while the expression of LacZ had no effect on migration. Because STAT3 was phosphorylated by HGF, and because HGF-induced migration was blocked by STAT3F, we concluded that the phosphorylation of STAT3 is essential for HGF-induced keratinocyte migration.

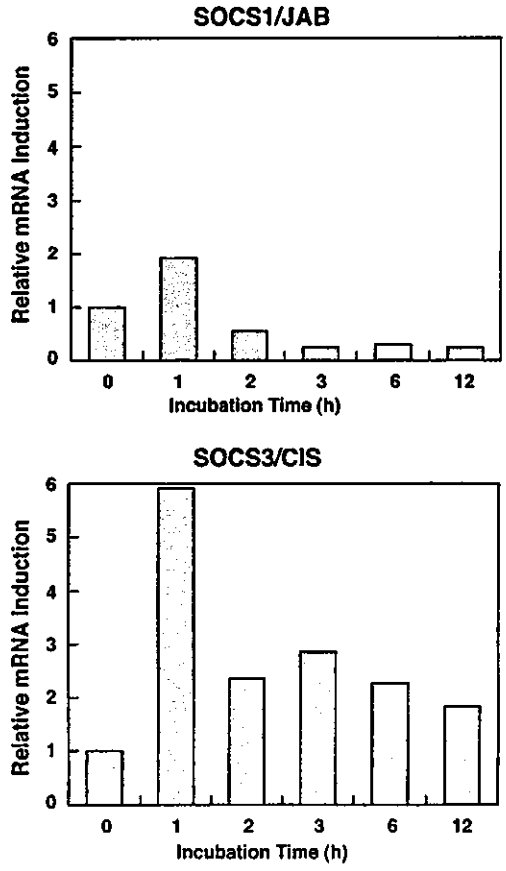

Fig. 3. Induction of SOCS3/CIS by HGF. Subconfluent keratinocytes were stimulated with 10 ng/ml HGF. Cells were harvested at the indicated time. The mRNA expression of SOCS1/JAB and SOCS3/CIS was analyzed using real-time PCR. The results were adjusted to relative values using GAPDH as an internal reference.

HGF induces SOCS3/CIS3

The SOCS/CIS family is inducible de novo by stimulation and negatively regulates the STAT family. Therefore, it is possible that the SOCS/CIS family regulates HGF-induced keratinocyte migration. To prove this, we first determined whether HGF induces the SOCS/CIS family in keratinocytes. As shown in Fig. 3, HGF enhanced the SOCS3/CIS3 mRNA expression sixfold at 1 h after stimulation, while the induction of SOCS1/JAB by HGF was not as significant as that of SOCS3/CIS3.

SOCS1/JAB and SOCS3/CIS3 inhibit HGF-induced STAT3 phosphorylation and keratinocyte migration

First, we determined the effects of SOCS1/JAB and SOCS3/CIS3 on HGF-induced STAT3 phosphorylation. AxCAJAB and AxCACIS3 were transfected into keratinocytes; the expression of either SOCS1/JAB or SOCS3 / CIS3 almost completely blocked HGF-induced STAT3 phosphorylation (Fig. 4A).

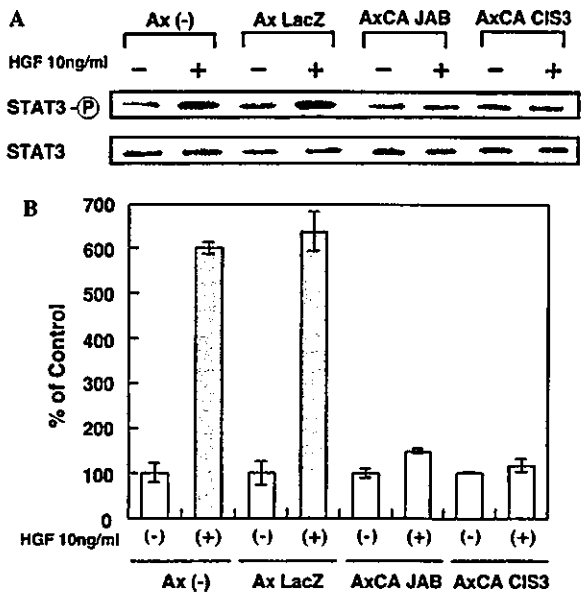

Fig. 4. Inhibition of HGF-induced STAT3 phosphorylation and keratinocyte migration by SOCS1/JAB and SOCS3/CIS3. (A) Inhibition of STAT3 phosphorylation. Ax LacZ, AxCAJAB, and AxCACIS3 were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were stimulated with 10 ng/ml HGF or vehicle alone for 25 min. Then, the cells were harvested and analyzed by Western blotting. (B) Inhibition of keratinocyte migration. Ax LacZ, AxCAJAB, and AxCACIS3 were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were harvested and transferred to a Boyden chamber for the analysis of migration, as described in Fig. 1B. HGF (10 ng/ml) was added to the lower chamber, and the set-up was incubated overnight. Each point shows the mean ± SD of quadruplicate measurements.

Using AxCAJAB and AxCACIS3, we analyzed the regulatory mechanism of STAT3 in HGF-induced keratinocyte migration. After the transfection of keratinocytes with AxCAJAB or AxCACIS3, the HGF-induced migration of the keratinocytes was analyzed quantitatively with the Boyden chamber assay (Fig. 4B). The expression of either SOCS1/JAB or SOCS3/CIS3 almost completely blocked HGF-induced keratinocyte migration. Because SOCS3/CIS3 was induced by HGF and SOCS3/CIS3 blocked HGF-induced phosphorylation of STAT3 and migration, we concluded that SOCS3/CIS3 regulates HGF-induced keratinocyte migration.

Discussion

The intracellular signaling mechanisms involving c-Met have been studied in hepatocytes primarily. Although it was demonstrated that HGF induces keratinocyte migration [8], the intracellular signaling mechanisms remained unclear. It was suggested that the major signaling pathway downstream from HGF/c-Met is the MAPK cascade [31]. However, the involvement of STAT signaling cascades in HGF-induced signal transduction was also reported [19]. Because EGF-induced keratinocyte migration was abolished in STAT3-disrupted keratinocytes [32], STAT pathways were thought to be involved in HGF-induced keratinocyte migration. Therefore, we studied whether SOCS/CIS regulates HGF-induced keratinocyte migration.

The SOCS/CIS family negatively regulates STAT pathways. However, the inhibitory functions of the SOCS/CIS family differ among cell types and cell conditions. In this study, we showed that SOCS3/CIS3 was induced and inhibited STAT3 phosphorylation and migration, indicating that SOCS3/CIS3 acts as a self-limiting factor to avoid overstimulation. Although HGF induces SOCS3/CIS3, EGF, a growth factor that strongly induces keratinocyte migration [33], does not induce SOCS1/JAB or SOCS3/CIS3 [29]. Therefore, EGF-induced keratinocyte migration does not involve the SOCS1/JAB- or SOCS3/CIS3-mediated self-regulatory mechanism of STAT3 activation in keratinocytes. This indicates that the intracellular regulatory mechanism of keratinocyte migration differs among growth factors.

In this study, we showed that SOCS1/JAB inhibited HGF-induced keratinocyte migration. However, SOCS1/JAB was not induced by HGF, suggesting that SOCS1/JAB has another role other than acting as a self-limiting factor. It is possible that cytokine-induced SOCS1/JAB or SOCS3/CIS3 affects HGF-induced keratinocyte migration. Although the inhibitory and negative regulatory mechanisms of cytokine signals in epidermal keratinocytes have not been assessed fully, cytokines such as IFN-γ, IL-4, and IL-6 are implicated in a variety of physiological and pathological conditions of the skin. IFN-γ enhances SOCS1/JAB and SOCS3/CIS3 expression [29]. In addition, IL-4 and IL-6 enhance the expression of SOCS1/JAB and SOCS3/CIS3, respectively [29]. Therefore, cytokines in various inflammatory skin conditions might affect wound healing by regulating keratinocyte migration via the induction of SOCS1/JAB or SOCS3/CIS3.

In conclusion, the SOCS3/CIS3 negative feedback mechanism of STAT3 activation is a key pathway of HGF-induced keratinocyte migration.

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