Differential Regulation by IL-1β and EGF of Expression of Three Different Hyaluronan Synthases in Oral Mucosal Epithelial Cells and Fibroblasts and Dermal Fibroblasts: Quantitative Analysis Using Real-Time RT-PCR

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Using ''real-time RT-PCR'', we assessed the expression of three different hyaluronan synthase genes, *HAS1, HAS2*, and *HAS3*, by measuring their mRNA amounts in cultured human oral mucosal epithelial (COME) cells, oral mucosal fibroblasts, and dermal fibroblasts, and investigated the effects of interleukin-1β (IL-1β) and epidermal growth factor (EGF). When COME cells were treated with IL-1β or EGF, early and marked increases and subsequent rapid decreases were observed for all HAS genes and, moreover, actual changes in hyaluronan synthesis subsequently occurred. The effects of IL-1β stimulation were concentration-dependent and the maximal response to the EGF stimulation was observed at a low concentration (0.1 ng per mL). When two different types of fibroblasts were treated with IL-1β or EGF, increased expression with different degrees and rates of three different HAS genes and subsequent increased synthesis of hyaluronan were also observed. In addition, HAS1 gene expression was not detectable in the mucosal fibroblasts, while weak HAS3 gene expression was detected in the dermal fibroblasts. Taken together, it is likely that the regulation of the expression of the three different HAS genes is different between oral mucosa and skin, which may be of significance for elucidating some of the differences between these tissues in wound healing.

Key words: hyaluronan/hyaluronan synthase genes/real-time RT-PCR analysis/tissue engineering
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The process of wound healing depends upon a variety of interactions between cells and the extracellular matrix (Clark and Henson, 1988). It is well known that hyaluronan not only supports tissue architecture as a passive structural component of the matrix in various connective tissues but is also involved in dynamic cellular processes such as cell migration and cell–cell recognition during wound healing and inflammation (Weigel *et al*, 1997; Knudson *et al*, 1989; Turley, 1989). Three different mammalian hyaluronan synthases, HAS1, HAS2, and HAS3, have been identified and characterized (Rosa *et al*, 1988; Itano and Kimata, 1996a,b; Shyjan *et al*, 1996; Spicer *et al*, 1996; Watanabe and Yamaguchi, 1996; Spicer *et al*, 1997). The three HAS genes show distinct expression patterns (Spicer and Mcdonald, 1998) and the synthases are significantly different in their enzymatic properties and in their role in the pericellular hyaluronan coat formation (Itano *et al*, 1999). The precise regulatory mechanism of the expression of each HAS is still unknown. It has been shown that the synthesis of hyaluronan is stimulated by some growth regulatory factors and anti-inflammatory cytokines such as epidermal growth factor (EGF) and IL-1β (Heldin *et al*, 1989; Yung *et al*, 1996; Kaback and Smith, 1999), which have also been shown to be agents that promote wound healing (Brown *et al*, 1986; Gailit *et al*, 1994). Considering the above findings on the existence of three different hyaluronan synthases, it is likely that the expression of each hyaluronan synthase is regulated in a different manner, depending on the difference of growth regulatory factors or cytokines.

Langer and Vacanti (1993) described a new technology for solid organ transplants called *tissue engineering*, which involves the morphogenesis of new tissue using constructs formed from isolated cells cultured with growth regulatory factors and biocompatible scaffolds. Ueda *et al* (1995) fabricated cultured epithelium sheets for skin repair using cultured human oral mucosal epithelial (COME) cells and attained good clinical results. Therefore, we focused on the relationship among the growth regulatory factors, extracellular matrix, and epidermal and dermal cells, with the aim of tissue regeneration without scarring.

Most skin lesions heal rapidly and efficiently within a week or two; however, scars remain where the collagen matrix has been poorly reconstituted. Oral mucosa, on the other hand, rarely suffers from scarring in the process of

Abbreviations: COME cells, cultured human oral mucosal epithelial cells; EGF, epidermal growth factor; HA, hyaluronan; hHAS, human hyaluronan synthase; IL-1β, interleukin-1β; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase

wound healing (Tsai *et al*, 1995) and appears to be different from skin in this regard. We have developed a method to fabricate cultured epithelium for skin repair using COME cells, which are a potential new source of cells for cultured epithelial grafts without scarring (Ueda *et al*, 1995). Therefore, comparison of the regulation of hyaluronan synthesis in oral mucosa with that in skin may yield important insights into the wound-healing processes characteristic of oral mucosa lesions. Histological analysis of the hyaluronan distribution in skin showed that this molecule is localized not only in the dermis but also in the epidermis (Tammi *et al*, 1984). The ability of keratinocytes to synthesize hyaluronan has been reported in both cell cultures (Brown and Parkinson, 1983) and organ cultures (Tammi *et al*, 1989; Agren *et al*, 1995). Skin grafts have been shown to promote wound closure by releasing a variety of cytokines (Krejci *et al*, 1991; Martin, 1997). Therefore, it is likely that the expression of three different hyaluronan synthases in the skin may be regulated by these factors. Actually, Sugiyama *et al* (1998) found that TGF-β upregulates *HAS 1* and *HAS2* expression independently in cultured human skin fibroblasts. Although HAS1, 2, and 3 mRNAs have been detected in various tissues by northern blot analyses (Spicer and Mcdonald, 1998), comparative studies on the distributions of HAS1, 2, and 3 expression as well as on the effect of cytokines on their expression between oral mucosa and skin have not yet been performed.

Real-time RT-PCR analysis enables one to detect quantitatively certain mRNAs in RNA samples, although careful attention must be paid to establishing suitable PCR conditions and primers (Gibson *et al*, 1996; Heid *et al*, 1996). In this study, we took advantage of this new method and also developed culture systems to assess the expression of the HAS1, HAS2, and HAS3 genes and hyaluronan synthesis in COME cells, oral mucosal fibroblasts, and skin dermal fibroblasts, and compared them before and after simulation by IL-1β or EGF. The current results may help to clarify some of the mechanisms of the different responses in wound healing between oral mucosa and skin, and define potential targets for specific therapy directed at modulating hyaluronan synthesis in tissue engineering.

Results

Stimulation of expression of HAS genes by IL-1β or EGF in human mucosal COME cells Using real-time RT-PCR analysis, we measured the absolute amounts of the mRNAs of the three different hyaluronan synthases, HAS 1, HAS2, and HAS3, in the COME cells. Comparisons of the expression among different samples by normalizing each of the absolute amounts enabled us to elucidate how the addition of IL-1β or EGF affected the expression of the HAS 1, HAS2, and HAS3 genes. Treatment of COME cells with 0.1–100 ng per mL of IL-1β or EGF for 3 h resulted in a marked increase in both HAS1 and HAS2 mRNAs (Figs 1 and 2). Maximal levels of HAS1 and HAS2 expression were attained at 100 ng per mL IL-1β and 0.1 ng per mL EGF, respectively. Since EGF at this concentration (0.1 ng per mL) is used as one of the supplements for HuMedia-KG2 medium (commercially available), our finding indicates that this medium is suitable at least for hyaluronan synthesis by

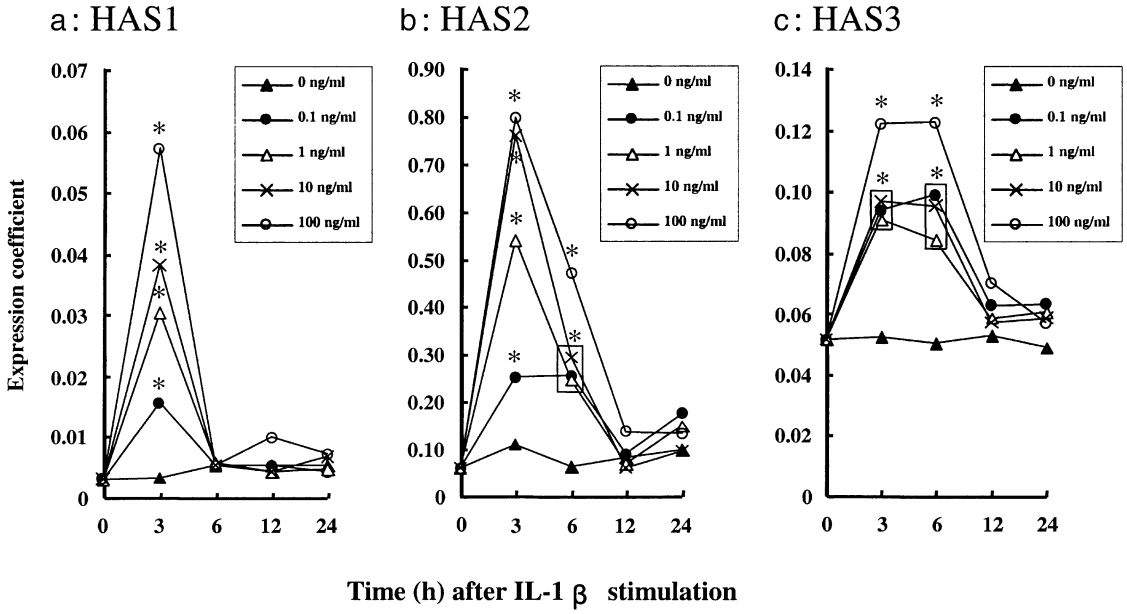

Figure 1
Stimulation by IL-1β of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression in COME cells. COME cells were cultured in the presence of IL-1β at the indicated concentrations: 0 ng per mL (▲), 0.1 ng per mL (•), 1 ng per mL (△), 10 ng per mL (×), and 100 ng per mL (○). After 3, 6, 12, and 24 h, cells were lysed and total RNA were extracted. Total RNA were also extracted from the cells just before adding IL-1β as a control (0 h). Equal amounts of total RNA (200 ng) were subjected to real-time RT-PCR analysis, and the absolute amounts of mRNA of HAS1, HAS2, HAS3, and GAPDH in each sample were quantitated by comparison with their standard curves, as described in *Materials and Methods*. The expression coefficient for each mRNA (plotted on the ordinate) was calculated by dividing the absolute amount of each mRNA (HAS1, HAS2, and HAS3) by the absolute amount of GAPDH mRNA in each sample so that quantitative comparisons of the expression of each HAS mRNA could be made among the different samples. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p < 0.01$. Boxed values are also significantly different ($p < 0.01$) from the control.

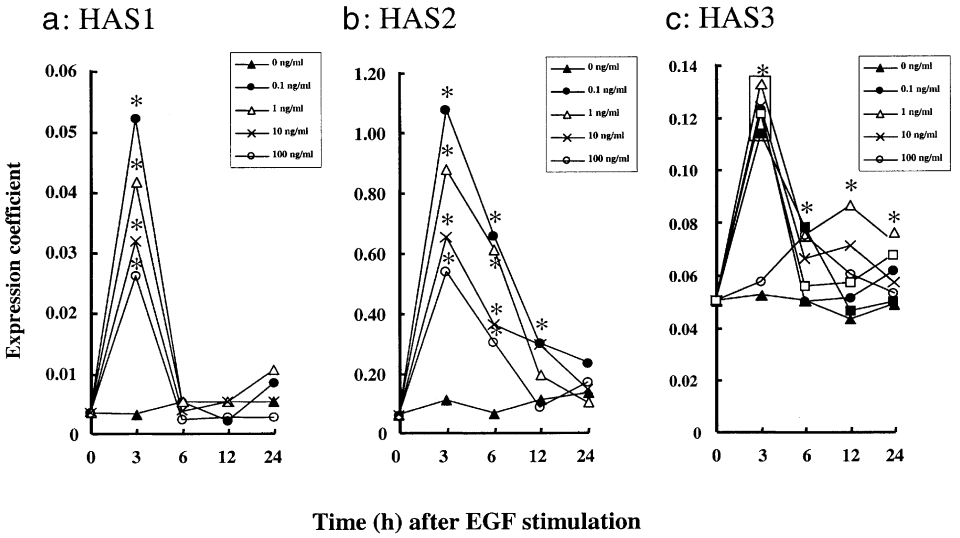

Figure 2
Stimulation by EGF of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression in COME cells. COME cells were cultured in the presence of EGF at the indicated concentrations: 0 ng per mL (▲), 0.1 ng per mL (•), 1 ng per mL (△), 10 ng per mL (×), and 100 ng per mL (○). After 3, 6, 12, and 24 h, cells were lysed and total RNA were extracted. Total RNA were also extracted from the cells just before the addition of EGF as a control (0 h). The ordinate represents the expression coefficient for each mRNA, which was calculated as described in Fig 1. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p<0.01$. Boxed values are also significantly different ($p<0.01$) from the control.

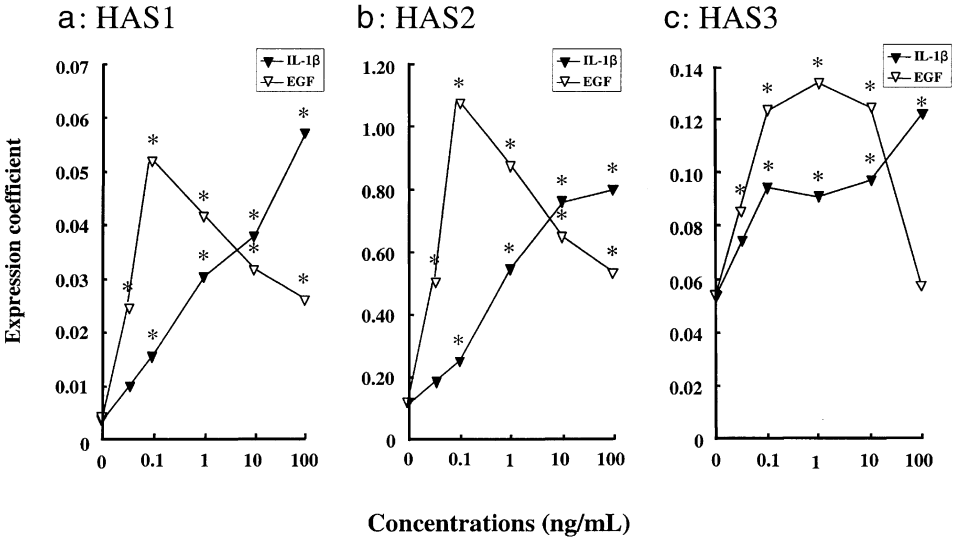

Figure 3
Comparison of concentration-dependent changes of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression between stimulation by IL-1β or EGF. COME cells were cultured in the presence of the indicated concentrations of 0.1–100 ng per mL IL-1β (▼) or EGF (▽). After 3 h, cells were lysed and total RNA were extracted. Total RNA were also extracted from the cells just before the treatment for the 0 h sample. Quantitation of each mRNA (HAS1, HAS2, and HAS3) was performed using equal amounts of total RNA (200 ng) by real-time RT-PCR as described in *Materials and Methods*. The ordinate represents the expression coefficient for each mRNA, which was calculated as described in Fig 1. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p<0.01$.

COME cells. The amounts of HAS1 and HAS2 mRNAs were increased in a concentration-dependent manner by IL-1β at all concentrations tested, while those in EGF-treated COME cells were maximal at 0.1 ng per mL EGF and decreased to almost the basal level at the higher concentrations of EGF in order of HAS1 and HAS expression (Fig 3). A much smaller increase of HAS3 than of HAS1 and HAS2 expression was observed in the early phase of the stimulation (Figs 1 and 2). The concentration-dependent changes in HAS3 expression after IL-1β or EGF stimulation were similar to, but less marked than, those in HAS1 and HAS2 expression (Fig 3).

Comparisons of expression coefficients at 3 h, when the maximal stimulation was observed, suggested that the absolute amount of HAS2 mRNA was about 20-fold that of HAS1 mRNA, and about 8-fold that of HAS3 mRNA in COME cells (Figs 1–3). Therefore, the HAS2 mRNA appeared to be responsible for most of the hyaluronan synthase expression in the early phase of IL-1β or EGF stimulation. The proportion of HAS3 mRNA, however, appeared to become more significant in the late phase of the stimulation (Figs 1 and 2).

Stimulation of HAS gene expression by IL-1β or EGF in cultured human oral mucosal and dermal fibroblasts We examined the effects of IL-1β or EGF on the expression of HAS1, HAS2, and HAS3 mRNAs in cultured oral mucosal fibroblasts and dermal fibroblasts using the same method. Stimulation of these fibroblasts with 0.1–100 ng per mL IL-1β or EGF for 3 h resulted in a marked increase of HAS1 mRNA in cultured dermal fibroblasts, HAS2 mRNA in both dermal and cultured oral mucosal fibroblasts, and HAS3 mRNA in cultured oral mucosal fibroblasts. Maximal levels of HAS1 and HAS2 expression were attained at 1 ng per mL IL-1β in dermal fibroblasts, while those of HAS2 and HAS3 expression were attained at 1 ng per mL EGF in oral mucosal fibroblasts (Fig 4). Neither significant HAS1 gene expression in cultured oral mucosal fibroblasts nor HAS3 gene expression in dermal fibroblasts was detected at any concentration of EGF or IL-1β (Fig 4). Interestingly, lower expression of the HAS2 gene was observed in both types of fibroblasts after EGF stimulation compared with that after IL-1β stimulation, whereas the opposite was observed in COME cells after EGF and IL-1β stimulation

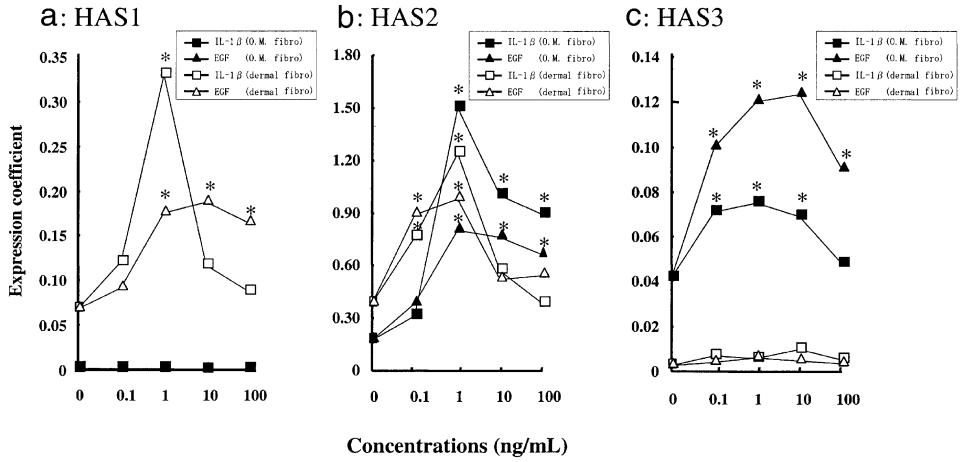

Figure 4
Comparison of concentration-dependent changes of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression in oral mucosal and dermal fibroblasts between stimulation by IL-1β or EGF. Oral mucosal fibroblasts and dermal fibroblasts were cultured in the presence of the indicated concentrations of 0.1–100 ng per mL IL-1β (■ for oral mucosal fibroblasts, □ for dermal fibroblasts) or EGF (▲ for oral mucosal fibroblasts, △ for dermal fibroblasts) After 3 h, cells were lysed and total RNA were extracted. Total RNA were also extracted from the cells just before the treatment for the 0 h sample. Quantitation of each mRNA (HAS1, HAS2, and HAS3) was performed using equal amounts of total RNA (200 ng) by real-time RT-PCR as described in *Materials and Methods*. The ordinate represents the expression coefficient for each mRNA, which was calculated as described in Fig 1. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p < 0.01$.

(compare concentration-dependency in Fig 4 with that in Fig 3).

We then investigated the upregulation of the expression of HAS1, HAS2, and HAS3 mRNAs when oral mucosal and dermal fibroblasts were stimulated with 1 ng per mL IL-1β or EGF for different periods. Treatment of oral mucosal fibroblasts with IL-1β or EGF resulted in marked and moderate increases, respectively, in the amount of HAS2 mRNA, with maximal stimulations at 6 and 3 h, respectively (Figs 5 and 6). On the other hand, the amount of HAS3 mRNA in mucosal fibroblasts was increased time dependently for at least 24 h by IL-1β stimulation, but reached a plateau with a 2-fold increase at 3 h in response to EGF stimulation (Figs 5 and 6). When dermal fibroblasts were treated with IL-1β or EGF, the upregulation of expression of HAS1 and HAS2 mRNAs was observed as early as 3 h after stimulation with either agent. The amount of HAS1 mRNA, however, was further increased time dependently up to 12 h, and then decreased to almost the basal level. The level of HAS2 mRNA was increased to a maximum at 6 h after IL-1β stimulation, decreased at 12 h, and again increased slightly thereafter. The maximal HAS2 expression level after IL-1β stimulation in dermal fibroblasts was 1.5 times higher than that after EGF stimulation (expression coefficient, 1.45 *vs* 0.93) (compare Fig 5 with Fig 6). The maximal stimulations by these cytokines of HAS1, HAS2, and HAS3 gene expression in both types of fibroblasts were mostly observed later than those in COME cells, as described above. The expression coefficients in Figs 5 and 6 suggest that in both types of cells, HAS2 mRNA was more abundant (about 5-fold) than the other HAS mRNAs.

These differences suggest that HAS1, HAS2, and HAS3 gene expression in oral mucosal and dermal fibroblasts is regulated by IL-1β and EGF in distinct manners that are cell-origin-specific and cytokine-specific.

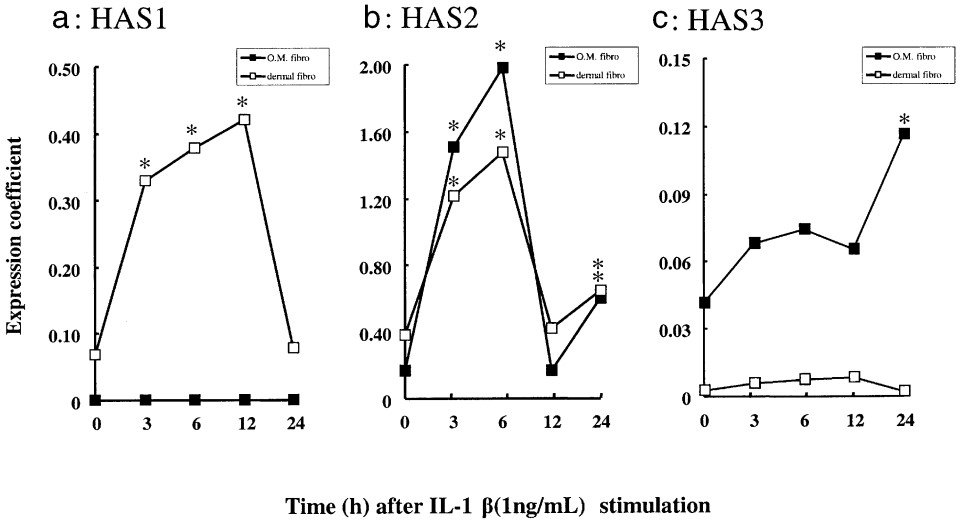

Figure 5
Comparison of stimulation by IL-1β of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression between oral mucosal and dermal fibroblasts. Oral mucosal (■) and dermal (□) fibroblasts were cultured in the presence of 1 ng per mL IL-1β. After 3, 6, 12, and 24 h, cells were lysed and total RNA were extracted. For controls (0 h sample), total RNA were also extracted from the cells just before the addition of IL-1β. Quantitation of each mRNA (HAS1, HAS2, and HAS3) was performed using equal amounts of total RNA (200 ng) by real-time RT-PCR as described in *Materials and Methods*. The ordinate represents the expression coefficient for each mRNA, which was calculated as described in Fig 1. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p < 0.01$.

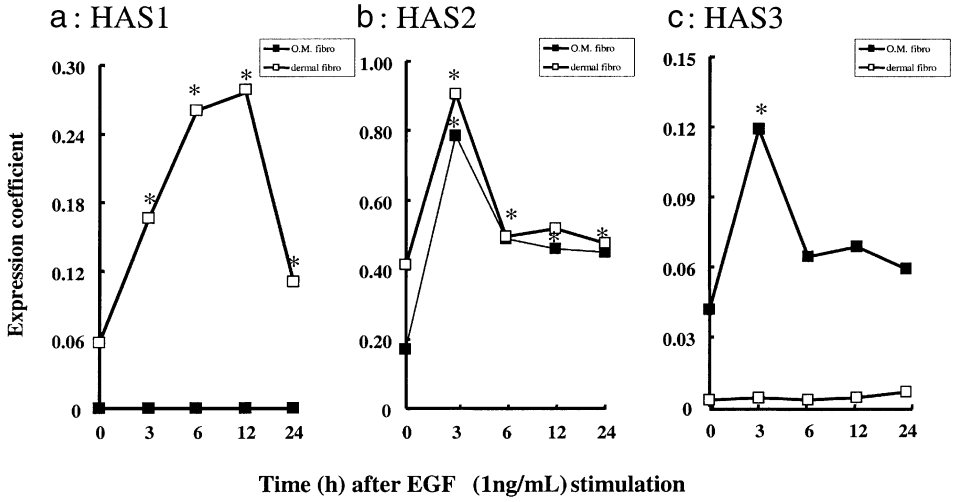

Figure 6
Comparison of stimulation by EGF of HAS1 (*a*), HAS2 (*b*), and HAS3 (*c*) gene expression between oral mucosal and dermal fibroblasts. Oral mucosal (■) and dermal (□) fibroblasts were cultured in the presence of 1 ng per mL EGF. After 3, 6, 12, and 24 h, cells were lysed and total RNA were extracted. For controls (0 h sample), total RNA were also extracted from the cells just before the addition of EGF. Quantitation of each mRNA (HAS1, HAS2, and HAS3) was performed using equal amounts of total RNA (200 ng) by real-time RT-PCR as described in *Materials and Methods*. The ordinate represents the expression coefficient for each mRNA, which was calculated as described in Fig 1. Each point is the mean value obtained from five independent experiments in which the differences were less than 10%. *Significantly different from the control value; $p < 0.01$.

Time course of IL-1β- or EGF-induced hyaluronan synthesis in cultured human COME cells, oral mucosal, and dermal fibroblasts To monitor the rate of hyaluronan synthesis at different times between 0 and 24 h after treatment with 1 ng per mL IL-1β or EGF, we measured the HA concentration in the culture medium at different times by a competitive ELISA-like assay, as shown in Fig 7. In COME cell cultures, IL-1β or EGF treatment induced an ∼ 2–7-fold increase of newly synthesized hyaluronan during the stimulation, compared to the level at 3 h (Fig 7*a*). The amounts of HA in the cultures treated with either agent further increased time dependently up to 24 h. On the other hand, the amounts of HA in cultures of oral mucosal fibroblasts treated with IL-1β or EGF showed an ∼ 23–47-fold increase, and that in cultures of dermal fibroblasts showed a 10–20-fold increase, compared to the levels at 3 and 6 h (Fig 7*b, c*). It is of note that neither oral mucosal fibroblasts nor dermal fibroblasts showed an IL-1β- or EGF-induced increase in hyaluronan synthesis until 6 h and that higher levels of HA were generated in oral fibroblasts than in dermal fibroblasts after IL-1β stimulation (Fig 7*b*), whereas higher levels of HA were observed in dermal fibroblasts than in oral mucosal fibroblasts after EGF stimulation (Fig 7*c*) Overall, it was interesting to see that the increased expression levels of HAS after IL-1β or EGF stimulation resulted in increased rates of HA synthesis, although concentration dependency of the increase of HA synthesis by these agents remains to be determined.

Discussion

A major goal of wound-healing biology is to discover how skin can be induced to reconstruct damaged parts more perfectly (Martin, 1997). In this regard, we firstly noted the previous observation that oral mucosa rarely suffers from

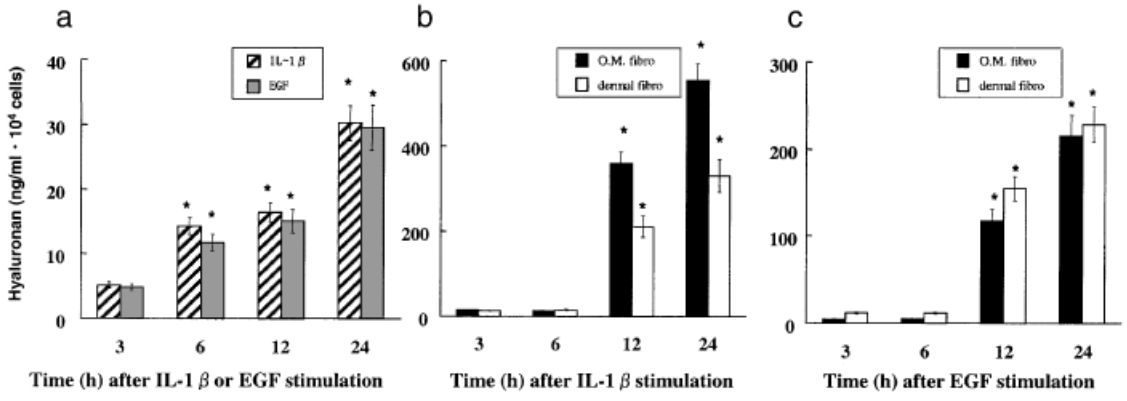

Figure 7
Time course of hyaluronan synthesis after the addition of IL-1β or EGF to COME cells (*a*), and after the stimulation of oral mucosal (■) and dermal fibroblasts (□) by IL-1β (*b*) or EGF (*c*). HA concentrations of the conditioned media of 3, 6, 12, and 24 h cultures were measured by a competitive ELISA-like assay as described in *Materials and Methods*. Each value was calculated by subtracting the control value from the sample value. The control HA activities were about 14.3 (ng per mL 10^4 cells) in COME cells (*a*), 93.6 (ng per mL 10^4 cells) in oral mucosal fibroblasts, 115 (ng per mL 10^4 cells) in dermal fibroblasts, and these values varied only slightly at different times (3, 6, 12, and 24 h). (*a*) COME cells were cultured in the presence of 1 ng per mL IL-1β or EGF (*b*) Oral mucosal and dermal fibroblasts were cultured in the presence of 1 ng per mL IL-1β. (*c*) Oral mucosal and dermal fibroblasts were cultured in the presence of 1 ng per mL EGF. Each column represents the mean ± SD of three separate experiments. *Significantly different from the value at 3 h; $p < 0.01$.

scarring in the process of wound healing and appears to be different from skin (Tsai *et al*, 1995), and therefore focused on differences in physiological responses to wound healing between oral mucosa cells and skin cells in this study. Wound healing involves many dynamic cellular processes, such as cell proliferation, cell migration, cell–cell interaction, and inflammation (Martin, 1997). It has been found that hyaluronan is deeply involved in these dynamic cellular processes during wound healing and inflammation (Knudson *et al*, 1989; Turley, 1989; Weigel *et al*, 1997). It is also known that some cell growth factors and cytokines facilitate wound healing and re-epithelialization by stimulating keratinocyte proliferation and migration (Clark and Henson, 1988; Gailit *et al*, 1994; Hubner *et al*, 1996; Martin, 1997; Tammi and Tammi, 1998; Pienimaki *et al*, 2001). Therefore, in this study we examined the relationships between hyaluronan synthesis and cellular responses to two cell growth regulatory factors involved in wound healing of the skin, namely IL-1β and EGF.

The present results demonstrated that the expression of the three different HAS genes was increased in COME cells by IL-1β or EGF treatment, and there was a corresponding increase of hyaluronan synthesis in these cells after the treatments. Therefore, such treatments may induce changes in the extracellular environment via the increased synthesis and accumulation of hyaluronan. The present data also showed that the stimulations varied with different concentrations and times of treatment with the cytokines and that the stimulation patterns were highly dependent upon cell origins. This may be important for understanding the difference in wound healing between oral mucosal epithelium and epidermis.

Our present study did not focus on human keratinocytes, because they have already been studied in detail in a few studies, with the following results. Sugiyama *et al* (1998) showed that human keratinocytes expressed HAS1 mRNA and a trace of HAS2 mRNA, and that when the culture was stimulated with TGF-β, HAS1 mRNA in keratinocytes was upregulated but HAS2 mRNA was not. A more recent study by Pienimaki *et al* (2001) showed that HAS2 mRNA was expressed in a rat keratinocyte cell line and EGF stimulation brought about an increase in HAS2 mRNA corresponding to about a 30-fold enhancement of hyaluronan production from the basal synthesis rate. They also showed that there was no increase in HAS1 or HAS3 in the cell line, but HAS2 mRNA increased 2–3-fold with less than 2 h following stimulation with EGF. Our present study on oral mucosal epithelium cells, however, demonstrated that in those cells the expression of HAS1 and HAS3 was upregulated after EGF stimulation, although these mRNAs were expressed at lower levels than HAS2 mRNA, and, in addition, HAS2 mRNA increased 2–11-fold depending on the concentration of the inducing agent (Fig 3). This finding may have depended on our use of the different cells or of the real-time RT-PCR method, which is more accurate than the methods used in earlier studies. Our finding also suggests that HAS1 and HAS3 might have distinctive effects on the wound healing of oral mucosal epithelia.

Sugiyama *et al* (1998) demonstrated that when human dermal fibroblasts were stimulated with TGF-β, both HAS1 and HAS2 mRNAs were upregulated. Zhang *et al* (2000) found by a quantitative study of HAS mRNA level that no HAS3 mRNA was detected in the RNA from human skin fibroblasts. We obtained similar results by quantitative real-time RT-PCR in our culture system of human dermal fibroblasts (Fig 4), although we used different cytokines. Our present study showed for the first time that HAS3 gene expression was detected in all of the mRNA samples obtained from human mucosal fibroblasts, but HAS1 gene expression was not detected. This unique HAS expression pattern of mucosal fibroblasts may be related to the unique feature of the lack of scarring in wound healing of oral mucosa epithelia.

It was interesting that the gradual increase in the amount of hyaluronan after the rapid and temporal increases in HAS mRNA expression after IL-1β or EGF treatment and the rates of hyaluronan synthesis differed, depending on the cell type and the stimulation agent (Fig 7). There have been several reports showing that the mRNA levels correlated with the levels of HAS proteins and with the production of hyaluronan (Jacobson *et al* (2000) for human mesothelial cells; Pienimaki *et al* (2001) for rat epidermal keratinocytes; Sayo *et al* (2002) for human keratinocytes). Pienimaki *et al* (2001) showed that the changes in HAS mRNA and hyaluronan synthesis levels showed a temporal correlation, suggesting tight transcriptional regulation of hyaluronan synthesis. In our study, however, in COME cells the hyaluronan synthetic rates increased gradually and time dependently with the maximal stimulation of the HAS mRNA expression at 3 h after IL-1β or EGF treatment, and the rate induced by IL-1β was higher than that induced by EGF. In both oral mucosal and dermal fibroblasts, the increased rates of hyaluronan synthesis were observed at later phases (12–24 h) than those of the mRNA expression. These differences suggest that the increased HAS enzyme proteins translated from the transiently increased amounts of the mRNAs resulting from the stimulation may have a long life span and produce hyaluronan continuously, especially in the cells stimulated by IL-1β. In addition, the turnover rates of the HAS mRNA may differ, depending on the cell type and way of stimulating the cells. In relation to the latter possibility, the gene structures, especially in the regulatory regions influencing the mRNA turnover, are different among the three HASs (Spicer and McDonald, 1998), and we previously characterized the promoter region of the mouse *HAS1* gene (Yamada *et al*, 1998). Investigation of the respective promoter sequences and identification of potential enhancer sequences for the three *HAS* genes and the respective stability of those HAS mRNAs will be necessary for understanding the differences in the responses of the genes to stimulation by various factors.

Jacobson *et al* (2000) pointed out that the cytokine inducibility of the transcripts of the three HAS genes might be widespread, but differ depending on the cell type. Heldin *et al* (1989) also showed that cytokines and growth factors regulate the rates of hyaluronan synthesis differently, depending on the cell type. Since there were differences in the experimental systems and conditions, exact comparisons between studies cannot be made. There are several examples, however, in previous reports suggesting that the above possibility is true. For example, Kaback *et al* (1999) showed substantial upregulation of hyaluronan synthesis in

orbital fibroblasts when they were activated with IL-1β. Hyaluronan synthesis in human synovial lining cells is stimulated by TGF-β and IL-1β, and to a lesser extent by TNF-α, and these cytokines have been suggested to be major factors leading to the development of joint swelling in inflammatory and degenerative joint diseases (Tammi and Tammi, 1998). In human keratinocytes, interferon-γ markedly upregulates HAS3 mRNA, whereas transforming growth factor β downregulates HAS3 transcript levels (Sayo *et al*, 2002). We found that in COME cells stimulated by IL-1β or EGF, there was a smaller increase of HAS3 expression compared to HAS1 and HAS2 expression at an early phase of the stimulation (Figs 1 and 2). Human peritoneal mesothelial cells also show a pronounced increase in hyaluronan synthesis upon IL-1β stimulation, and the resultant increase in the hyaluronan level in the peritoneal cavity was suggested to be relevant to the inflammation in peritonitis (Yung *et al*, 1996). In the mesothelial cells, the IL-1β stimulation caused a continuous time-dependent increase in hyaluronan synthesis, which appeared to be different from our present results demonstrating rapid and temporary stimulation of the expression of all of the HAS genes by IL-1β (Figs 1, 3, and 5). The effect of EGF on the hyaluronan synthesis in human foreskin fibroblasts did not show a sharp dependency on the EGF concentration (Heldin *et al*, 1989), but we found that the effect on HAS mRNA expression in oral mucosal fibroblasts and dermal fibroblasts did (Fig 4). Taken together, these facts imply that it is likely that different regulatory mechanisms for the expression of each of the three HAS genes and the stability of their transcripts control hyaluronan synthesis in different cells, which might lead to important differences in the physiological responses between oral mucosa cells and skin cells.

The general tendency for EGF to inhibit terminal differentiation and the ability of EGF to increase the synthesis of hyaluronan, which we observed in the present experiments, may be in accord with the previous results on the treatment of keratinocytes with hydrocortisone (Agren *et al*, 1995) and retinoic acid (Tammi *et al*, 1989), further strengthening the concept that enhanced hyaluronan synthesis is associated with the delayed cornification of keratinocytes and subsequent epidermal thickening (Clark and Henson, 1988; Tammi and Tammi, 1998). Therefore, regulating hyaluronan synthesis by using the cytokines EGF or IL-1β might be useful for adjusting the levels of hyaluronan in skin cells to levels equal to those in oral mucosal cells, and might thereby promote wound healing without scarring in the skin if this is applied to tissue engineering technology using living cells, cytokines, and growth factors.

Materials and Methods

Cell culture and RNA isolation Skin and oral mucosa (superfluous tissue obtained during surgery) were obtained from five patients treated in the Department of Oral Surgery and Plastic Surgery, Nagoya University Hospital. Donors gave informed consent. The cells were isolated by the method of Hata *et al* (1995). COME cells were routinely grown in HuMedia-KG2 medium (Kurabo, Osaka, Japan) (Boyce *et al*, 1983), supplemented with insulin (10 μg per mL), hydrocortisone (0.5 μg per mL), 4% (v/v) bovine pituitary extract, and EGF (0.1 ng per mL) plus various antibiotics.

To study the effects of IL-1β and EGF, COME cells were expanded by culturing in HuMedia-KG2 medium with the above supplements for 5 d. The cells were thoroughly washed with the medium without the supplements, dissociated by treatment with trypsin, and inoculated into plastic tissue culture dishes (35 mm) containing fresh medium at a density of 5.0×10^5 cells in the medium without EGF. At 48 h, the medium was exchanged and IL-1β or EGF (0.1–100 ng per mL) was added to the medium, and the cells were continuously cultured for the indicated times (0, 3, 6, 12, and 24 h). Total RNAs were extracted from the cells with an RNeasy Mini Kit (QIAGEN, GmbH, Hiden, Germany).

Human oral mucosal and dermal fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Rockville, MD) containing 10% (v/v) fetal bovine serum and various antibiotics. In order to make the culture conditions identical to those used for studies on the stimulation of COME cells by IL-1β or EGF, the cells were grown for 14 d, dissociated with trypsin, and inoculated into plastic culture dishes (35 mm in diameter) at a density of 5.0×10^5 cells with HuMedia-KG2 medium as described above. At 48 h, the medium was refreshed and IL-1β or EGF was added to the medium at 0.1–100 ng per mL. The cells were then cultured for 0, 3, 6, 12, and 24 h and total RNAs were extracted as described above.

Preparation of absolute amounts of HAS1, HAS2, and HAS3 mRNAs Samples containing known amounts of HAS1, HAS2, and HAS3 RNAs were prepared for absolute comparison by real-time RT-PCR analysis. HAS1 cDNA was prepared as described previously (Itano and Kimata, 1996b). HAS2 and HAS3 cDNAs were obtained by PCR with the following pairs of primers—dGTGTTATACATGTCGAGTTTACTTCC (position 1475–1496 of the human *HAS2* ORF sequence) and dGTCATATTGTTGTCCCTTC-TTCCGC (position 1789–1766), and dGGTACCATCAGAAGTTCC-TAGGCAGC (position 83–108 of the human *HAS*3 ORF sequence) and dGAGGAGAATGTTCCAGATGCG (position 411–391), respectively—and subcloned into pBluescript II KS vector (Promega, Madison, Wisconsin). The PCR amplification was carried out by 35 cycles of incubation, with each cycle consisting of denaturation at 94°C for 20 s, annealing at 58°C for 30 s, and extension at 68°C for 8 min. The PCR products were checked by electrophoresis on a 2% (w/v) agarose gel. The cDNAs were confirmed by sequencing these products. The DNAs containing the respective HAS cDNAs downstream of the T7 promoter were constructed and used as templates to synthesize RNAs *in vitro* by incubation with T7 RNA polymerase (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. The RNAs were purified with an RNeasy Mini Kit (QIAGEN). The concentration of each RNA sample was determined by measuring the absorption (A_{260}) with a spectrophotometer.

Real-time RT-PCR analysis Real-time RT-PCR analysis was performed according to the reported method (Gibson *et al*, 1996; Heid *et al*, 1996). Briefly, within a gene-specific PCR oligonucleotide primer pair, an oligonucleotide probe labeled with a reporter fluorescent dye (FAM) at the 5′-end and a quencher fluorescent dye (TAMURA) at the 3′-end was designed. When the probe was intact, the reporter dye emission was quenched. During the extension phase of the PCR cycle, the nucleolytic activity of the DNA polymerase cleaved the hybridization probe and released the reporter dye from the probe. Fluorescence intensity produced during PCR amplifications was monitored by a sequence detector directly in the reaction tube ("real time"). A computer algorithm compared the amount of reporter dye emission with the quenching dye emission and calculated the threshold cycle number (C_T) when signals reached ten times the standard deviation of the baseline, from which the levels of the mRNAs transcribed from the various genes tested were obtained (Gibson *et al*, 1996).

Total RNA samples (200 ng of each) were added to a 50 μL RT-PCR reaction (PCR-Access, Promega). The "reaction master

Table I. Sequences of oligonucleotide primers and real time RT-PCR probes

Primer or Probe	Sequence	Position
hHAS1 900F	TGTGTATCCTGCATCAGCGGT	900–920
hHAS1 1072R	CTGGAGGTGTACTTGGTAGCATAACC	1072–1047
hHAS1 probeF	TAACCTCTTGCAGCAGTTTCTTGAGGCC	941–968
hHAS2 1475F	GTGTTATACATGTCGAGTTTACTTCC	1475–1496
hHAS2 1789R	GTCATATTGTTGTCCCTTCTTCCGC	1789–1766
hHAS2 probeF	TGGAACGTTGCTCTATGCATGCTATTGG	1692–1719
hHAS3 83F	GGTACCATCAGAAGTTCCTAGGCAGC	83–108
hHAS3 411R	GAGGAGAATGTTCCAGATGCG	411–391
hHAS3 probeF	TGGCTACCGAACTAAGTATACCGCGCGCTC	158–188

mixture" was prepared according to the manufacturer's protocol to give final concentrations of 1 × avian myeloblastosis virus Tfl reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 0.1 U per mL avian myeloblastosis virus reverse transcriptase, 0.1 U per μL Tfl DNA polymerase, 250 nM concentration of the primers, and 200 nM concentration of the corresponding probe, as described by Gibson *et al* (1996). Primers and probes for real-time PCR analysis of the hHAS1, hHAS2, and hHAS3 mRNAs were designed using the Oligo version 4.0 program (National Bioscience, Plymouth, Minnesota) according to Heid *et al* (1996). It has been shown that two or three transcripts that might be generated using alternative polyadenylation signals in the 3′-untranslated region exist for every HAS mRNA (Spicer and McDonald, 1998). In addition, there were differences in the 5′-terminal region sequence between the human HAS1 cDNAs cloned by us (Itano and Kimata, 1996b) and by Shyjan *et al* (1996). Therefore, this was taken into consideration when we designed the combinations of the primers. The sequences of all oligonucleotides used are shown in Table I. For the hHAS1 mRNA analysis, the primers hHAS1 900F and hHAS1 1072R were used and the probe was hHAS1TaqMan FP. For hHAS2 mRNA analysis, the primers hHAS2 1475F and hHAS2 1789R were used and the probe was hHAS2 TaqMan FP. For hHAS3 mRNA analysis, the primers hHAS3 83F and hHAS3 411R were used and the probe was hHAS3 TaqMan FP. The GAPDH primer and probe (TaqMan GAPDH detection reagents) were purchased from Perkin-Elmer and Applied Biosystems, Foster City, CA. RT-PCR reactions and the resulting relative increases in reporter fluorescent dye emissions were monitored in real time using a 7700 sequence detector (Perkin-Elmer). Signals were analyzed using the sequence detector 1.0 program (Perkin-Elmer). The conditions for PCR were as follows: an initial incubation at 50°C for 2 min, at 60°C for 30 min, and at 95°C for 5 min, followed by 50 cycles of incubation at 95°C for 20 s and 60°C for 1 min.

The absolute amount of each HAS mRNA in a given sample was obtained by comparison with the respective standard curves. Standard curves for each HAS mRNA were obtained using human heart t-RNA (Clontech, Palo Alto, California) with different concentrations (4000, 2000, 1000, 500, 250, 125, and 62.5 ng). Comparisons of the absolute amount of each mRNA for the three different HAS synthases among the different samples were made by using factors that were obtained by dividing the absolute amount of each mRNA by that of the GAPDH mRNA in each sample, and were designated as expression coefficients in this study.

Determination of HA concentrations by competitive ELISA-like assay Exponentially growing cells were cultured in fresh medium for 3, 6, 12, or 24 h, and the conditioned medium was recovered from each culture. The HA content of the conditioned medium was measured by a competitive ELISA-like assay as described previously (Itano *et al*, 1999). Briefly, the conditioned medium was mixed with biotinylated HA binding protein (b-HABP) and incubated at 4°C for 20 h. The mixture was added to the HA-coated wells of 96-well plates, followed by incubation for 6 h at room temperature. Alkaline phosphatase-conjugated streptavidin was used as the secondary probe, and the enzymatic activity was measured by using *p*-nitrophenyl phosphate as the substrate. HA contents were calculated by using a standard curve.

Statistical analysis The Dunnett test was used for comparisons between control and experimental groups (Dunnett, 1964).

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