Decreased Prostaglandin E_2 Production by Inflammatory Cytokine and Lower Expression of EP2 Receptor Result in Increased Collagen Synthesis in Keloid Fibroblasts

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We investigated the metabolism of arachidonic acid in normal skin-derived fibroblasts (NF) as well as in keloid-derived fibroblasts (KF) in response to macrophage migration inhibitory factor (MIF), a pluripotent cytokine. We found that MIF enhanced cyclooxygenase-2 activity in NF more than in KF. Consistent with this finding, prostaglandin E_2 (PGE_2), an antifibrogenic molecule, was more significantly increased in NF than in KF by MIF treatment. As regarding E prostanoid receptor 2, the level of expression was significantly lower in KF than in NF. On the other hand, Forskolin, a direct activator of adenylcyclase, decreased collagen synthesis in both NF and KF, which indicates that cAMP plays an important role in regulating collagen synthesis. As PGE_2 induces cAMP production, it is conceivable that increased collagen synthesis in KF might be owing to decreased PGE_2 and cAMP production. These findings may aid in the development of a therapeutic strategy for the regulation of collagen synthesis in keloid fibroblasts.

Journal of Investigative Dermatology (2006) **126,** 990–997. doi:10.1038/sj.jid.5700227; published online 9 March 2006

INTRODUCTION

Keloids are benign dermal tumors that invade normal skin beyond the boundaries of an original wound. Keloids are characterized by the overproliferation of fibroblasts and an increase in collagen synthesis (Peltonen *et al.*, 1991; Babu *et al.*, 1992; Lee *et al.*, 1999; Chodon *et al.*, 2000; Chin *et al.*, 2001; Funayama *et al.*, 2003), and they are reflective of a pathologic wound healing response with proliferative dermal growth. The pathologic features are known to be caused by the excessive deposition of collagen; however, the etiology remains elusive. Keloids can be considered as the end result of an excessive wound healing response, in which inflammatory cells play a major role. In this context, it is conceivable that the interaction between keloid fibroblasts and inflammatory cells is essential for keloidogenesis. In fact, a significant amount of infiltrated T lymphocytes and macrophages have been detected in the keloid dermis, in comparison to that in the dermis of normal skin (Boyce *et al.*, 2001).

Macrophage migration inhibitory factor (MIF) was originally identified as a T-cell-derived cytokine (Bloom and Bennett, 1966; David, 1966). This cytokine has been recognized as a pituitary hormone that is released in response to an array of stimuli, a proinflammatory cytokine released primarily by macrophages, and a T-cell activator essential for immune responses (Bernhagen *et al.*, 1993; Calandra *et al.*, 1994; Bacher *et al.*, 1996). MIF is a unique protein induced by glucocorticoids and counteracts their anti-inflammatory and immunosuppressive functions (Calandra *et al.*, 1995). Recent studies have revealed that MIF is ubiquitously expressed in various types of cells, and has been re-evaluated as a pluripotent cytokine involved in the broad-spectrum immune system (Bucala, 1996; Nishihira, 2000). MIF is known to be involved in angiogenesis, tumor growth, and metastasis (Sun *et al.*, 2003). MIF also activates cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase-2 (COX-2) in cultured fibroblast-like synoviocytes. Moreover, anti-MIF antibody significantly reduced IL-1β-induced COX-2 activity and COX-2 mRNA expression, suggesting that MIF acts as an essential component for the upregulation of $cPLA_2$ and COX-2 activity induced by IL-1β (Sampey *et al.*, 2001). These previous observations prompted us to investigate the role of MIF in normal skin-derived fibroblasts (NF) and keloid-derived fibroblasts (KF), relevant to arachidonic acid metabolites.

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Abbreviations: ANOVA, analysis of variance; $cPLA_2$, cytosolic phospholipase A_2; COX-2, cyclooxygenase-2; EP2, E prostanoid receptor 2; ERK, extracellular signal-regulated kinase; KF, keloid-derived fibroblasts; MIF, macrophage migration inhibitory factor; NF, normal skin-derived fibroblasts; PGE_2, prostaglandin E_2; PICP, procollagen type I C-peptide; RT-PCR, reverse transcriptase-polymerase chain reaction

Received 17 June 2005; revised 12 December 2005; accepted 4 January 2006; published online 9 March 2006

As regards the metabolism of arachidonic acid, $cPLA_2$ is known to play a central role in providing arachidonic acid from membrane phospholipids for the subsequent synthesis of prostaglandins and leukotrienes. Among the reported arachidonic acid metabolites, prostaglandin E_2 (PGE_2) inhibits fibroblast proliferation and collagen synthesis (Korn *et al.*, 1980; Goldstein and Polgar, 1982; Bitterman *et al.*, 1986; Fine and Goldstein, 1987; Elias, 1988; Fine *et al.*, 1989; Kawamoto *et al.*, 1995; Wilborn *et al.*, 1995). In this context, phospho-p44/42 mitogen-activated protein kinase (extracellular signal-regulated kinase (ERK)1/2) can phosphorylate $cPLA_2$, which then induces an increase in $cPLA_2$ activity (Lin *et al.*, 1993). It has been reported that MIF regulates $cPLA_2$ by the activation of mitogen-activated protein kinase (Mitchell *et al.*, 1999). In this study, we examined the induction of $cPLA_2$ by MIF in NF and KF, and we investigated the mechanism of the fibrogenesis in keloids. A better understanding of the pathophysiology of keloids might help in the development of an appropriate strategy for the treatment of keloids.

RESULTS

Immunohistochemical localization of MIF

Immunohistochemical analysis was carried out using tissues obtained during surgical excision. The keloid dermal skin showed significant MIF-positive staining. In particular, higher levels of expression of MIF were observed in KF than in NF (Figure 1). On the other hand, in normal skin and normal scar, endothelial cells exhibited the physiological expression of MIF, but no such expression in NF or in the extracellular matrix was detected.

Activation of $cPLA_2$ by MIF

$cPLA_2$ activity was expressed as the release of [^{3}H]arachidonic acid from fibroblasts. In the prelabeled NF and KF, the release of [^{3}H]arachidonic acid in non-stimulated both fibroblasts showed the similar level. To determine an influence of MIF on $cPLA_2$ activity in NF and KF, each type of fibroblast was stimulated with recombinant human MIF (500 ng/ml). The addition of MIF was associated with an increase in the release of [^{3}H]arachidonic acid from both NF and KF. Interestingly, the release of [^{3}H]arachidonic acid was higher in KF than in NF when these cells were stimulated with MIF (Figure 2).

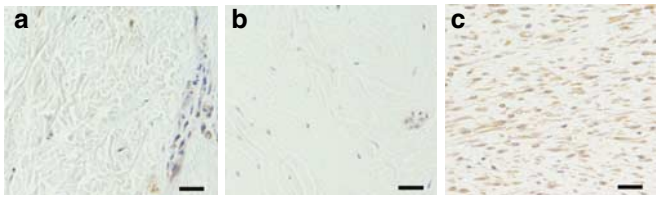

Figure 1. (a–c) Immunohistochemistry of MIF in keloid fibroblasts. We performed an immunohistochemical analysis of (**a**) normal skin or (**b**) normal scar or (**c**) keloid samples using an anti-MIF antibody. Dermis obtained from each sample was formalin-fixed and stained with rabbit anti-human MIF antibody. After incubation with secondary antibody conjugated with horseradish peroxidase, the sections were developed with diaminobenzidene. We repeated immunohistochemical analyses on normal skin ($n=6$), normal scar ($n=6$), and keloids ($n=6$), which showed the similar immunostaining patterns. We demonstrated representatives for these samples. Bar = 50 μm.

$cPLA_2$ mRNA induction in NF and KF by MIF

To examine in detail the effects of MIF on $cPLA_2$ activity, the induction of $cPLA_2$ mRNA by MIF in NF and KF was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Figure 3, MIF upregulated $cPLA_2$ mRNA in both NF and KF, but there was no significant difference in the levels of $cPLA_2$ mRNA induced by MIF. These findings suggested that the specific induction of $cPLA_2$ activity in KF by MIF was not transcriptionally regulated.

ERK1/2 phosphorylation by MIF

The ERK cascade is a convergent pathway in the mitogenic activity of various growth factors. The phospho-p44/42 mitogen-activated protein kinase (ERK1/2) phosphorylates $cPLA_2$, leading to an increase in $cPLA_2$ activity. Therefore, the release of arachidonic acid depends on the phosphorylation and activation of $cPLA_2$. MIF has been shown to stimulate the phosphorylation of p44/p42 ERK MAP kinase (Mitchell *et al.*, 1999; Fukuzawa *et al.*, 2002). Here, we analyzed the expression of phosphorylated ERK with MIF (100 or 500 ng/ml) in NF and KF by immunoblot analysis using anti-phospho-ERK1/2. In both NF and KF, the phosphorylated form of ERK1/2 was significantly enhanced at 15 minutes by MIF, and returned to the initial level at 1 hour (Figure 4). Interestingly,

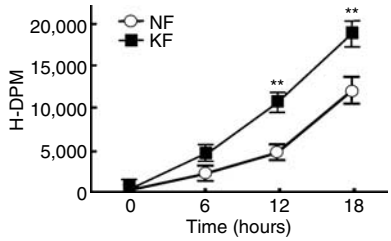

Figure 2. Time course of [^{3}H]arachidonic acid release induced by MIF stimulation from keloid fibroblasts. Each type of fibroblast was treated with recombinant human MIF (500 ng/ml) for 6, 12, and 18 hours. $cPLA_2$ activity was represented by [^{3}H]arachidonic acid released (c.p.m.). *$P<0.05$; **$P<0.01$ (ANOVA with Scheffe's *post hoc* test). Data shown are the mean ± s.d. of six independent experiments.

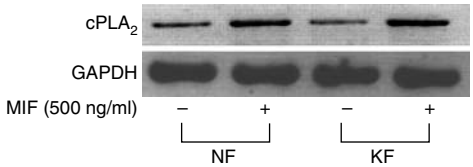

Figure 3. Expression of $cPLA_2$ mRNA after treatment with MIF. Fibroblasts were incubated in the presence or absence of recombinant human MIF (500 ng/ml) in DMEM/1% FBS. Expression of $cPLA_2$ and GAPDH mRNA was examined by RT-PCR as described in Materials and Methods. Similar results were obtained from three independent experiments.

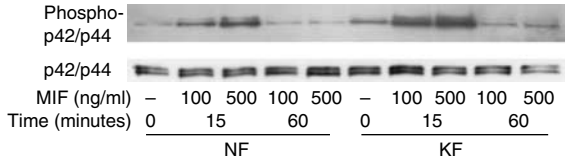

Figure 4. MIF-induced ERK1/2 activation in NF and KF. NF and KF were stimulated with recombinant human MIF (100 or 500 ng/ml). In order to examine ERK1/2 activity, the cells were lysed and subjected to immunoblot analysis using anti-phospho-specific ERK1/2 (upper panel) and anti-ERK1/2 (lower panel) antibodies, as described in Materials and Methods. Similar results were obtained from three independent experiments.

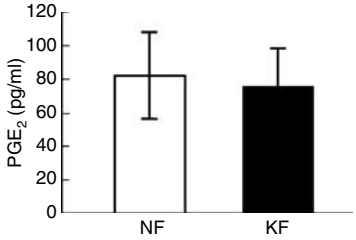

Figure 5. PGE_2 accumulation in unstimulated NF and KF. NF and KF were incubated for 24 hours in DMEM/1% FBS. Both NF and KF were grown to 80% confluence. Aliquots of the supernatants were then collected for PGE_2 ELISA as described. Data shown are the mean ± s.d. of six independent experiments.

KF showed a more strongly phosphorylated band of ERK than that of NF at 15 minutes when the cells were stimulated with MIF.

PGE_2 production in response to MIF and IL-1β in NF and KF

We assessed PGE_2 production in order to detect COX-2 activity in non-stimulated fibroblasts. There was no significant difference between NF and KF in terms of PGE_2 production (Figure 5). We then measured PGE_2 production in NF and KF upon treatment with MIF (500 ng/ml) or IL-1β (5 ng/ml) for 6 and 12 hours. COX-2 activity was detected by ELISA of PGE_2 production in the cellular supernatants. PGE_2 production was upregulated in response to MIF as well as to IL-1β in NF (Figure 6a and b). In contrast, the induction of PGE_2 accumulation by MIF and IL-1β was significantly higher in NF than KF. Thus, the difference in PGE_2 production in the stimulated cells was reflective of the differences between NF and KF with respect to COX-2 activity.

COX-2 mRNA induction by MIF or IL-1β in NF and KF

To examine whether or not the observed differences in PGE_2 production depended on COX-2 activity, we compared the levels of COX-2 mRNA expression in NF and KF using RT-PCR analysis after the cells had been treated with MIF (500 ng/ml) or IL-1β (5 ng/ml) for 12 hours. We found significantly higher levels of expression of COX-2 mRNA in the stimulated NF than in the stimulated KF. In KF, no differences in the levels of expression of COX-2 mRNA were observed. With the addition of MIF or IL-1β, the levels

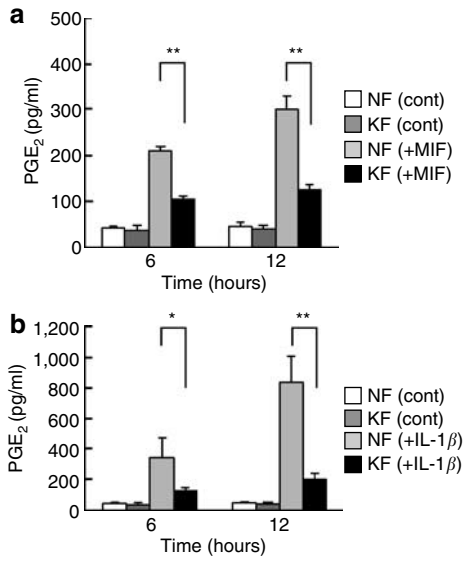

Figure 6. PGE_2 accumulation in NF and KF stimulated with MIF or IL-1β. NF and KF were incubated for 6 or 12 hours in the presence or absence of (**a**) recombinant human MIF (500 ng/ml) or (**b**) IL-1β (5 ng/ml) in DMEM/1% FBS. Aliquots of the supernatants were collected for PGE_2 ELISA as described. $*P<0.05$; $**P<0.01$ (ANOVA with Scheffe's *post hoc* tests). Data shown are the mean ± s.d. of six independent experiments.

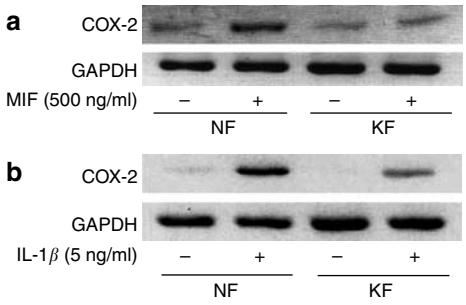

Figure 7. COX-2 mRNA expression after treatment with MIF or IL-1β. NF and KF were incubated for 12 hours in the presence or absence of (**a**) recombinant human MIF (500 ng/ml) or (**b**) IL-1β (5 ng/ml) in DMEM/1% FBS. Expression of COX-2 and GAPDH mRNA was examined by RT-PCR. Similar results were obtained from three independent experiments.

of COX-2 mRNA expression clearly increased in NF and in KF as well, albeit to a lesser extent than in NF (Figure 7a and b).

Effect of anti-MIF antibody on PGE_2 production and COX-2 mRNA expression induced by IL-1β

PGE_2 production and the levels of COX-2 mRNA expression clearly increased in response to IL-1β (5 ng/ml) in NF. To examine the involvement of MIF in the activation, we added an anti-MIF monoclonal antibody. We found that anti-MIF monoclonal antibody (50 μg/ml) inhibited both PGE_2 production and COX-2 mRNA expression induced by IL-1β in NF (Figure 8a and b). Similar results were obtained in KF (data not shown).

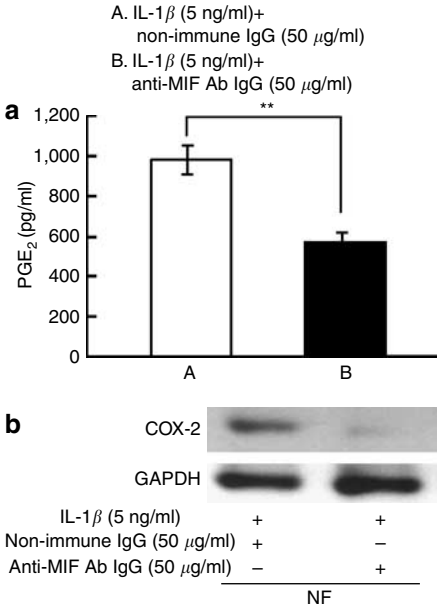

Figure 8. Effect of anti-MIF antibody on COX-2 activity induced by IL-1β in NF. (**a**) NF were treated with anti-MIF monoclonal antibody (50 μg/ml) or non-immune IgG (50 μg/ml), IL-1β (5 ng/ml) in DMEM/1% FBS and were incubated for 12 hours. Aliquots of the supernatants were collected for PGE_2 ELISA as described in Materials and Methods. *$P<0.05$; **$P<0.01$ (ANOVA with Scheffe's *post hoc* tests). Data shown are the mean±s.d. of six independent experiments. (**b**) Expression of COX-2 and GAPDH mRNA was examined by RT-PCR. Similar results were obtained from three independent experiments.

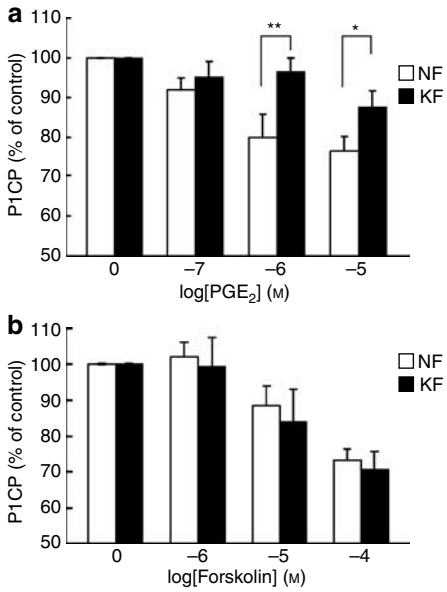

Figure 9. Regulation of collagen synthesis by treatment with PGE_2 and Forskolin. Cells were treated with (**a**) PGE_2 or (**b**) Forskolin in DMEM/0.5% FBS for 24 hours, and PICP levels were measured. The control level (100%) refers to the values obtained in the absence of PGE_2 or Forskolin. *$P<0.05$; **$P<0.01$ (ANOVA with Scheffe's *post hoc* tests). Data shown are the mean±s.d. of nine independent experiments.

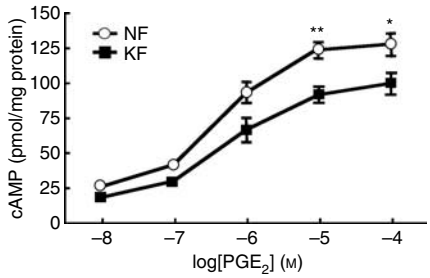

Figure 10. cAMP production in NF and KF. Cells were treated with various concentrations of PGE_2. cAMP production was measured by ELISA. *$P<0.05$; **$P<0.01$ (ANOVA with Scheffe's *post hoc* tests). Data shown are the mean±s.d. of six independent experiments.

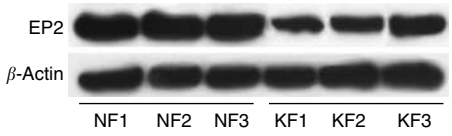

Figure 11. EP2 expression in keloid fibroblasts. Cells were lysed and subjected to immunoblot analysis using anti-EP2 polyclonal antibody (upper panel). The results were standardized with β-actin. NF1, 2, and 3: fibroblasts from normal skin samples obtained from three different patients. KF1, 2, and 3: fibroblasts from the keloid lesions of three different patients.

Effects of PGE_2 or Forskolin on PICP production

The addition of PGE_2 to the culture media decreased procollagen type I C-peptide (PICP) levels in the cellular supernatants in NF (Figure 9a). On the other hand, PGE_2 had no significant effect on PICP levels in KF. The addition of Forskolin to the culture media clearly decreased PICP levels in both NF and KF (Figure 9b).

cAMP production in NF and KF with PGE_2

To investigate why the addition of PGE_2 was associated with decreased PICP levels in the cellular supernatants in NF, but less so in KF, we measured cAMP production in NF and KF in response to various concentrations of PGE_2. The results demonstrated that cAMP production by PGE_2 was significantly higher in NF than KF (Figure 10).

EP2 expression in NF and KF

It is known that PGE_2 transduces its biological signal through its specific receptor, E prostanoid receptor 2 (EP2). We compared the expression levels of the EP2 in NF and KF using Immunoblot analysis. We found significant differences in the expression of the EP2 receptor between NF and KF. The expression of the EP2 receptor was significantly lower in KF than in NF (Figure 11).

DISCUSSION

A number of studies have demonstrated that MIF plays a pivotal role in the systemic as well as in the local inflammatory and immune responses. As for the role of MIF in association with arachidonic acid metabolism, several reports have been published. MIF stimulated mitogen-activated protein kinase and cPLA2, which indicates unique action of this protein in cell proliferation (Mitchell *et al.*,

1999). MIF also activates $cPLA_2$ and COX-2 in cultured fibroblast-like-synoviocytes (Sampey *et al.*, 2001). Moreover, this protein was found to be involved in cell proliferation in response to IL-1β and tumor necrosis factor-α (Lacey *et al.*, 2003). Accordingly, we used MIF as a stimulatory factor for the activation of the arachidonic acid cascade. We here observed abundant MIF in keloid tissues by immunohistochemical analysis.

In this study, we demonstrated that the release of [^{3}H]arachidonic acid from KF was greater than that from NF. Immunohistochemical analysis revealed the abundant expression of MIF protein in the cytosol of keloid fibroblasts. In KF but not in NF, the induction of phosphorylated ERK was obtained at 15 minutes when the cells were stimulated with MIF. PGE_2 production induced by MIF or IL-1β treatment was significantly higher in NF than KF, suggesting the suppression of COX-2 induction in KF, but not in NF. The difference in PGE_2 production between NF and KF was found to depend on the levels of expression of COX-2 mRNA. The expression of COX-2 mRNA was more markedly upregulated in NF than KF in response to MIF or IL-1β treatment. Moreover, we found that the sensitivity to exogenous PGE_2 with respect to the inhibition of collagen synthesis was more downregulated in KF than in NF. These results suggested that differences between NF and KF in terms of collagen synthesis might be associated with difference in PGE_2 production in NF and KF, and also with the respective sensitivity of these two distinct fibroblast types to exogenous PGE_2.

The typical characteristics of keloids are the excessive production and fibrotic deposition of collagen (Peltonen *et al.*, 1991; Babu *et al.*, 1992; Lee *et al.*, 1999; Chodon *et al.*, 2000; Chin *et al.*, 2001; Funayama *et al.*, 2003). Prostaglandin has the ability to downregulate fibroblast proliferation and collagen synthesis, and fibroblasts are known to synthesize PGE_2 (Korn *et al.*, 1980; Goldstein and Polgar, 1982; Bitterman *et al.*, 1986; Fine and Goldstein, 1987; Elias, 1988; Fine *et al.*, 1989; Kawamoto *et al.*, 1995; Wilborn *et al.*, 1995). We hypothesized that KF might have a diminished capacity to synthesize PGE_2 in comparison with that of NF. This hypothesis was tested using primary cultures of fibroblasts isolated from fresh keloid tissues obtained by surgical excision.

We found more enhanced levels of release of [^{3}H]arachidonic acid from KF than from NF in response to MIF, indicating that KF have greater $cPLA_2$ activity than NF. It was reported that $cPLA_2$ was activated by phosphorylation via the activation of p44/42 ERK1/2 (Lin *et al.*, 1993). As regards MIF, it is known to be able to stimulate the phosphorylation of p44/p42 ERK MAP kinase (Mitchell *et al.*, 1999; Fukuzawa *et al.*, 2002). Here, we revealed the expression of phosphorylated ERK by MIF in NF and KF by immunoblot assay using anti-phospho-ERK1/2 antibody. In both NF and KF, the levels of the phosphorylated form of ERK1/2 were significantly enhanced at 15 minutes following MIF pre-treatment, and the levels returned to the baseline at 1 hour. Interestingly, when KF was compared to NF, KF was found to exhibit higher levels of phosphorylation of ERK at 15 minutes after the addition of MIF. These data provide support for the hypothesis that the difference between NF and KF in terms of [^{3}H]arachidonic acid release may depend on differences in their respective levels of activated ERK. The endogenous levels of PGE_2 production were similar in NF and KF lacking exogenous stimulation; however, as regards PGE_2 production in response to MIF (500 ng/ml) or IL-1β (5 ng/ml), the induction of PGE_2 production was significantly more pronounced in NF than KF. It was considered likely that the differences in PGE_2 production in the stimulated cells might have been reflective of differences between NF and KF in terms of COX-2 activity.

Unlike in NF, in KF, the increase in COX-2 activity was not clearly observed after pretreatment of the cells with MIF or IL-1β. Although COX-2 mRNA levels were similar under non-stimulated conditions, these levels were indeed higher in NF than in KF in the cases involving MIF or IL-1β stimulation. The failure to augment COX-2 metabolic activity in KF correlated with COX-2 mRNA levels. Our current results demonstrated that MIF directly induces COX-2 expression in NF. Moreover, COX-2 activity in NF was increased to a greater degree by MIF or IL-1β than was the case in KF. In other words, only a negligible increase in COX-2 mRNA was induced in KF by these stimuli.

The autostimulatory pathway showing the biological link between IL-1β and MIF has been reported in association with activation of the arachidonic acid pathway (Sampey *et al.*, 2001). In consistent with this finding, we found that anti-MIF monoclonal antibody (50 μg/ml) inhibited COX-2 activity induced by IL-1β in NF. This fact indicates that MIF might be involved in collagen production in response to IL-1β via PGE_2 and COX-2.

PGE_2, a final product in arachidonic acid metabolism, was produced to a lesser extent in KF than in NF; however, arachidonic acid availability as a source of this metabolic pathway was less in NF than KF. These contradictory results between NF and KF suggest the possibility that, for the PGE_2 production in dermal fibroblasts, COX-2 activity is more critical than the amount of arachidonic acid released.

Despite a higher level of MIF expression in KF compared to NF demonstrated by immunohistochemistry, PGE_2 production in response to MIF was significantly reduced in KF than in NF. In this context, inhibition of collagen synthesis by PGE_2, an antifibrogenic molecule, is more intense in NF than in KF. Namely, it is suggested that decreased PGE_2 production in KF leads to enhanced collagen synthesis. On other hand, KF showed a more intense phosphorylated band of ERK than that of NF in response to MIF. The activation of ERK by MIF might induce cell proliferation in KF in addition to $cPLA_2$ stimulation. Further investigation is in progress to better understand the effect of MIF on ERK pathway in keloidogenesis.

We here demonstrated that PGE_2 inhibited collagen synthesis in NF, and less so in KF. PGE_2 has been reported to exert suppressive effects on collagen production in fibroblasts. These suppressive effects are mediated by increases in cAMP; such results are based on those of previous reports, which demonstrated that cAMP is an antifibrotic second messenger in fibroblasts (Kohyama *et al.*, 2001; Liu *et al.*,

2004). As regards the signal transduction associated with PGE_2, the biological activity of PGE_2 was found to be exerted via four receptor types: EP1, EP2, EP3, and EP4 (Coleman *et al.*, 1994; Negishi *et al.*, 1995; Narumiya, 1996). As regards fibrosis, recent publications have indicated that EP2 transduces PGE_2 signaling and results in the downregulation of collagen synthesis (Choung *et al.*, 1998; Kolodsick *et al.*, 2003).

We hypothesized that the differences between NF and KF in response to PGE_2 were caused by differences in EP2 expression levels, and found that the expression of EP2 in KF was lower than that in NF. Forskolin is known as an agent that increases cAMP in a receptor-independent manner by directly activating adenylate cyclase. Forskolin led to decreases in the levels of collagen produced in both types of fibroblasts within a range of 10^{-4}–10^{-5} M. These results indicated that the cAMP pathway is an important regulator of collagen synthesis in KF.

We demonstrated more enhanced MIF in KF than NF as shown in Figure 1. We examined only a few cases of normal skin fibroblasts obtained from early wound skin lesion during the course of this study. It showed higher MIF expression than fibroblasts obtained from late stage wound, as in the case of rat skin (Abe *et al.*, 2000). According to these facts, it is considered that MIF expression level sustains in KF, whereas it transiently increased and returns to the minimal level in NF. We believe that MIF may play an important role for hyperproliferation of KF.

In conclusion, we demonstrated here that exogenous MIF enhanced COX-2 activity and PGE_2 to a greater degree in NF than in KF, and that cAMP, when elevated by Forskolin, suppressed collagen synthesis in both types of fibroblast. These findings suggest that the reduced capacity of KF to downregulate collagen synthesis via PGE_2 contributes to cellular hyperproliferation in keloids. Our current results indicate that the regulation of cellular levels of cAMP might be useful as a therapeutic strategy in the regulation of collagen synthesis and deposition in keloids.

MATERIALS AND METHODS

Tissue samples

Keloid is a benign dermal tumor as it invades normal skin beyond the boundaries of the original wound and dose not regress spontaneously. Recurrence is common after surgical excision, which often exacerbates the condition. Hypertrophic scars, on the other hand, remain within the boundaries of the original wound, frequently regress spontaneously, and recurrence is rare after surgical excision (Rockwell *et al.*, 1989; Chodon *et al.*, 2000). Based on these data, we differentiated keloid from hypertrophic scars.

A total of six keloid samples were obtained from six Japanese patients. Only clinically typical samples were included in this study. In addition, six normal age- and site-matched skin samples were obtained during unrelated surgical operations. None of the keloid patients had received previous treatment other than pressure therapy. Written informed consent was obtained from all patients. Primary fibroblasts from fresh keloid tissue and from normal tissue obtained at the time of surgical excision were cultured for the present experiments. All procedures were approved by the ethical code of Hokkaido University School of Medicine in accord with the Declaration of Helsinki Principles.

Fibroblast culture

Fibroblasts were grown in DMEM (Life Technologies Inc., Gaithersburg, MD) supplemented with 20% and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) for the primary culture and subsequent cultures, respectively, and 5 mg/ml L-glutamine in an atmosphere of 5% CO_2. Fibroblasts in the primary cultures were trypsinized with 0.05% trypsin/0.53 mM EDTA/4Na (Life Technologies Inc., Carlsbad, CA). The three to four passed cells were used for the experiments.

MIF

Recombinant MIF was prepared as described previously (Sun *et al.*, 2003).

Anti-MIF monoclonal antibody

We raised monoclonal antibodies against human MIF (Mizue *et al.*, 2000), and used them for the current experiments.

Assessment of $cPLA_2$ activity

To measure the release of arachidonic acid from NF and KF, fibroblasts (2×10^5 cells) were labeled at 37°C for 18 hours with [^{3}H]arachidonic acid (3 μCi/3 ml per dish) (Amersham, Arlington Heights, IL) in DMEM/1% bovine serum albumin as described previously (Sakamoto *et al.*, 1993; Croxtall *et al.*, 1995; Sampey *et al.*, 2001).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Funayama *et al.*, 2003), using anti-phospho-ERK antibody (1:2,000; specific for the phosphorylated forms of ERK1 and ERK2 activated by dual phosphorylation in the Thr202/Tyr204 region; Cell Signaling Technology, Beverly, MA), or anti-EP2 polyclonal antibody (1:500; Cayman Chemical, Ann Arbor, MI).

RT-PCR for $cPLA_2$ and COX-2

The PCR was performed as follows: $cPLA_2$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 94°C for 2 minutes for one cycle, 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute for 35 cycles, and 72°C for 7 minutes for one cycle; COX-2 and GAPDH, 94°C for 2 minutes for one cycle, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute for 35 cycles, and 72°C for 7 minutes for one cycle using a thermal cycler (PC808, ASTEC, Tokyo, Japan). The following PCR primers were used: for $cPLA_2$, 5′-GAG-CTG-ATG-TTT-GCA-GAT-TGG-GTT-G-3′ (forward) and 5′-GTC-ACT-CAA-AGG-AGA-CAG-TGG-ATA-AGA-3′ (reverse); for COX-2, 5′-TTC-AAA-TGA-GAT-TGT-GGG-AAA-ATT-GCT-3′ (forward) and 5′-AGA-TCA-TCT-CTG-CCT-GAG-TAT-CTT-3′ (reverse); and for GAPDH, 5′-CGT-CTT-CAC-CAC-CAT-GGA-GA-3′ (forward) and 5′-CGG-CCA-TCA-CGC-CAC-AGT-TT-3′ (reverse). After PCR, an aliquot of each amplification mixture was subjected to electrophoresis on 2% agarose gel, and the DNA was stained with ethidium bromide.

Measurement of PGE_2 accumulation in unstimulated NF and KF

NF and KF (2×10^5 cells) were seeded and incubated overnight, washed twice with PBS, and then incubated again for 24 hours in

DMEM/1% FBS. The amount of PGE_2 in the cellular supernatants was then determined by ELISA (Cayman Chemical) according to the manufacturer's instructions. PGE_2 accumulation was used to reflect the activity of both $cPLA_2$ and COX-2.

Measurement of PGE_2 accumulation in NF and KF with MIF or IL-1β

NF and KF (2×10^5 cells) were seeded and incubated overnight. For the inhibition of endogenous COX, fibroblasts were incubated with serum-free DMEM containing 10 μM indomethacin for 15 minutes at 37°C in an atmosphere of 5% CO_2, and then the cells were washed twice with PBS. Then, the indomethacin-treated fibroblasts were treated with recombinant human MIF (500 ng/ml) or recombinant human IL-1β (5 ng/ml) (BioVision, Palo Alto, CA) in DMEM/1% FBS and were incubated for 6 or 12 hours at 37°C in an atmosphere of 5% CO_2. PGE_2 formation in the supernatant was then measured by ELISA.

Effect of anti-MIF antibody on IL-1β bioactivity

NF (2×10^5 cells) were treated with anti-MIF monoclonal antibody (50 μg/ml) or non-immune IgG (50 μg/ml) followed by the addition of recombinant human IL-1β (5 ng/ml) in DMEM/1% FBS, and were incubated for 12 hours at 37°C in an atmosphere of 5% CO_2. PGE_2 formation in the supernatant was then measured by ELISA specific for PGE_2, and COX-2 mRNA levels in NF was examined by RT-PCR.

Assay of human PICP

Type I collagen is derived from a large protein, type I procollagen, which has propeptide extensions at both ends of the molecule. Specific enzymes remove these propeptides before the collagen molecules are assembled into fibers. A fragment removed from the carboxy-terminus, PICP, is secreted by cells, and its level reflects the level of synthesis of type I collagen. For detection of PICP in the supernatants, NF and KF (3×10^5 cells) were incubated at 37°C for 24 hours in DMEM/0.5% FBS after the addition of PGE_2 or Forskolin (Sigma, St Louis, MO). The medium in each well was then collected and frozen until use. The amount of PICP in the cellular supernatants was determined by radioimmunoassay (Chugai, Tokyo, Japan) according to the manufacturer's instructions.

cAMP determination

The amount of cAMP produced by the cells in response to PGE_2 was quantified. At confluence, the cells were washed three times with PBS and then were preincubated in DMEM containing 0.2 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, for 30 minutes. The media were removed, and the cells were further incubated with test agents for 10 minutes. At the end of the treatment period, the cell cultures were extracted with 0.1 M HCl. The cAMP content was determined by ELISA (Cayman Chemical) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical analysis was performed as described (Nishio *et al.*, 1999). In brief, normal skin, normal scar, and keloid skin samples were fixed with 4% formalin, and then paraffin-embedded. The embedded tissues were cut into 3-μm-thick sections. Skin tissues were stained immunohistochemically for MIF with a Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) followed by Scheffe's *post hoc* analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid from the Japan Ministry of Education and Sports. We are also grateful for the technical assistance of Mrs S. Tone.

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