# Different roles of PKC and PKA in effect of interferon-γ on proliferation and collagen synthesis of fibroblasts

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**KEY WORDS** signal transduction; protein kinase C; protein kinase A; interferon type II; hypertrophic scar; fibroblasts; collagen synthesis

## ABSTRACT

**AIM:** To study the signal roles of protein kinase C (PKC) and protein kinase A (PKA) in the influence of interferon-γ (IFN-γ) on proliferation and collagen synthesis of fibroblasts derived from hypertrophic scar (HS-FB) and normal skin (NS-FB). **METHODS:** HS-FB and NS-FB were cultured and passaged in Dulbecco's modified Eagle's medium (DMEM). Activity of PKC and PKA were assayed by transferring phosphorus ($^{32}P$) into substrate after treatment with IFN-γ 1000 kU/L at 10, 30, 60, and 120 min. Cell proliferation was determined with MTT assay. The collagen synthesis was measured with [$^{3}H$]proline incorporation and Type III pre-collagen was determined with radioimmunoassay. **RESULTS:** After exposure to IFN-γ 1000 kU/L for 30 min, PKC activity of HS-FB and NS-FB increased from 2.57±0.14 and 2.13±0.12 nmol·min$^{-1}$·g$^{-1}$ of control to 3.75±0.19 and 3.36±0.16 nmol·min$^{-1}$·g$^{-1}$ respectively ($P$<0.05). After exposure to IFN-γ 1000 kU/L for 60 and 120 min, PKA activities of HS-FB increased gradually from 0.82±0.04 nmol·min$^{-1}$·g$^{-1}$ of control to 1.03±0.05 and1.23±0.06 nmol·min$^{-1}$·g$^{-1}$, respectively ($P$<0.05). The PKA activities of NS-FB also increased from 0.52±0.03 nmol·min$^{-1}$·g$^{-1}$of control to 0.68±0.03 and 0.89±0.05 nmol·min$^{-1}$·g$^{-1}$, respectively ($P$<0.05). The proliferation and collagen synthesis were enhanced by PKC activator (containing phosphatidylserine, diacylglycerol and $Ca^{2+}$) and PKA inhibitor [$H_7$ 250 μmol/L, 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine], and inhibited by PKC inhibior (GF109 250 μmol/L) and PKA activator (cAMP 25 μmol/L) ($P$<0.01). GF109 abrogated increased proliferation and collagen synthesis by IFN-γ but it did not affect the inhibitory effects of IFN-γ. At 120 min $H_7$ reversed the inhibitory functions of IFN-γ. **CONCLUSION:** IFN-γ transiently increased proliferation and collagen synthesis of HS-FB and NS-FB by activation of PKC and subsequently inhibited proliferation and collagen synthesis by activation of PKA.

## INTRODUCTION

Hypertrophic scar, a common fibroproliferative disorder of human dermis, was characterized by erythematous and pruritic lesions in the healing skin when wounds required more than 3 weeks to heal. The physical derangement in the affected tissue was associated with increases in the amount of type III and type I collagen. Fibroblasts played a crucial role in increased deposition of extracellular matrix.

Activation of protein kinase A (PKA) increased apoptosis and differentiation and inhibited proliferation and collagen synthesis in endothelial cells[1] and vascular smooth muscle cells[2,3]. Our previous work showed

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that PKA activity did not change in tissues of hypertrophic scar and keloid[4].

Protein kinase C (PKC) had biphasic effects on fibroblast proliferation and collagen synthesis. It facilitated fibroblast proliferation by increasing transcription and translation of c-*myc* and c-*fos* mRNA[5]. On the other hand, it inhibited cell proliferation by decreasing intracellular $[Ca^{2+}]_i$[6]. PKC activated Ras signal pathway[7], which mediated cell proliferation by activating extracellular regulated kinase (ERK). PKC activated heat-shock protein-25/27, which affected formation and secretion of actin[8]. It might be the mechanisms that phosphorylated-PKC mediated the hyperplasia and contracture of cicatrix. Our previous study showed that PKC activity was high in tissues from hypertrophic scar and keloid. Moreover, the increased PKC activity was parallel to the level of scar hyperplasia[9].

Many cytokines regulated proliferation and collagen synthesis of fibroblasts. IFN-γ was demonstrated as an inhibitory factor of proliferation and collagen synthesis of fibroblasts[10,11] and was used to treat hypertrophic scars and keloids[12,13]. IFN-γ was capable of activating Janus kinase transducer and activator of transcription signal pathway[14].

However, up to date, little has been known about the co-modulatory role of PKC and PKA in inhibitory effects of IFN-γ on hypertrophic scar fibroblasts (HS-FB) and normal fibroblasts (NS-FB) proliferation and cross-talk between PKC or PKA. In the present study, the role of PKC and PKA in effects of IFN-γ on normal and hypertrophic scar fibroblasts were investigated.

## MATERIALS AND METHODS

**Reagents** Leupeptin, aprotinin, MTT(dimethylthiazol-diphenyltetrazolium bromide), β-aminopropionitrile, $H_7$[1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], and GF109203 X (bisindoly-maleimide, GF109) were purchased from Sigma Chemical Co. PKC assay kit, PKC activator [containing 1.6 g/L phosphatidylserine (PS) and 0.16 g/L diacylglycerol (DG)], PKA assay kit, and IFN-γ were purchased from Promega Co. Protein assay kit was purchased from Bio-RAD Co. [$^3$H]proline was purchased from Beijing Atomic Energy Inc. [γ-$^{32}$P] ATP was purchased from Beijing Yahui Bio-tech Co Ltd. Type III pre-collagen assay kit was purchased from Chunqing Tumor Inc. Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone Co.

**Cell culture and treatment** Fibroblasts were established from biopsies of HS-FB in four patients from thermal injures and NS-FB in four normal human (mated for location and age). The experiments were permitted by Ethics Committee of Fourth Military Medical University. Cells were cultured in DMEM with 10 % heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5 % $CO_2$ and 95 % air at 37 ºC. Confluent cells were subcultured by trypsin digestion. HS-FB/NS-FB at same passage (passage 4-10) were used in the following experiment.

HS-FB and NS-FB were seeded in 96-well plates and grown to 80 % confluence in DMEM with 10 % FBS. Cultured cells were starved for 24 h in DMEM with 0.5 % FBS, and then treated for 10, 30, 60, and 120 min with fresh DMEM containing 0.5 % FBS, IFN-γ 1000 kU/L, GF109 250 μmol/L, PS+DG, $H_7$ 250 μmol/L, cAMP 25 μmol/L, IFN-γ 1000 kU/L+GF109 250 μmol/L, and IFN-γ 1000 kU/L+$H_7$ 250 μmol/L, respectively.

**Cell proliferation assay** Cell proliferation was measured by MTT assay. Briefly, cells were incubated for 20 h in DMEM containing 0.5 % FBS and then treated with MTT 5 g/L for 3 h. The culture medium was removed carefully then $Me_2SO$ was added. Cultures were vibrated for 10 min. The metabolic activity was quantified by light absorbance at 490 nm.

**[$^3$H]Proline incorporation assay** Total collagen synthesis was determined with [$^3$H]proline incorporation. The cells were incubated with DMEM containing 10 % FBS, [$^3$H]proline 5 mCi/L, vitamin C 50 mg/L, and β-aminopropionitrile 100 mg/L for 24 h. The cells were digested with trypsin and collected to Whatman paper with multiple cellular collectors. Radioactivity was measured with liquid scintillation counter.

**Type III pre-collagen assay** Type III pre-collagen assay in cultured medium was determined by radioimmunoassay. Post-treated cells were cultured for 48 h with DMEM containing 10 % FBS. The cultured medium was collected carefully and preserved at -20 ºC. The assay was performed in subsequent two weeks. The cultured medium was pre-incubated with polyclonal antibodies against human type III pre-collagen (hPCIII) at 4 ºC for 20 h. $^{125}$I-hPCIII was then added and incubated at 4 ºC for 6 h. Subsequently, the separated medium was added and treated for 30 min at room temperature. After centrifugation at 1400×*g* for 30 min, precipitates were counted.

**Preparation of lysates** For PKC and PKA

detection, HS-FB and NS-FB cultured in 25-$cm^2$ flask were grown to $1\times10^6$ and then starved for 24 h in DMEM containing 0.5 % FBS. After treatment with different time the cells were washed three times with ice-cold PBS and the reaction was terminated. The cells were collected with a rubber policeman on ice. They were pelleted by centrifugation (2 min at 250×*g*) and disrupted in 100 μL of extraction buffer containing leupeptin 1 mg/L and aprotinin 1 mg/L. Harvested cells were sonicated on ice with 10-s bursts each preceded by 10-s pause. Homogenates were centrifuged at 14 000×*g* at 4 ºC for 5 min. The supernatant fraction was used to measure PKA activity. But, for PKC detection, the extraction buffer must contain 0.05 % Triton X-100 and PKC inhibitor and phosphatase was separated from the supernatant fraction by DEAE-cellulose chromatography. The samples were loaded onto individual columns pre-equilibrated with extraction buffer. Following washing three times with 5 mL of extraction buffer, the fraction of PKC was eluted with extraction buffer containing NaCl 200 mmol/L, stored at 4 ºC, and generally assayed within 24 h.

**PKC activity assay** PKC activity was assayed by measuring the incorporation of $^{32}P$ from [γ-$^{32}P$]ATP (111PBq/mol) into peptide Neurogranin ($_{28}$AAKIQAS$^*$-FRGHMA-RKK$_{43}$), a specific substrate of PKC. The standard assay mixture (25 μL) contained extraction buffer 5 μL, PKC co-activation buffer (5×) 5 μL, 5 μL PKC substrate peptide 0.5 mmol/L, and 5 μL of ATP mixture [containing ATP 0.5 mmol/L and [γ-$^{32}P$]ATP 18.5G Bq (0.5 Ci)]. Control reaction used control buffer instead of PKC substrate peptide. The reaction was terminated by adding ice-cold 7.5 mol/L guanidine hydrochloride 12.5 μL at 30 ºC after 5 min. Each terminated reaction mixture 10 μL was then spotted onto a SAM$^{2TM}$ membrane. The membrane was rinsed with 2 mol/L NaCl and 2 mol/L NaCl in 1 % phosphoric acid and deionized water, respectively. Moreover, 5 μL of aliquots in any 2 reactions were spotted onto SAM$^{2TM}$ membrane. All membranes were dried at room temperature and put into liquid scintillator. Radioactivity was counted. All values represented the mean from three separate culture dishes with an average difference between duplicates of <±5 %. Protein quantity was measured on triplicates by protein quantity assay.

**PKA activity assay** Specific substrate peptide for PKA was Kemptide (LRRASLG). The standard assay mixture (25 μL) was consisted of PKA assay buffer 5 μL, 0.5 mmol/L Kemptide 5 μL, ATP mixture 5 μL, and deionized water 5 μL. Control reaction used control buffer instead of PKA substrate peptide. The following procedure was the same with PKC assay.

**Statistics** All results were expressed as mean±SD and assessed by *t*-test. $P<0.05$ was considered to be statistically significant.

## RESULTS

**PKC activity** After exposure to IFN-γ 1000 kU/L for 30 min, PKC activity of HS-FB and NS-FB increased from 2.57±0.14 and 2.13±0.12 nmol·min$^{-1}$·g$^{-1}$ of control to 3.75±0.19 and 3.36±0.16 nmol·min$^{-1}$·g$^{-1}$ respectively ($P<0.05$). After 60 min, they recovered to control level (Fig 1A). PKC activity was decreased after GF109 treatment compared with NS control or HS control group at 30 min ($P<0.05$).

**PKA activity** After exposure to IFN-γ 1000 kU/L for 60 and 120 min, PKA activities of HS-FB increased gradually from 0.82±0.04 nmol·min$^{-1}$·g$^{-1}$ of control to 1.03±0.05 and1.23±0.06 nmol·min$^{-1}$·g$^{-1}$, respectively ($P<0.05$). The PKA activities of NS-FB also increased from 0.52±0.03 nmol·min$^{-1}$·g$^{-1}$of control to 0.68±0.03 and 0.89±0.05 nmol·min$^{-1}$·g$^{-1}$, respectively ($P<0.05$). PKA activity was not affected after $H_7$ treatment from 10-120 min compared with NS-control and HS control group ($P>0.05$, Fig 1B).

**Cell proliferation** Proliferation of NS-FB and HS-FB was increased at 30 min ($P<0.01$) but decreased at 120 min after IFN-γ 1000 kU/L treatment ($P<0.05$, Fig 2A, 2C).

The proliferation of NS-FB was also enhanced by PKC activator (PS+DG) alone at 30, 60, and 120 min ($P<0.01$) and inhibited by PKC inhibitor, GF109 alone, at 60 and 120 min compared with control ($P<0.01$, Fig 2A). The proliferation of HS-FB was also enhanced by PKC activator (PS+DG) alone at 30 min ($P<0.01$) and inhibited by PKC inhibitor, GF109, at 60 and 120 min compared with control ($P<0.01$, Fig 2C). GF109 abrogated increased proliferation and collagen synthesis by IFN-γ but it did not affect the inhibitory effects of IFN-γ (Fig 2A, 2C).

Proliferation of NS-FB was inhibited at 60 and 120 min by cAMP and HS-FB were inhibited at 30, 60, and 120 min by cAMP ($P<0.01$) and increased by $H_7$ alone at 30, 60, and 120 min ($P<0.01$). At 120 min $H_7$ reversed the inhibitory functions of IFN-γ (Fig 2B, 2D).

**[$^3$H]Proline incorporation** The [$^3$H]proline incorporation in NS-FB was increased at 30 and 60 min

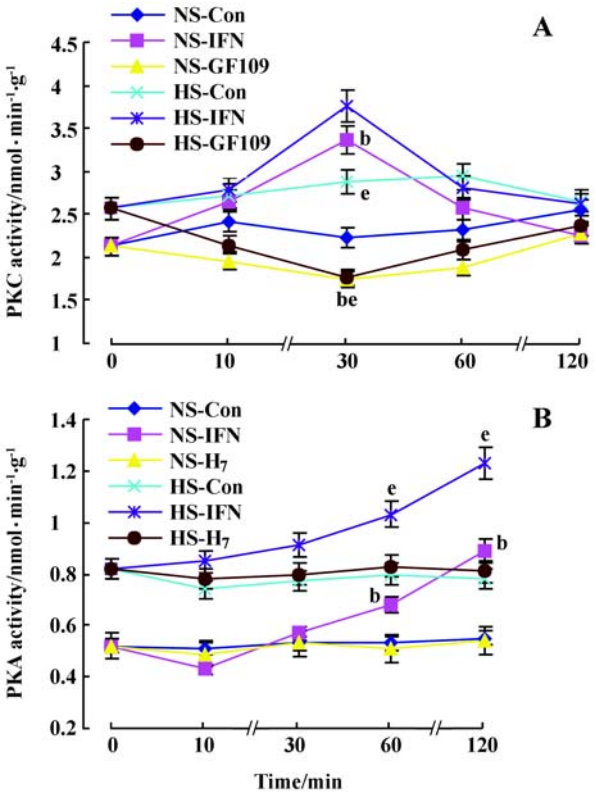


**Fig 1. Effect of IFN-γ, GF109, and $H_7$ on PKC (A) and PKA (B) activity of fibroblasts. *n*=8. Mean±SD. $^bP$<0.05 *vs* NS-Con. $^eP$<0.05 *vs* HS-Con.**

(*P*<0.01) but decreased to normal level at 120 min after IFN-γ 1000 kU/L treatment (*P*>0.05, Fig 3A). [$^3$H]proline incorporation in HS-FB was increased at 30 and 60 min (*P*<0.01) but decreased at 120 min after IFN-γ 1000 kU/L treatment compared with control (*P*<0.01, Fig 3C).

[$^3$H]proline incorporation was enhanced by PKC activator (PS+DG) alone at 30, 60, and 120 min for NS-FB and at 30 and 60 min for HS-FB (*P*<0.01) and inhibited by PKC inhibitor, GF109 alone, at 60 and 120 min compared with control (*P*<0.01). GF109 abrogated increased proliferation and collagen synthesis by IFN-γ but did not affect the inhibitory effects of IFN-γ (Fig 3A, 3C).

[$^3$H]proline incorporation in NS-FB and HS-FB was inhibited by cAMP at 60 and 120 min (*P*<0.01) and increased by $H_7$ at 30, 60, and 120 min (*P*<0.01), an inhibitor of PKA. At 120 min $H_7$ reversed the inhibitory functions of IFN-γ (Fig 3B, 3D).

**Type III pre-collagen** Type III pre-collagen synthesis in NS-FB was increased at 10 and 30 min (*P*<0.05, *P*<0.01) but decreased at 120 min after IFN-γ 1000 kU/L treatment (*P*<0.05). In NS-FB it was increased at 30 min but decreased at 60 and 120 min

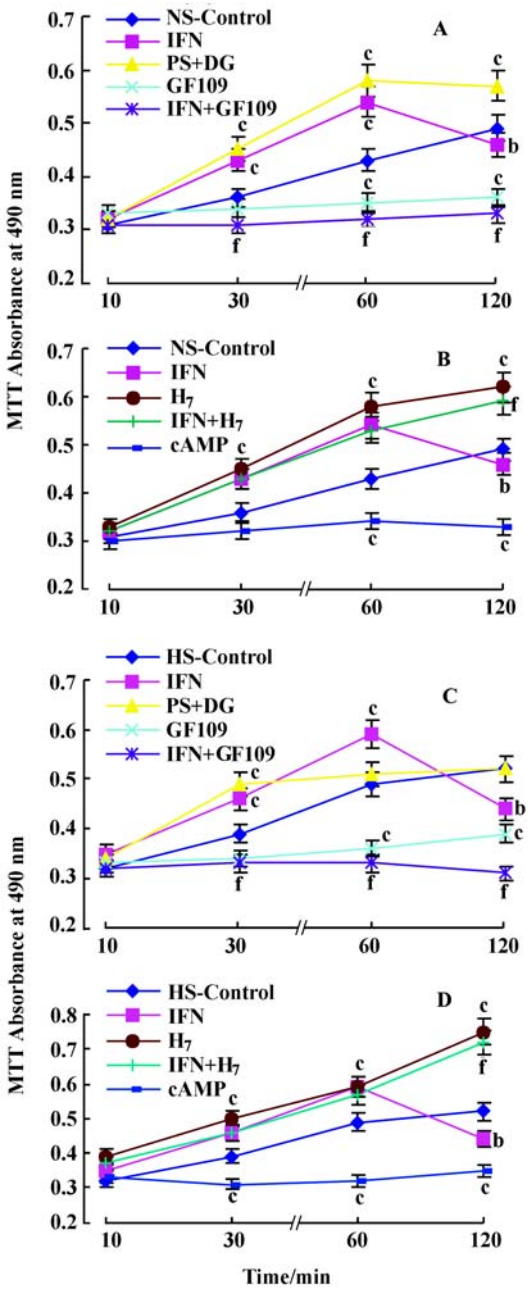


**Fig 2. Effect of IFN-γ, PS+DG, GF109, $H_7$, and cAMP on proliferation of NS-FB (A, B) and HS-FB (C, D). *n*=8. Mean±SD. $^bP$<0.05, $^cP$<0.01 *vs* control group. $^fP$<0.01 *vs* IFN-γ.**

after IFN-γ 1000 kU/L treatment (*P*<0.01).

Type III pre-collagen synthesis in NS-FB and HS-FB was enhanced by PKC activator (PS+DG) alone from 30-120 min (*P*<0.01) and inhibited by PKC inhibitor, GF109 alone, at 60 and 120 min (*P*<0.05). GF109 abrogated increased proliferation and collagen synthesis by IFN-γ but it did not affect the inhibitory effects of IFN-γ.

Type III pre-collagen synthesis in NS-FB and HS-

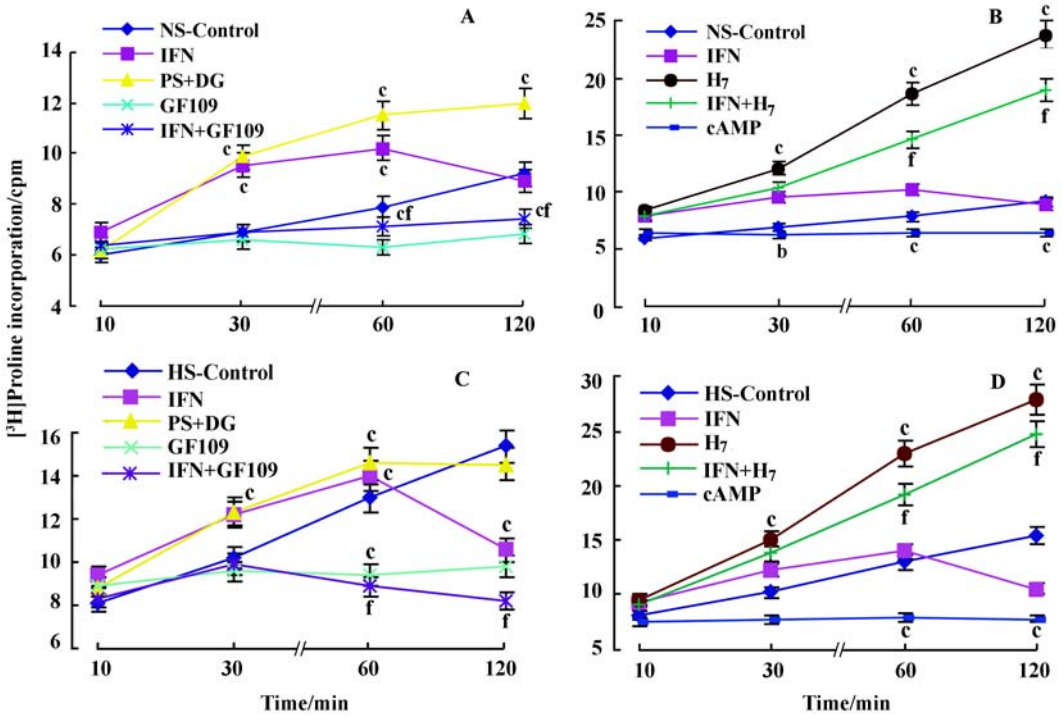


**Fig 3. Effect of IFN-γ, PS+DG, GF109, $H_7$, and cAMP on [$^3$H]-proline incorporation by NS-FB (A, B) and HS-FB (C, D). *n*=8. Mean±SD. $^b$*P*<0.05, $^c$*P*<0.01 *vs* control group. $^f$*P*<0.01 *vs* IFN-γ.**

FB was inhibited by cAMP alone at 60 and 120 min (*P*<0.05) and increased by $H_7$ alone at 30, 60, and 120 min compared with control (*P*<0.01). At 120 min $H_7$ reversed the inhibitory functions of IFN-γ (Tab 1).

**Tab 1. Effect of IFN-γ, PS+DG, GF109, $H_7$, and cAMP on type III pre-collagen content in cultured medium. *n*=8. Mean±SD. $^b$*P*<0.05, $^c$*P* <0.01 *vs* control group. $^f$*P* <0.01 *vs* IFN-γ.**

| | | Type III pre-collagen content/μg·L$^{-1}$ | | | |
|---|---|---|---|---|---|
| | | 10 min | 30 min | 60 min | 120 min |
| NS-FB | Control | 74±10 | 84±17 | 126±15 | 144±21 |
| | IFN-γ | 102±20$^b$ | 170±21$^c$ | 138±15 | 101±19$^b$ |
| | PS+DG | 84±12 | 151±21$^c$ | 185±25$^c$ | 188±25$^b$ |
| | GF109 | 80±10 | 88±11 | 98±13$^b$ | 110±15$^b$ |
| | IFN+GF109 | 86±10 | 89±11$^f$ | 90±12$^f$ | 86±11$^f$ |
| | cAMP | 74.4±1.6 | 78±14 | 71±11$^c$ | 75±11$^c$ |
| | $H_7$ | 78±12 | 201±29$^c$ | 245±32$^c$ | 342±33$^c$ |
| | IFN-γ+$H_7$ | 89±14 | 178±20 | 225±23$^f$ | 286±31$^f$ |
| HS-FB | Control | 77±12 | 94±12 | 131±19 | 154±23 |
| | IFN-γ | 93±13 | 143±23$^b$ | 93±14$^b$ | 90±16$^b$ |
| | PS+DG | 96±11 | 197±30$^c$ | 232±32$^c$ | 244±34$^c$ |
| | GF109 | 83±10 | 96±12 | 109±15$^b$ | 128±15$^b$ |
| | IFN+GF109 | 88±12 | 92±12$^f$ | 97±13$^f$ | 91±12$^f$ |
| | cAMP | 76±12 | 78±14 | 79±14$^c$ | 79±12$^c$ |
| | $H_7$ | 77±12 | 180±20 | 290±32$^c$ | 398±35$^c$ |
| | IFN-γ+$H_7$ | 76±12 | 155±24 | 248±26$^f$ | 302±32$^f$ |

IFN-γ: 1000 kU/L; GF109: 250 μmol/L; cAMP: 25 μmol/L; $H_7$: 250 μmol/L.

The difference in PKC and PKA activity, cell proliferation, and collagen synthesis was not significant between NS-FB and HS-FB.

## DISCUSSION

IFN-γ inhibited proliferation and collagen synthesis of fibroblasts[10,11]. But in this study IFN-γ transiently increased proliferation and collagen synthesis of HS-FB and NS-FB. The different results may be related to the experimental condition. In previous study HS-FB and NS-FB were incubated with IFN-γ for more than 12 h but only 120 min in present study. Biological behavior of IFN-γ resulted from integration of many signaling pathways. IFN-γ transiently activated PKC and simultaneously increased proliferation and collagen synthesis of HS-FB and NS-FB. The proliferation and collagen synthesis were enhanced by PS+DG, PKC activator, and inhibited predominantly by GF109, a specific PKC inhibitor. At 30 min PKC activity reached the peak but PKA activity was not markedly increased. Proliferation of HS-FB and NS-FB began to increase at 30 min and reached the peak at 60 min. It indicated that at 30 min the transitory increased proliferation and collagen synthesis by IFN-γ were attributed, at least in part, to activation of PKC.

When HS-FB and NS-FB were incubated with IFN-γ for 120 min, both proliferation and collagen synthesis of HS-FB and NS-FB were reduced. PKC activity recovered to the normal level in IFN-γ or GF109 treated groups. GF109 did not influence the inhibitory effects of IFN-γ. It suggested that PKC was not involved in inhibitory effects of IFN-γ on HS-FB and NS-FB proliferation and collagen synthesis.

Our previous study showed that PKA played a key role in collagen synthesis of normal and hypertrophic fibroblasts after stimulation with TGF-β1[15]. In present study PKA activity was increased by IFN-γ accompined with reduced proliferation and collagen synthesis of HS-FB at 120 min. The proliferation and collagen synthesis of fibroblast was inhibited by cAMP alone but stimulated by $H_7$ alone from 10-120 min. Furthermore $H_7$ partialy reversed stimulating effects of IFN-γ at 120 min. It suggested that cAMP-PKA pathway played a key role in this process. Many scientists demonstrated that PKA was involved in proliferation of macrophages[16, 17], human thyroid follicles[18], and nomal human dermal fibroblasts [19]. Our results were in accordance with these reports.

In this study PKC and PKA activity did not change in HS-FB or NS-FB control from 10-120 min. Because inflammatory factors and cytokines as *in vivo* were absent in the culture medium.

Comparisons of the rate of proliferation and collagen synthesis of fibroblasts from hypertrophic scar and normal skin generally showed no significant difference[11]. As well, the effect of IFN-γ on the proliferation and collagen production was apparently similar in HS-FB and their normal controls in culture[11]. But, Tredget *et al* found that HS-FB from dermal fibroblasts was different with uninjured normal skin in the same patients characterized by increased amounts of mRNA and protein expression of type I and III procollagen, reduced mRNA and active collagenase, and more tissue inhibitor of metalloproteinase, which was likely due to excessive production of extracellular matrix *in vitro*[20]. In this study we strictly controlled the experimental conditions, especially the cell density inoculated at initialization and found that the activities of PKC and PKA, and the proliferation and collagen synthesis of HS-FB were higher that of NS-FB but the difference was not significant ($P>0.05$). HS-FB *in vitro* was different from hypertrophic scar *in vivo*. Although HS-FB may be originated from the normal skin of wound margin, cytokines *in vivo* will directly affect the behavior of the fibroblasts within it. Furthermore, the milieu *in vivo* probably changed over the course of the wound evolution.

In summary, IFN-γ transiently increased proliferation and collagen synthesis of HS-FB and NS-FB by activating PKC but subsequently inhibited proliferation and collagen synthesis by activating PKA.

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