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Connective Tissue Growth Factor Causes Persistent Proα2(I) Collagen Gene Expression Induced by Transforming Growth Factor-β in a Mouse Fibrosis Model

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Abbreviations: b-FGF, basic fibroblast growth factor; COL1A2, α2 chain of type I collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; PDGF, platelet derived growth factor; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; MCP-1, macrophage chemoattractant protein-1

Key words: systemic sclerosis/fibrosis/transforming growth factor-β/connective tissue growth factor.

Running head: CTGF persists COL1A2 gene expression in vivo

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ABSTRACT

Skin fibrotic disorders such as systemic sclerosis (SSc) are characterized by an excessive production of extracellular matrix (ECM) and understood to develop under the influence of certain growth factors. Connective tissue growth factor (CTGF) is a cysteine-rich mitogenic peptide that is implicated in various fibrotic disorders and induced in fibroblasts after activation with transforming growth factor-β (TGF-β). To better understand the mechanisms of persistent fibrosis seen in SSc, we previously established an animal model of skin fibrosis induced by exogenous application of growth factors. In this model, TGF-β transiently induced subcutaneous fibrosis and serial injections of CTGF after TGF-β caused persistent fibrosis. To further define the mechanisms of skin fibrosis induced by TGF-β and CTGF *in vivo*, we investigated in this study, the effects of growth factors on the promoter activity of the proα2 (I) collagen (COL1A2) gene in skin fibrosis. For this purpose, we utilized transgenic reporter mice harboring the -17 kb promoter sequence of the mouse COL1A2 linked to either a firefly luciferase gene or a bacterial β-galactosidase gene. Serial injections of CTGF after TGF-β resulted in a sustained elevation of COL1A2 mRNA expression and promoter activity compared with consecutive injection of TGF-β alone on day 8. We also demonstrated that the number of fibroblasts with activated COL1A2 transcription was increased by serial injections of CTGF after TGF-β in comparison with the injection of TGF-βalone. Furthermore, the serial injections recruited mast cells and macrophages. The number of mast cells reached a maximum on day 4 and remained relatively high up to day 8. In contrast to the kinetics of mast cells, the number of macrophages was increased on day 4 and continued to rise during the subsequent consecutive CTGF injections until day 8. These results suggested that CTGF maintains TGF-β-induced skin fibrosis by sustaining COL1A2 promoter activation and increasing the number of activated fibroblasts. The infiltrated mast cells and macrophages may also contribute to the maintenance of fibrosis.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by excessive fibrosis and vascular changes in the skin and various internal organs, with an autoimmune background. The central event in the pathogenesis of SSc is an abnormal accumulation of extracellular matrix (ECM) components, predominantly types I and III collagens. The mechanisms that cause excessive fibrosis in SSc remain incompletely understood. However, many studies have suggested that several growth factors and cytokines released from inflammatory cells, endothelial cells, fibroblasts, and other cells in the involved organs play an important role in the initiation and maintenance of connective tissue fibrosis (Jimenez et al., 1996)

Among these factors, transforming growth factor-β (TGF-β) has gained much attention as an essential factor in the pathogenesis of SSc (Leroy *et al.*, 1989), since TGF-β is a very potent stimulator of collagen synthesis by fibroblasts (Miles, 1987; Roberts and Sporn, 1993). However, studies have suggested that TGF-β expression at the fibrotic sites is limited to the early inflammatory stage in various fibrotic disorders in the skin, including SSc and localized scleroderma (Falanga et al., 1992; Higley et al., 1994). Furthermore, there is little difference in the ability to produce TGF-βbetween fibroblasts taken from SSc lesions and their normal counterparts (Needleman et al., 1990), thus implicating other growth factors as additional pathogenetic factors in the development of fibrosis in SSc.

Connective tissue growth factor (CTGF) is a cysteine-rich peptide, originally identified in human umbilical endothelial cell supernatants that exhibit platelet-derived growth factor (PDGF)-like chemotactic and mitogenic activities toward mesenchymal cells, and appears to be antigenically related to PDGF A and B chain peptides (Bradham et al., 1991). Because of its ability to induce the expression of ECM molecules as one of the mediators of TGF-β (Igarashi et al., 1993), CTGF has emerged as an additional candidate responsible for excessive production of ECM proteins in SSc (Takehara, 2000). Previously, CTGF expression was detected *in vivo* in many fibrotic diseases, including SSc, localized scleroderma, and keloid as well as in renal, hepatic, and lung fibrosis (Igarashi *et al.*, 1995; Igarashi *et al.*, 1996; Ito *et al.*, 1998; Lasky *et al.*, 1998; Paradis *et al.*, 1999). In addition, Sato et al. reported that serum CTGF levels were increased in patients with SSc, and correlated with the extent of skin sclerosis and the severity of pulmonary fibrosis (Sato et al.,

2000).

We recently established an animal model of skin fibrosis by exogenous injection of growth factors. In 1986, Roberts et al. reported that the subcutaneous injection of TGF-β in newborn mice caused granulation tissue formation followed by fibrosis (Roberts et al., 1986). We conducted similar experiments, and found that TGF-β caused skin fibrosis after 3 consecutive days of injection. However, this change was transient and disappeared after 7 consecutive days of TGF-β injection (Shinozaki *et al.*, 1997; Mori *et al.*, 1999). In contrast, irreversible fibrosis was observed upon the simultaneous injection of TGF-β and CTGF or by the injection of TGF-β for the first 3 days followed by CTGF for the next 4 days. Irreversible fibrosis was not observed when TGF-β was injected after CTGF. These observations suggested that CTGF maintains skin fibrosis induced by TGF-β.

Based on the results with TGF-β and CTGF described above, we hypothesized that a two-step process of fibrosis occurs in SSc (Takehara, 2003). We postulate that TGF-β induces fibrosis in the early stage of SSc, and then CTGF acts to maintain tissue fibrosis. To further define the mechanisms of skin fibrosis induced by TGF-β and CTGF *in vivo*, we investigated in this study, the effects of growth factors on the promoter the activity of proα2 (I) collagen gene (COL1A2) in skin fibrosis by utilizing transgenic reporter mice harboring the COL1A2 gene upstream promoter sequence. We also examined the role of TGF-β and CTGF in inflammatory cell recruitment to the injection site in this animal model of skin fibrosis.

MATERIALS AND METHODS

Transgenic mice

All animals used in the present study received humane care in compliance with the guidelines of Kanazawa University School of Medicine. The transgenic mouse strains, GB17/3 and GB17/6, were kindly provided by Dr. B. de Crombrugghe, and contain the -17 kb to +54 sequence of the mouse proα2 (I) collagen gene linked to either a firefly luciferase gene or a bacterial β-galactosidase gene (Bou-Gharios et al., 1996). Strong enhancer activity was observed between the –17 kb and –13.5 kb of the promoter (Bou-Gharios et al., 1996). The phenotype expression of these mice was normal and the general wound healing process was not impaired (Kinbara *et al*, 2002). We have previously confirmed that the expression levels of transgenes were similar between the two germlines of transgenic mice (Inagaki et al., 1998). In accordance with this, there was essentially no difference in the results of the following experiments between the two germlines.

Growth factors

Human recombinant TGF-β3 was kindly provided by Novartis Pharmaceutical Co. (Basel, Switzerland). Human recombinant CTGF was a generous gift from JT Industrial Co. (Tokyo, Japan).

Experimental model

TGF-β3 and CTGF were dissolved in phosphate-buffered saline (PBS), to obtain final concentrations of 40 and 20 ng/μl, respectively. Newborn mice were injected with 20 μl of TGF-β3 (800 ng), CTGF (400 ng) or with PBS as a control into the neck subcutaneous tissue once a day for 3 or 7 consecutive days. Serial injections of the two growth factors (TGF-β3 for the first 3 days and CTGF for 4-7 days) were also conducted. The amounts of growth factors used in these experiments were previously shown to be optimal in our skin fibrotic animal models using BALB/C mice (Shinozaki *et al.*, 1997; Mori *et al.*, 1999).The mice were killed with an overdose of diethyl ether 24 hours after the final injection, and tissue samples were obtained from the site of injection. Parts of the tissue samples were fixed in 10% neutral buffered formalin and processed for routine histological examination and the measurement of collagen contents. The remaining tissue was embedded in

Tissue-Tek OCT compound (Miles, IN), snap frozen in liquid nitrogen, and stored at –70 ^{O}C prior to use. Ten serial 6-μm sections were cut from each frozen sample embedded in OCT compound. One section was used for X-gal staining and the remaining sections were subjected to RNA extraction.

Measurement of collagen contents in tissue samples

Tissue samples were embedded in paraffin and sections, approximately 15 μm thick, were obtained. Groups of 10 to 20 sections were deparaffinized after incubation with xylol, xylol:ethanol (1:1), ethanol, water:ethanol (1:1), and water. We used only granulated areas of the sections. Individual samples were placed in small test tubes and covered with 0.2 ml of a saturated solution of picric acid in distilled water that contained 0.1 % Fast green FCF and 0.1 % Sirius red F3BA. The samples were rinsed several times with distilled water until the fluid was colorless. One milliter of 0.1 N NaOH in absolute methanol (1:1; v:v) was added and the eluted color was read in a spectrophotometer at 540 nm and 605 nm. The method is based on the selective binding of Sirius red F3BA and Fast green FCF to collagens and non-collagenous proteins, respectively (Lopez-De Leon and Rojkind, 1985).

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen granulation tissue samples using RNeasy (Qiagen, Valencia, CA) spin columns, according to the manufacturer's protocol, with the addition of a DNase digestion step. RNA concentration and purity was determined spectrophotometrically by measuring fluorescence at 260 nM and 280 nM. Total RNA (100 □g) was reverse transcribed into cDNA in a total volume of 20 μ□ using a Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions.

Taqman quantitative real-time PCR analysis

Four microliters was used as a template for real-time PCR, which was carried out in an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Foster city, CA). The sequences of the mouse α 2 chain of the type I collagen (COL1A2) primers and probe used are 5'-CAC CCC AGC GAA GAA CTC ATA-3' (forward), 5'-GCC ACC ATT GAT AGT CTC TCC TAA C-3'(reverse) and 5'-CGC CCA GGC CAA CAA GCA TGT C-3' (probe). Intron-spanning primers were used to minimize the possibility of co-amplifying

genomic DNA. The primers for macrophage chemoattractant protein-1(MCP-1) were purchased from Applied Biosystems. We used the Taqman rodent GAPDH control reagent (PE Applied Biosystems) as an internal control. PCR (1 x (50 ^{O}C, 2 min, 95 ^{O}C, 10 min), 40 x (95 ^{O}C, 15 sec, 60 ^{O}C, 1 min) was performed in the presence of 0.6 x Taqman Universal PCR master mix (PE Applied Biosystems), forward and reverse primers and a sequence-specific fluorescent probe. Optimal probe and primer concentrations were determined for each assay to ensure maximum specificity. Relative units (RU) were calculated by the comparative C_T method. First, the C_T for the target amplification (FAM) and the C_T for the endogenous control (VIC; GAPDH) were determined for each sample. The Difference between the C_T for the target and the C_T for the internal control, called ΔC_T, was calculated to normalize for the differences in the amounts of total nucleic acid added to each mixture. The ΔC_T of the calibrator was subtracted from the ΔC_T of each experimental sample to give $\Delta\Delta C_T$. The amount of target normalized to an endogenous control and relative to the calibrator, was then calculated using the equation $2^{(-\Delta\Delta CT)}$.

Luciferase assay

The luciferase assay was performed as described previously (Kinbara et al., 2002). Briefly, granulation tissues were homogenized in a buffer containing 25 mM Tris (pH 7.8) and 2 mM DTT, and then subjected to three cycles of freezing and thawing. After centrifugation, the supernatant was assayed for luciferase activity in the presence of excess 470 □M D-luciferin, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 □M CoA, and 530 □M ATP using a luminometer (Microtech Nition, Funabashi, Japan). Luciferase activity was expressed as counts per minute per □g tissue homogenate.

β-galactosidase expression

Granulation tissue blocks were cryo-sectioned 6 μm thick. Sections were fixed with 4 % paraformaldehyde at room temperature and subsequently stained with 0.1 % 4-chloro-5-bromo-3 indolyl-β-galactoside (X-Gal) in 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM $MgCl_2$ at 37 ^{O}C for 12-16 hrs. The sections were counterstained with eosin. The positively stained fibroblasts were counted in ten high power fields at x 400 (Bonnerot and Nicolas, 1993).

Histological examination and immunohistochemistory

The formalin-fixed and paraffin-embedded specimens were cut to a thickness of 4 μm and stained with hematoxylin and eosin or toluidine blue for mast cell staining. Immunohistochemical analysis using antimouse macrophage antibody (F4/80, clone A3-1, ACM, UK) at a dilution 1:100 was performed with biotinylated antimouse immunoglobulin G as secondary antibody. Visualization was done with the standard streptavidin-biotin-coupled immunoperoxidase technique (Histofine kit, Nichirei, Tokyo, Japan). The measurement of mast cells and macrophages was performed by averaging the number of cells identified by toluidine blue staining and positive for the F4/80 staining in nine high power fields (magnification, x 400), respectively.

Statistical Analysis

Values were expressed as the mean ± SEM. Student's *t* test was used to evaluate the statistical differences between the groups.

RESULTS

Quantitative analysis of the amounts of collagen in an animal model of skin fibrosis induced by exogenous injection of TGF-β and CTGF

We previously showed that serial injections of CTGF after TGF-β caused persistent fibrotic tissue formation in newborn mice (Mori et al., 1999). In this experiment, we injected 800 ng of TGF-β on days 1-3 followed by 400 ng of CTGF on days 4-7. Histological examination revealed the formation of granulation tissue with fibrosis on day 4 and this response became relatively weaker but persisted on day 14. Since the profibrogenic effects of TGF-β1, -β2,and -β□ were almost identical in previous experiments (Mori et al., 1999), we used TGF-β□ (henceforth referred to as TGF-β) in all the experiments described in the present study.

To better understand the mechanisms of fibrosis induced by multiple cytokines *in vivo*, we first semi-quantified the amounts of collagen depositedin tissue samples obtained from this animal model of skin fibrosis. Collagen levels in the granulation tissue were significantly increased by TGF-βinjection on day 4 as compared with those in PBS-treated control mice and in CTGF-injected mice (**Fig. 1A**). After injecting TGF-β for seven consecutive days, a decrease in the collagen contents was found on day 8. In contrast, serial injections of CTGF after TGF-β caused a more prominent collagen accumulation than did TGF-β injection alone (**Fig. 1B**). These semi-quantitative results were for the most part consist ent with our previous histological findings.

We next compared the influence of the exogenous addition of TGF-β and CTGF on COL1A2 mRNA expression in fibroblasts. **Figure 2** shows the expression levels of COL1A2 mRNA in granulation tissue determined by quantitative real-time PCR. TGF-β induced a significant increase in the amounts of COL1A2 mRNA on day 4 (**Fig. 2A**). The mRNA expression levels on day 8 after serial injections of CTGF and TGF-β were relatively lower than those on day 4 after injection of TGF-β alone, but significantly higher than those on day 8 after the consecutive injection of TGF-β (**Fig. 2B**). Therefore, it is suggested that the serial injection of CTGF and TGF-β is important to maintain a certain level of COL1A2 mRNA expression and cause persistent fibrosis.

CTGF maintains the TGF-β-induced activation of COL1A2 promoter *in vivo*

To confirm whether the increase in COL1A2 mRNA levels described above was attributed to the activation of COL1A2 transcription, we utilized transgenic reporter mice harboring the –17 kb enhancer and promoter sequence of the mouse COL1A2 linked to either a firefly luciferase or a bacterial β-galactosidase gene. We obtained tissue samples from the site of the injections on the indicated days and investigated the kinetics of luciferase activity, which represented the activity of the COL1A2 promoter in granulation tissue. As shown in **Figure 3**, the injection of TGF-β for 3 days resulted in a 6-fold increase in the luciferase activity on day 4 compared with the control (PBS injection). This activity returned to the control level after TGF-β for an addional 4 days. In contrast, sustained luciferase activity was observed until day 8 after the injection of TGF-βon days 1-3 followed by CTGF on days 4-7 (**Fig. 3B**). CTGF injection alone for three days or for seven consecutive days did not increase the promoter activity. These results reflected the expression levels of COL1A2 mRNA determined by real-time PCR.

To investigate the numbers of fibroblasts in which the COL1A2 promoter was activated, we used X-gal staining to detect β-galactosidase expresion driven by the COL1A2 enhancer and promoter. TGF-βinjections for 3 days increased the number of X-gal-positive fibroblasts compared with the control (**Fig. 4A, B and E**). In spite of additional TGF-β injections for 4 more days, the number of X-gal-positive fibroblasts was decreased dramatically to the control level (**Fig. 4C**). In contrast, serial injections of CTGF after TGF-β significantly increased the number of X-gal-positive fibroblasts on day 8 compared with the injection of TGF-β alone (**Fig. 4D and F**). Based on these findings, it is suggested that the persistent fibrosis induced by the cytokines *in vivo* resulted mostly from the sustained COL1A2 promoter activation and the increase in the number of activated fibroblasts.

Profile of inflammatory cells in this animal model of skin fibrosis

It has been reported that numbers of mast cells and macrophages increase in fibrotic tissues (Nishioka *et al.*, 1987; Ishikawa and Ishikawa, 1992). To test whether these inflammatory cells are associated with the progression of fibrosis in our model, we examined the numbers of mast cells and macrophages in fibrotic tissues. The number of mast cells was significantly increased after TGF-β injections for 3 days compared with the control (**Fig 5**). The number of mast cells returned to the basal level after TGF-β injections

for seven consecutive days. However, it persisted to show a relatively high level after serial injections of CTGF and TGF-β(**Fig 5**). TGF-β also increased the number of macrophages on day 4 (**Fig 6**). In contrast to the kinetics of mast cells, the number of macrophages continued to rise after subsequent CTGF injections on day 4-7 (**Fig 6**).

Chemokine MCP-1 mRNA expression is elevated in an animal model of skin fibrosis

We found that mast cells and macrophages infiltrate skin in our animal model of skin fibrosis induced by exogenous injections of TGF-βand CTGF. To examine whether chemokines produced partly by inflammatory cells play a role in skin fibrosis, we performed real-time PCR analysis to determine the expression of MCP-1 mRNA in our animal model. On day 8, MCP-1 mRNA levels following serial injections of CTGF after TGF-β were elevated 8-times as much as those after the single injection of TGF-β or CTGF (**Fig 7**).

DISCUSSION

TGF-β promotes fibroblast proliferation as well as matrix synthesis, and thus is generally considered to play a key role in the development of skin fibrotic disorders such as SSc (Leroy *et al.*, 1989; Roberts and Sporn, 1993). The importance of TGF-β is also supported by animal models of scleroderma; treatment with anti-TGF-β antibodies was effective in preventing dermal sclerosis in the graft-versus-host disease (GVHD) scleroderma model (McCormick et al., 1999) and deletion of one TGF-β allele resulted in decreased dermal thickness in tight skin mice (McGaha et al., 2001). Our animal model of skin fibrosis further demonstrated that the injection of TGF-β was required for the initiation of skin fibrosis. However, a single application of TGF-β caused transient fibrosis, and was not sufficient to induce persistent fibrosis, even if applied for 7 consecutive days (Mori et al., 1999). Furthermore, we conclusively found that the injection of CTGF after the initiation of fibrosis with TGF-β injection was necessary for developing persistent fibrosis (Mori et al., 1999). Herein, we show that the persistent fibrosis induced by these cytokines *in vivo* is related to the sustained promoter activation of the gene for type I collagen, which is the most abundant component of the ECM in the skin. Our data also support that the increased number of fibroblasts with accelerated COL1A2 transcription contributes to maintaining fibrosis.

Type I collagen is the major structural component of the ECM and consequently, the main contributor to connective tissue physiology and pathology. The regulation of collagen expression by TGF-β appears to be controlled mostly at the level of transcription. Recent research demonstrated that TGF-β stimulation of COL1A2 promoter activity is mediated by synergistic cooperation between Sp1 and Smad3/Smad4, which are components of the TGF-β signaling pathways (Zhang et al., 2000). On the other hand, the role of CTGF in collagen expression remains controversial. The CTGF responsive element is localized between nucleotides –376 and +58 of the COL1A2 promoter and CTGF stimulates collagen synthesis transcriptionally *in vitro* (Shi-wen et al., 2000). However, Gore-Hyer *et al.* demonstrated that both normal and SSc fibroblasts showed no induction of collagen expression after stimulation with CTGF alone. They found that CTGF-mediated collagen synthesis requires the co-stimulatory activation of insulin signaling pathways

(Gore-Hyer et al., 2003). Our *in vivo* data also revealed that injection of CTGF alone did not stimulate COL1A2 promoter activity. However, serial injection of CTGF after TGF-β stimulated COL1A2 promoter activity. Thus, TGF-β may induce not only collagen synthesis, but also co-stimulatory molecules necessary for CTGF-mediated collagen expression. Another possible explanation is that CTGF can bind TGF-β directly through the cysteine-rich domain. Therefore this binding may enhance the activity of TGF-β to bind its receptor and potentiate the downstream signaling (Abreu et al., 2002).

Fibroblasts are heterogeneous in their capacity to synthesize a matrix (Jelaska et al., 1996). Since TGF-β induces resistance to apoptosis in SSc fibroblasts, it may drive fibrosis in SSc by providing a survival advantage to clonal populations of high-collagen-producing fibroblasts (Jelaska and Korn, 2000). COL1A2-expressing fibroblasts can be easily detected in our transgenic mice using X-gal staining. This staining revealed that the number of fibroblasts with accelerated COL1A2 transcription was increased by the serial injection of CTGF after TGF-β compared with the injection of TGF-βalone. Therefore, CTGF may contribute to clonal selection by increasing the number of collagen-expressing fibroblasts.

Although we showed that injection of CTGF after TGF-β induced skin fibrosis in newborn mice, the precise mechanism of maintaining fibrosis is still unclear. Since the recruitment of inflammatory cells to the injection site releases a battery of cytokines and chemokines other than TGF-β and CTGF, these inflammatory cells such as mast cells and macrophages may contribute to the initiation or maintenance of skin fibrosis. Increased numbers of mast cells have been observed in SSc and hypertrophic scars (Kischer *et al.*, 1978; Hawkins *et al.*, 1985; Nishioka *et al.*, 1987). Further evidence has been obtained in a murine sclerodermatous graft-versus-host disease (GVHD) model for SSc. McCormick *et al.* showed that TGF-β1 mRNA up-regulation and mononuclear cell infiltration are observed by day 7 after bone marrow transplantation, preceding detectable increases in collagen mRNA and skin thickening (McCormick et al., 1999). They also demonstrated that antibodies to TGF-β prevent not only skin thickening and lung fibrosis, but also the infiltration and activation of mononuclear cells into skin (Zhang et al., 2002). Thus, the sustained number of mast cells and increased number of macrophages seen at the site of injection of CTGF following TGF-β may contribute to the maintenance of fibrosis via production of cytokines and chemokines.

In this context, MCP-1 has become a topic of interest. MCP-1 belonging to the C-C

chemokine supergene family, has stimulatory effects on collagen expression via the up-regulation of TGF-β expression in addition to stimulating recruitment activities of monocyte/macrophages and lymphocytes (Gharaee-Kermani et al., 1996). Previous studies have demonstrated that MCP-1 gene expression increases in Bleomycin-induced pulmonary fibrosis(Zhang *et al.*, 1994) or interstitial kidney fibrosis(Lloyd *et al.*, 1997). Hasegawa et al showed that serum MCP-1 levels were significantly elevated in patients with SSc in comparison with normal controls(Hasegawa *et al.*, 1999). A recent study showed that expression of MCP-1 in infiltrating mononuclear cells and fibroblasts in the lesional skin of SSc was increased(Yamamoto *et al.*, 2001). These findings suggest that MCP-1 plays an important role in the pathogenesis of SSc. Our *in vivo* data also revealed that serial injections of CTGF after TGF-β increased MCP-1 mRNA expression levels. Thus, MCP-1 might be a candidate for a co-stimulatory molecule necessary for CTGF-mediated induction of collagen synthesis our mouse fibrosis model.

TGF-β stimulates production of matrix proteins like collagen and inhibits that of matrix metalloproteinase. TGF-β also can induce the expression of other fibrotic cytokines such as CTGF, which leads to the proliferation of fibroblasts and further synthesis of matrix (Igarashi et al., 1993). However, consecutive injections of TGF-β alone down-regulated CTGF mRNA expression in fibroblasts in this model (Mori et al., 1999), and addition of CTGF was needed for persistent fibrosis. Since SSc fibroblasts constitutively express significant levels of CTGF (Shi-wen et al., 2000), abnormal persistence of CTGF gene expression may account for the excessive collagen synthesis seen in SSc. In addition to directly stimulating matrix gene expression, TGF-β is a very strong chemoattractant for mast cells, monocyte/macrophages and fibroblasts (Wahl *et al.*, 1987; Gruber *et al.*, 1994). These infiltrating cells responding to TGF-β□□□ also capable of producing TGF-β (Zhang et al., 2002), which may coordinate with chemokines in attracting more monocyte/macrophages and T cells to skin. Therefore, stimulatory signals released from these inflammatory cells may be necessary for CTGF-mediated collagen production in our animal model. In fact, inflammatory cells may contribute to the pathogenesis of SSc, as we previously showed that production of the profibrotic cytokine IL-13 by activated T-cells was increased in patients with SSc (Hasegawa et al., 1997).

Fibrosis is a complex process that may involve multiple cytokines and chemokines, which crosstalk with effector cells. Our animal model provides a unique opportunity to

further investigate these pathways *in vivo*, to better understand the mechanism of fibrosis and pathogenesis of SSc. For example, experiments with antibodies to these cytokines or to macrophage surface markers will be useful in preventing disease progression in SSc. Investigations of this nature are currently under way in our laboratory.

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LEGENDS

Figure1. Semi-quantitative analysis of the amounts of collagen deposited in granulation tissues at 4 days (**A**) and 8 days (**B**) after injections. **A:** Newborn mice were injected with 20 □l of TGF-β3 (800 ng), CTGF (400 ng) or PBS as a control into subcutaneous tissue of the neck once a day for 3 consecutive days. Injection of TGF-β for 3 days significantly increased the collagen content of granulation tissues as compared with the control or CTGF alone. **B:** Newborn mice were serially injected with two growth factors or PBS as indicated. The collagen content of granulation tissue after serial injections of TGF-β for the first 3 days and CTGF for 4-7 days was significantly more than that after TGF-β injections for 3 days. Values are the mean of 5 mice in each group. *$p<0.001$

Figure 2. mRNA expression levels of the α2 chain of the type I collagen in granulation tissues at 4 days (**A**) and 8 days (**B**) after injections. Total RNA was extracted from frozen tissue samples. The expression levels of COL1A2 mRNA were determined by real-time PCR and normalized against the GAPDH mRNA expression level in each sample. TGF-β significantly increased the expression of COL1A2 mRNA on day 4 (**A**), and serial injections of TGF-β and CTGF caused a persistent elevation of the mRNA expression on day 8 (**B**). Values are the mean of 5 mice in each group. *$p<0.05$.

Figure 3. CTGF maintains the TGF-β-induced activation of the COL1A2 promoter *in vivo*.
A: Newborn transgenic mice were injected with TGF-β3, CTGF or PBS as a control into subcutaneous tissue of the neck once a day for 3 or 7 consecutive days. In some cases, we injected CTGF for 4 days after injecting TGF-β for the first 3 days. Luciferase activity, which represents the activity of the proα2 (I) collagen promoter in granulation tissue, was determined on day 2, 4, 6, and 8 using a luminometer. Closed circles, squares and triangles express control, TGF-β and CTGF, respectively. Open triangles represent serial injections of CTGF after TGF-β. Luciferase activity of the TGF-β̃injected mouse reached maximum on day 4 and returned to the control level on day 8. In contrast, a high level of COL1A2 promoter activity was maintained after serial injections of TGF-β and CTGF. **B:** The *bar graph* shows the levels of COL1A2 promoter activity on day 8 following consecutive or serial injections. Serial injections of CTGF after TGF-β resulted in a 12-fold increase in the

luciferase activity on day 8 compared with consecutive injections of PBS or TGF-β. Values are the mean of 5 mice in each group. *p<0.01, **p<0.0001.

Figure 4. Effects of TGF-β and CTGF on the numbers of X-gal-positive fibroblasts. Frozen tissue samples were stained with X-gal after the injection of PBS (**A**), TGF-β (**B**) or CTGF for 3consecutive days, TGF-β for 7 days (**C**), and CTGF for 4 days following TGF-β injections for the first 3 days (**D**). The sections were counterstained with eosin. The X-gal-stained fibroblasts were counted in ten high power fields at x 400. TGF-βinjection for 3 days significantly increased the number of X-gal-positive fibroblasts compared with control on day 4 (**E**). Serial injections of CTGF after TGF-β further increased the number of X-gal-positive cells on day 8 (**F**). Values are the mean of 5 mice in each group. *p<0.01, **P<0.05. Original magnification; x400.

Figure 5. Kinetics of mast cell recruitment in this animal model of skin fibrosis. We stained sections with toluidine blue for counting mast cells. The cells positive for toluidine blue were enumerated in three areas at x400 (**A**). Closed circles, squares and triangles express control, TGF-β and CTGF, respectively. Open triangles represent serial injections of CTGF after TGF-β. Note that the number of mast cells was significantly increased by TGF-β injections for 3 days compared with the control . Cell numbers remained relatively high on day 8 after serial injections of CTGF and TGF-β. Values are the mean of 5 mice in each group. *p<0.001, **p<0.005. Representative photographs are also shown; control (**B**), TGF-β for 3 days (**C**), TGF-β for 7 days (**D**), and CTGF for 4 days after TGF-β for the first 3 days (**E**). Original magnification; x400.

Figure 6. Kinetics of macrophage recruitment in this animal model of skin fibrosis. Immunohistochemical analysis using antimouse macrophage antibody (F4/80) diluted 1:100 was performed with biotinylated antimouse immunoglobulin G as the secondary antibody. Visualization was done with the standard streptavidin-biotin-coupled immunoperoxidase technique. The positively stained cells were counted in ten high power fields at x400 (**A**). Closed circles, squares and triangles express control, TGF-β and CTGF, respectively. Open triangles represent serial injections of CTGF after TGF-β. Note that the number of macrophages was significantly increased by TGF-β injections for 3 days compared with

control. In addition, the number of macrophages continued to increase after additional CTGF injections for 4 days after the TGF-β injections. Values are the mean of 5 mice in each group. *$p<0.05$, **$p<0.0005$. Representative photographs are also shown; control (**B**), TGF-β for 3 days (**C**), TGF-β for 7 days (**D**), and CTGF for 4 days after TGF-β for the first 3 days (**E**). Original magnification; x400.

Figure 7. MCP-1 mRNA expression levels in granulation tissues on day 8. The levels of MCP-1 mRNA were determined by real-time PCR. Relative expression levels of MCP-1 mRNA were normalized against those of GAPDH mRNA. Newborn mice were injected with TGF-β, CTGF or PBS once a day for 7 consecutive days or with serial injections of the two growth factors (TGF-β for the first 3 days and CTGF for 4-7 days). MCP-1 mRNA levels following serial injections of CTGF after TGF-β were 8-times as high as those after consecutive injections of TGF-β or CTGF. Values are the mean of 5 mice in each group. *$p<0.05$.

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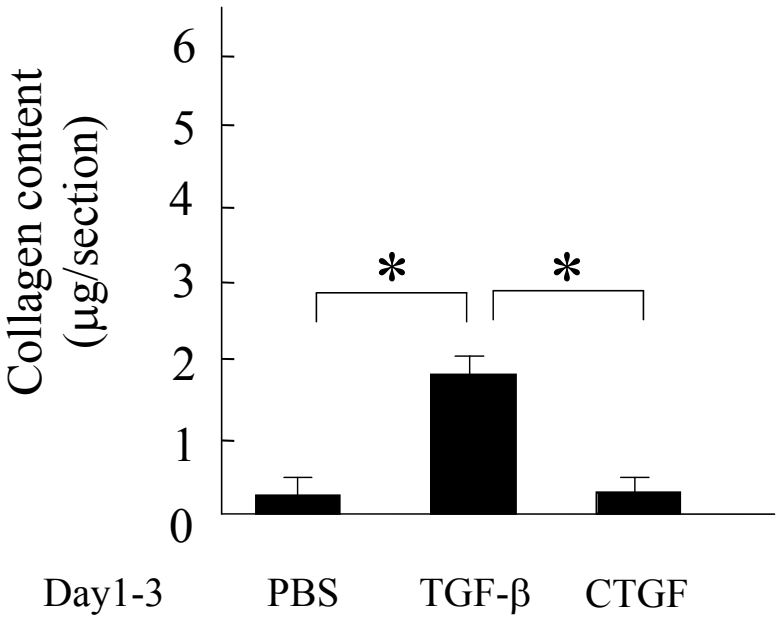
Fig.1

A


Collagen content
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4
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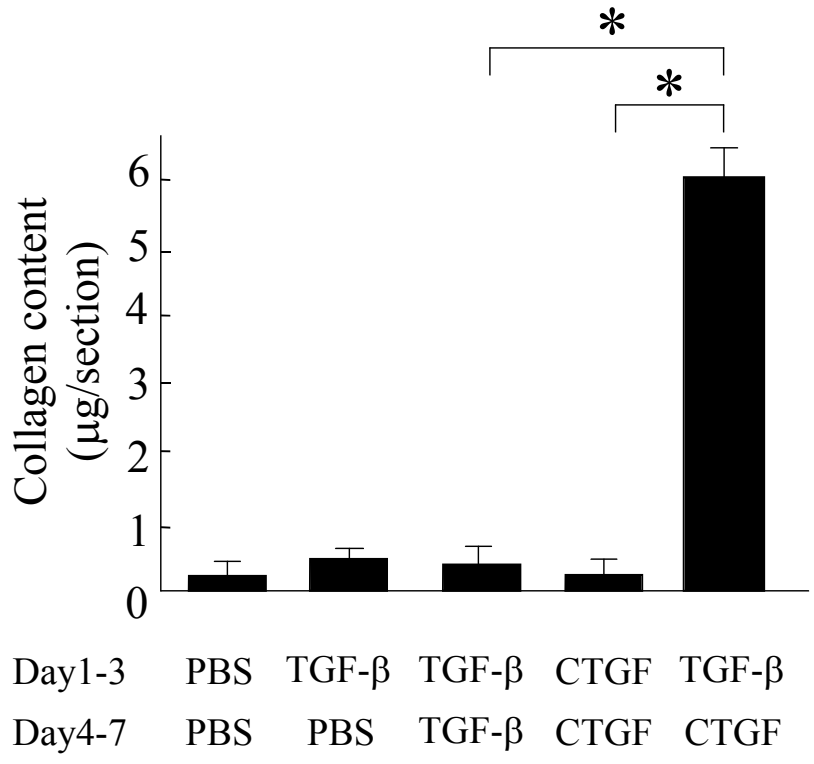
2
1
0
*
*
Day1-3
PBS
TGF-β
CTGF

B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Day1-3
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Day4-7
PBS
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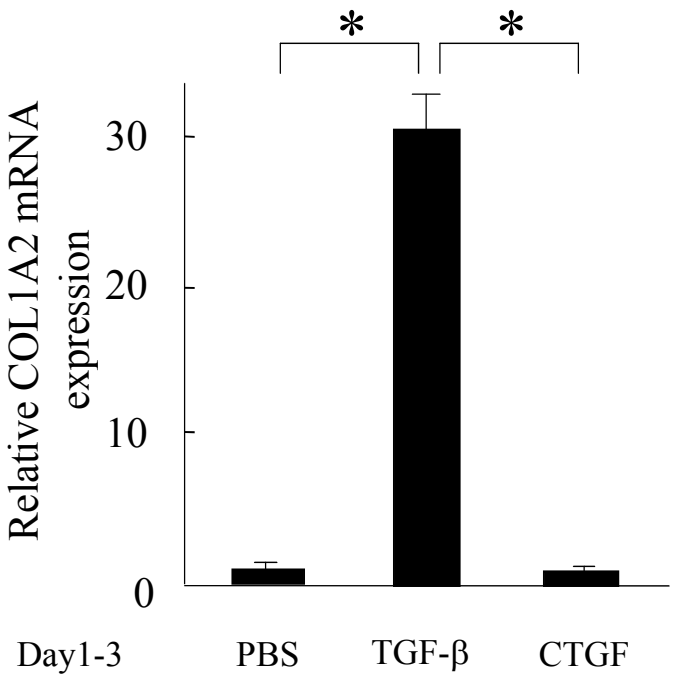
CTGF
CTGF

Fig.2

A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*
Relative COL1A2 mRNA expression
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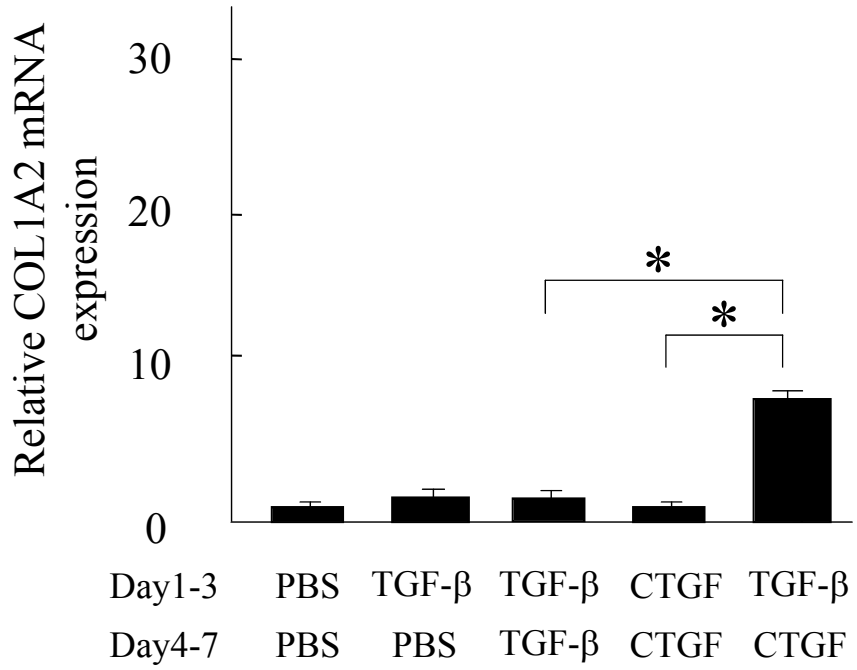
20
10
0
Day1-3
PBS
TGF-β
CTGF

B


Relative COL1A2 mRNA expression
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20
10
0
*
*
Day1-3
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CTGF
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Day4-7
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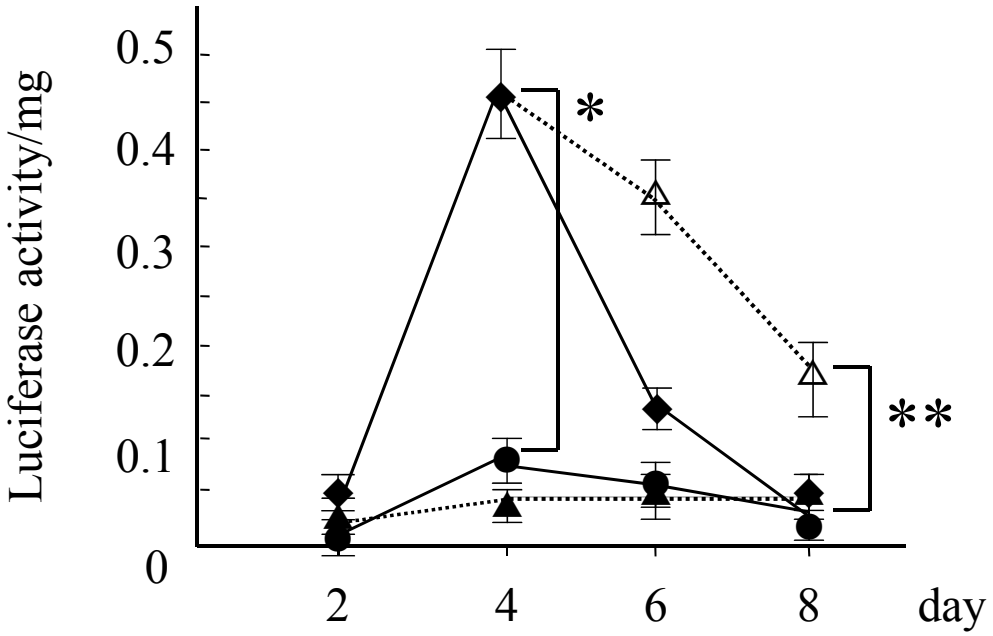
PBS
TGF-β
CTGF
CTGF

Fig.3

A


Luciferase activity/mg
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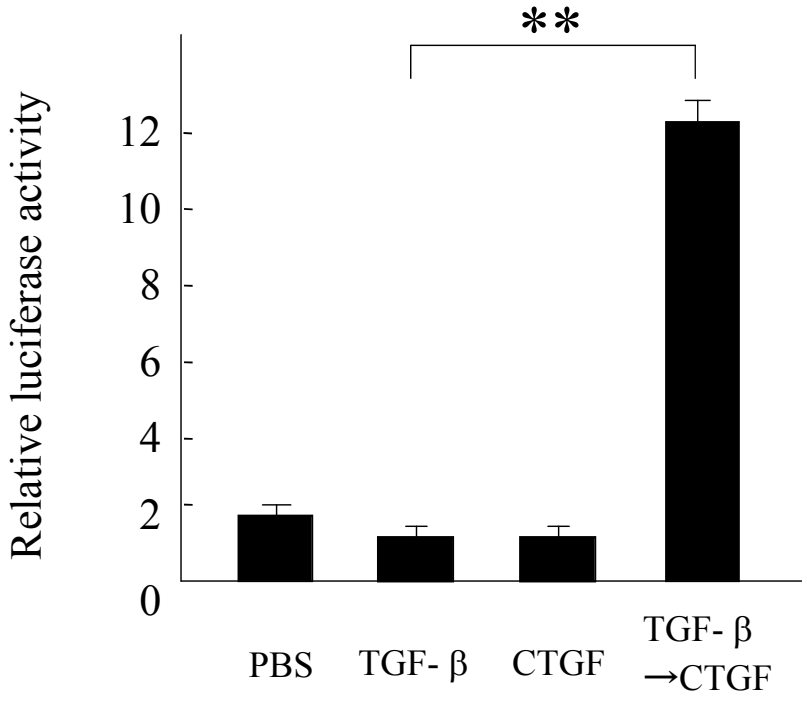
0.3
0.2
0.1
0
2
4
6
8
day
*
**

B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0
**
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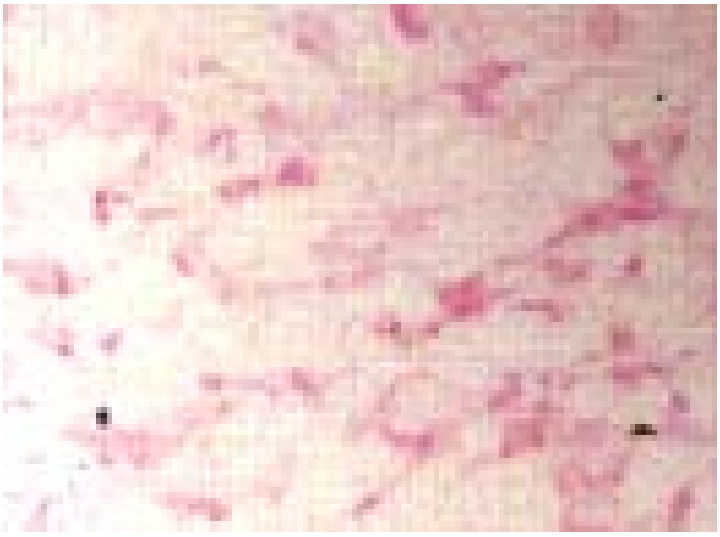
TGF- β
CTGF
TGF- β
→CTGF

Fig.4

A



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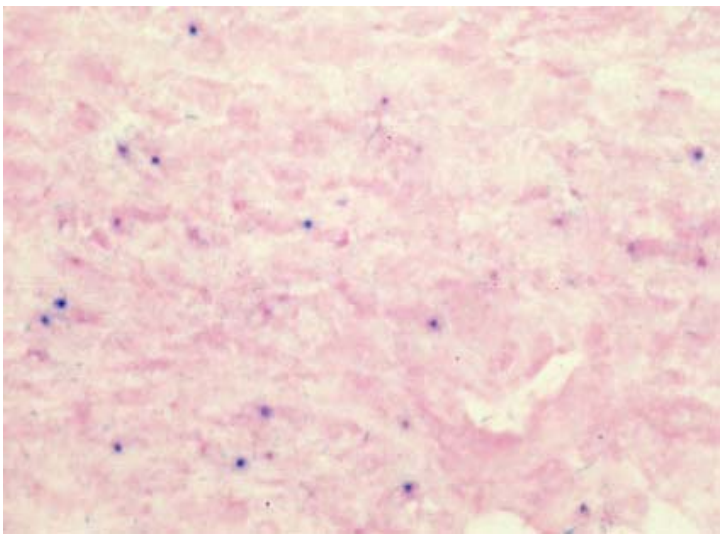

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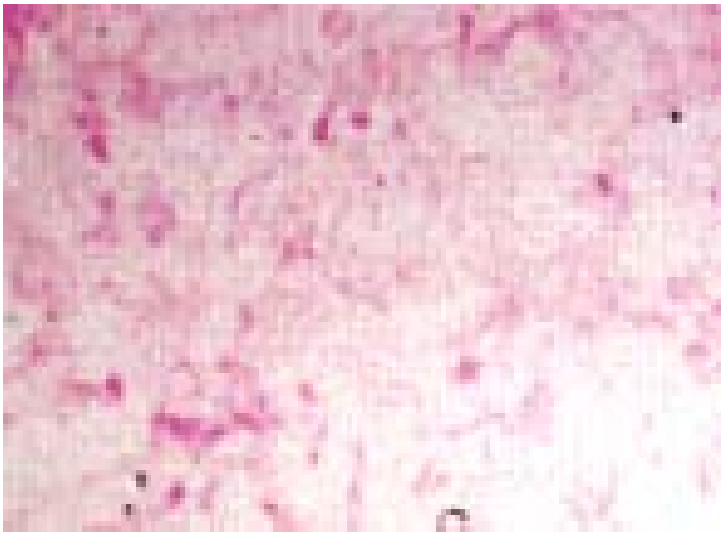

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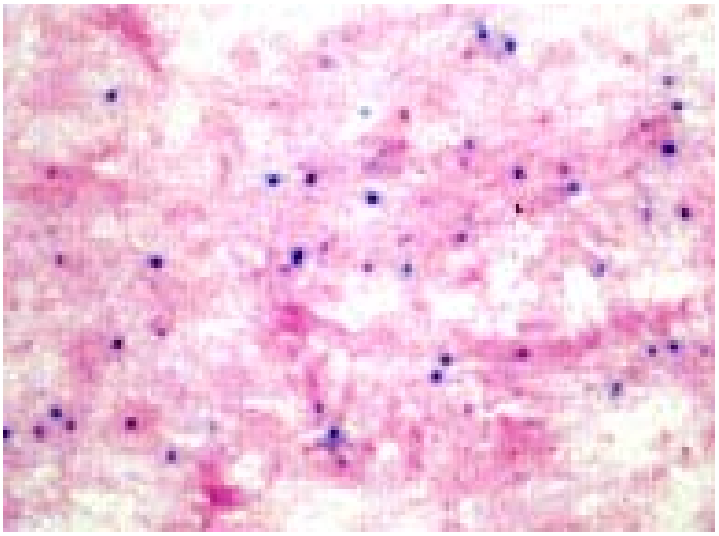

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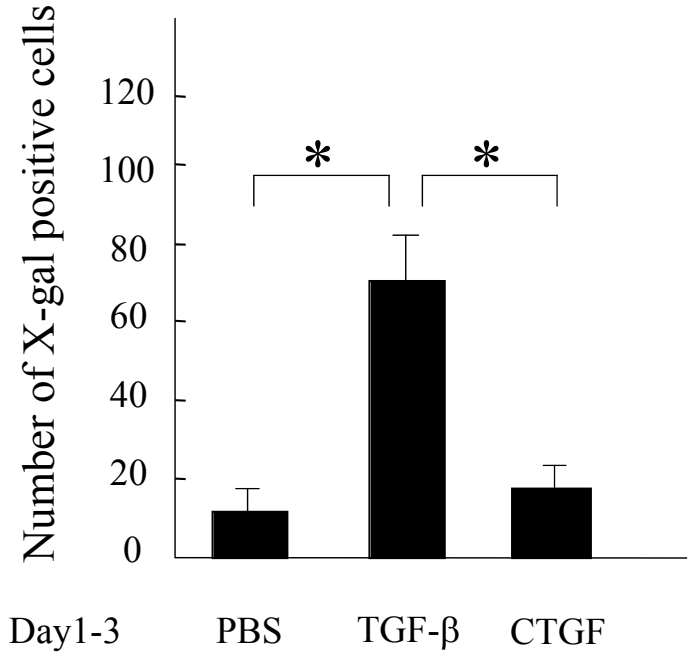
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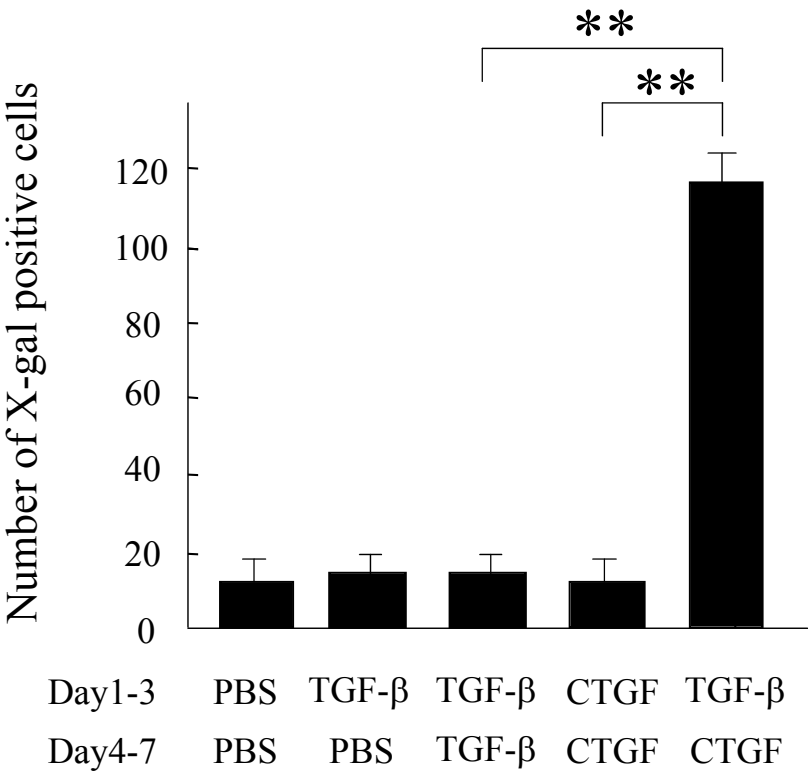
80
60
40
20
0
*
*
Day1-3
PBS
TGF-β
CTGF

F


Number of X-gal positive cells
120
100
80
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40
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**
**
Day1-3
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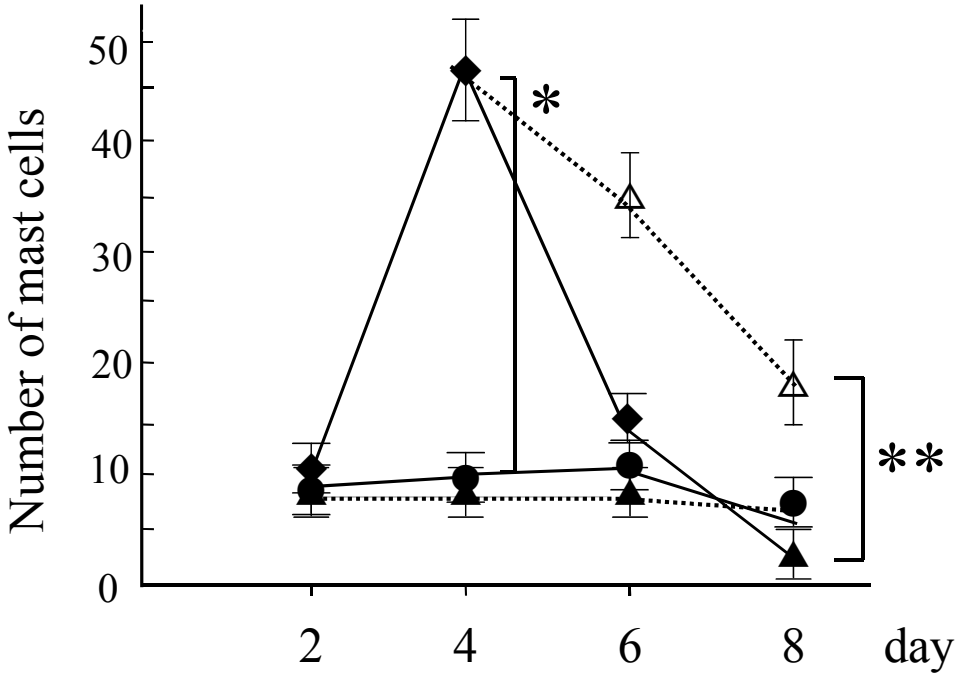
TGF-β
TGF-β
CTGF
TGF-β
Day4-7
PBS
PBS
TGF-β
CTGF
CTGF

Fig.5

A



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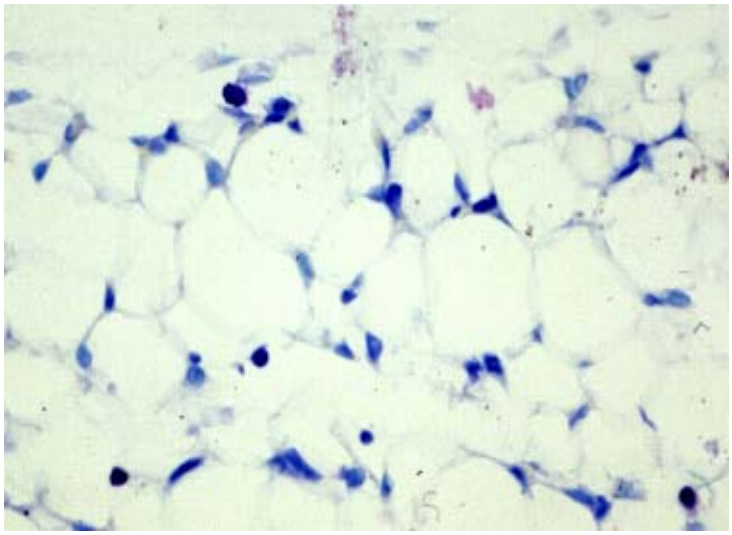

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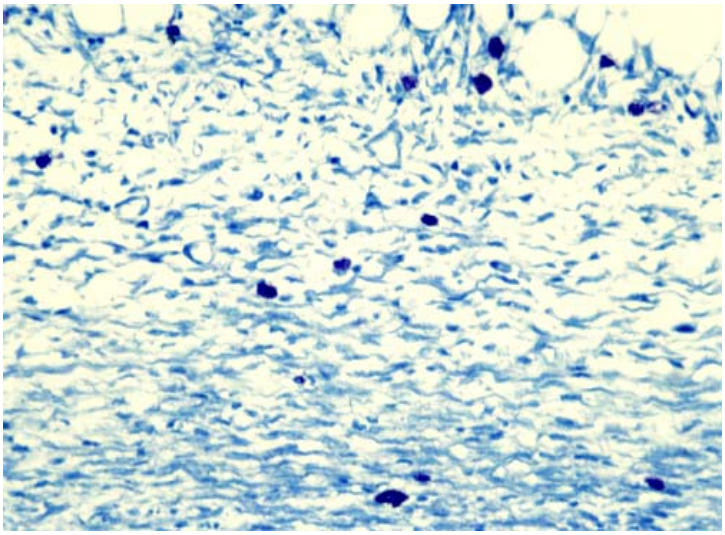

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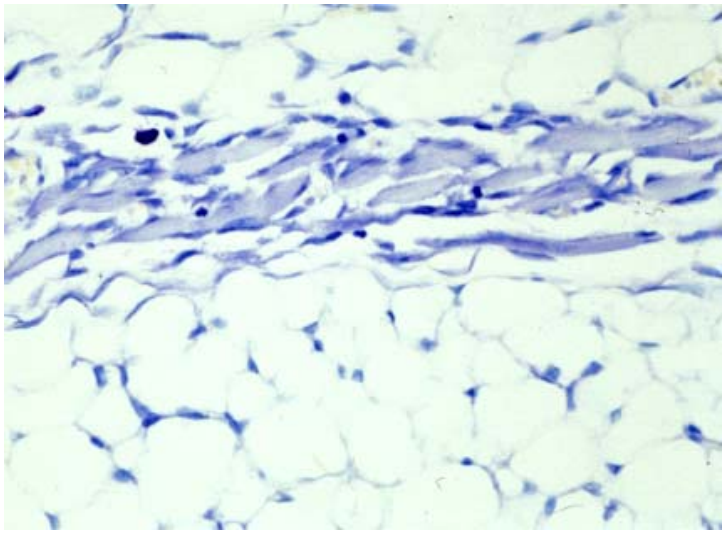

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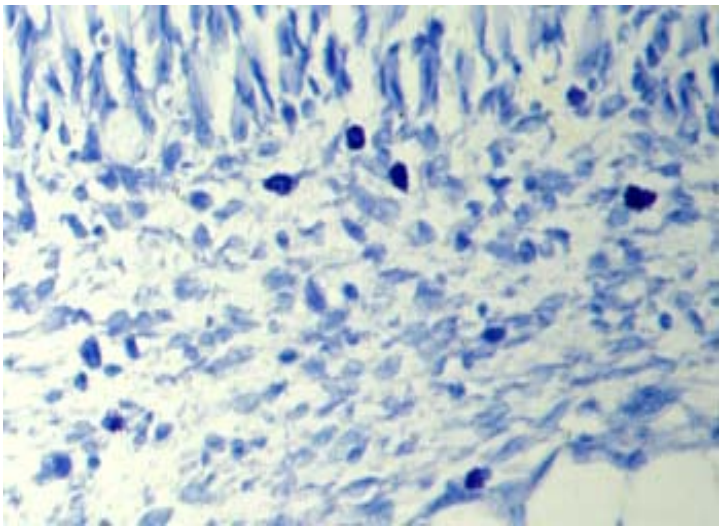

Fig.6

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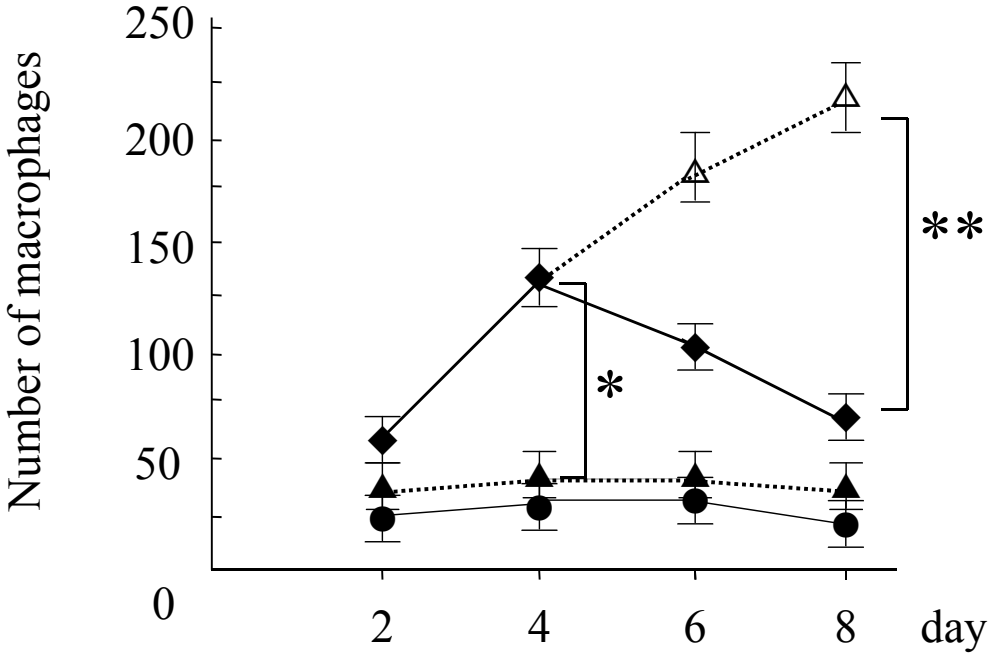

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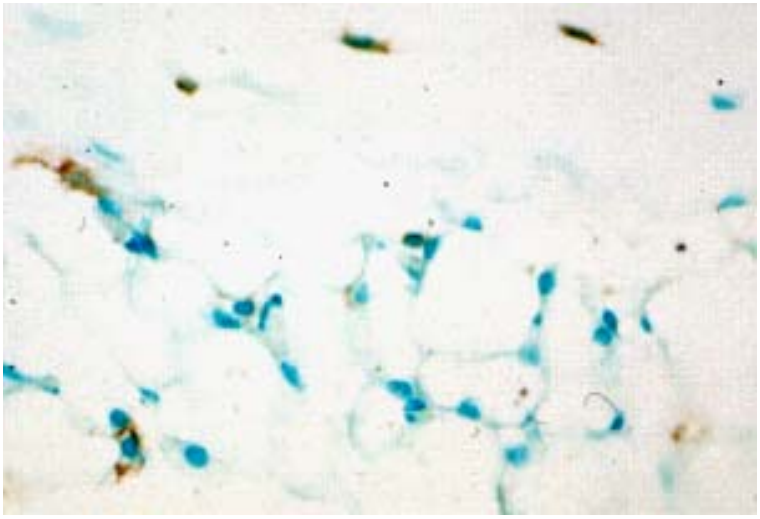

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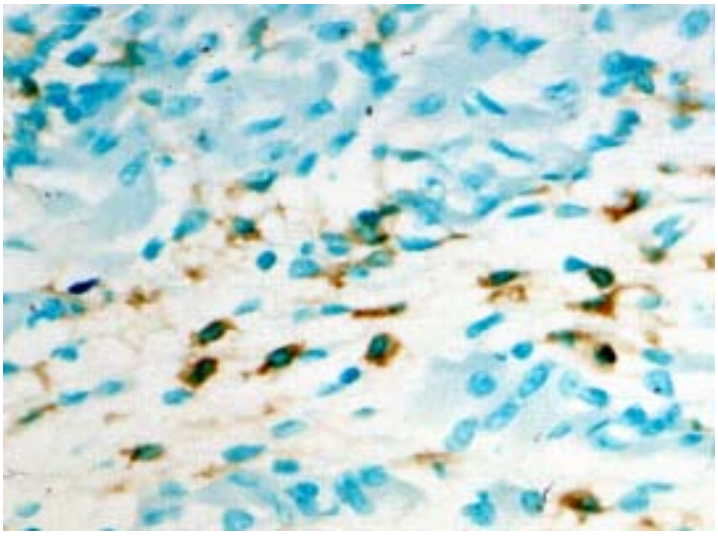

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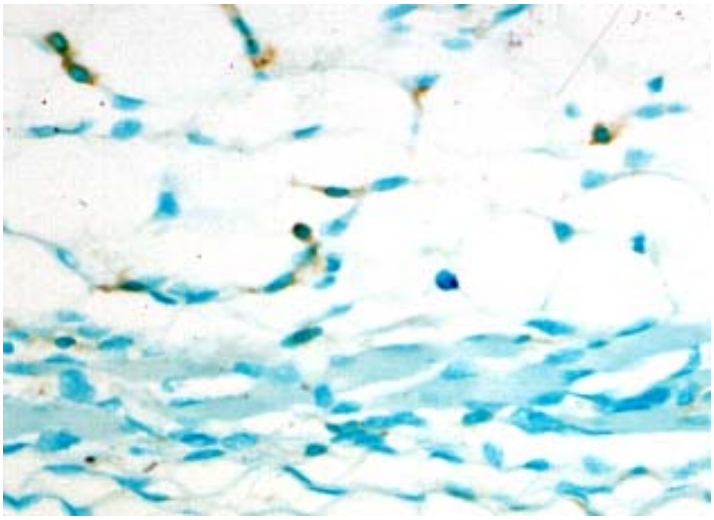

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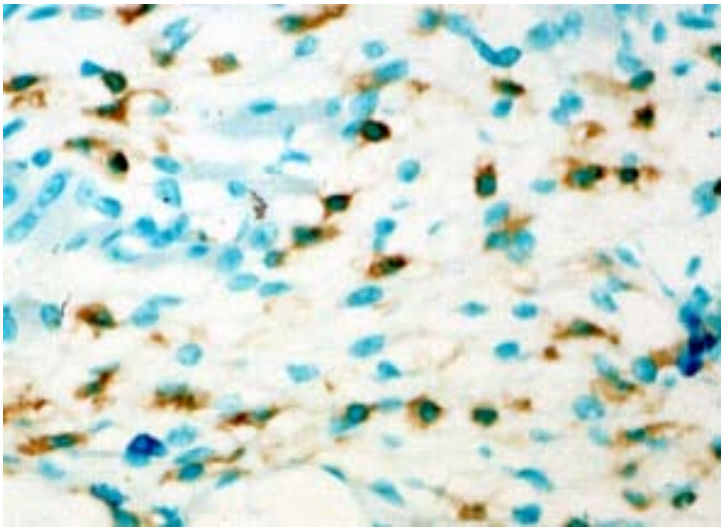

Fig.7