Dexamethasone Is a Novel Potent Inducer of Connective Tissue Growth Factor Expression

IMPLICATIONS FOR GLUCOCORTICOID THERAPY*

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Johanna Dammeier, Hans-Dietmar Beer, Maria Brauchle‡, and Sabine Werner§

From the Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, 82152 Martinsried, Germany

Due to its potent effect on fibroblast proliferation and extracellular matrix deposition, connective tissue growth factor (CTGF) seems to play an important role in the pathogenesis of fibrotic disease. Since glucocorticoids are frequently used for the therapy of these disorders, we determined a potential effect of these steroids on CTGF expression. In cultured fibroblasts, a striking induction of CTGF expression was observed after dexamethasone treatment and occurred in a time- and dose-dependent manner. This effect was obviously not mediated by the CTGF inducer transforming growth factor-β1, since expression of this factor was down-regulated by the glucocorticoid. Most importantly, CTGF expression levels also increased substantially in various tissues and organs by systemic glucocorticoid treatment of mice. After cutaneous injury, a strong induction of CTGF expression was seen in the wounds of nontreated mice. However, no further increase in the levels of CTGF mRNA occurred in wounded skin compared with unwounded skin of glucocorticoid-treated animals, suggesting the presence of other factors in the wound that might compensate for the effect of the steroids. Tumor necrosis factor-α was identified as a possible mediator of this effect because this factor suppressed CTGF expression in cultured fibroblasts and also blocked the glucocorticoid-induced CTGF production by these cells. These findings indicate that glucocorticoids stimulate CTGF expression in normal tissues and organs but not in highly inflamed areas.

Connective tissue growth factor (CTGF) is a potent and ubiquitously expressed growth factor that has been shown to play a unique role in fibroblast proliferation and in the stimulation of extracellular matrix production by these cells (1). The 38-kDa protein was originally identified in conditioned medium from human umbilical vein endothelial cells (2) and is also secreted by fibroblasts in vitro. CTGF belongs to a cysteine-rich protein family that includes serum-induced immediate-early gene products such as Fisp12/BIGM2, the mouse homologue of CTGF (3), Cyr61, whose expression correlates with chondrocyte proliferation and extracellular matrix production by these cells, CTGF might play an important role in the induction of these processes. In addition to the physiological process of wound repair, CTGF also seems to be an important player in the pathogenesis of various fibrotic disorders. Thus it was shown to be overexpressed in scleroderma, keloids, and other fibrotic skin disorders (11), as well as in stromal-rich mammary tumors (12) and in advanced atherosclerotic lesions (13).

Recently, we demonstrated a strong overexpression of CTGF mRNA in affected areas of inflammatory bowel disease patients, whereby a strong correlation between the levels of CTGF mRNA and the production of extracellular matrix molecules was observed. Moreover, areas displaying stenosis showed a particularly strong overexpression of CTGF mRNA. Chronic fibrosis of major organs causes severe medical problems ranging from disfigurement to progressive disability and death. Considering that chronic inflammation may induce some forms of fibrosis, it seems logical to apply anti-inflammatory therapy, whereby glucocorticoids are frequently used. However, the use of these steroids is somewhat controversial in treating fibrosis, because these drugs are frequently ineffective in blocking the progress of the disease. Although they have been shown to be beneficial in the treatment of some cases of liver and lung fibrosis, they seem to aggravate other conditions such as cardiac fibrosis (15, 16).

Since CTGF is highly overexpressed in several fibrotic diseases, we investigated whether glucocorticoids had any effect on the expression of CTGF. Here we identified dexamethasone,
Dexamethasone Is a Novel Inducer of CTGF Expression

To determine a potential effect of dexamethasone on the expression of CTGF, we treated exponentially growing 3T3 fibroblasts with 1 μM dexamethasone for different time periods and analyzed total cellular RNA by RNase protection assay with a CTGF antisense probe (Fig. 1). In comparison to solvent-treated controls, dexamethasone treatment resulted in a strong induction of CTGF mRNA expression. Maximal induction was seen as early as 1 h after addition of the steroid, and increased CTGF mRNA levels were still observed after 10 h. A similar kinetics of CTGF induction was seen in three independent experiments. As shown in Fig. 2, this dexamethasone-mediated induction was dose-dependent, whereby a concentration of 100 nM dexamethasone was sufficient to elicit a maximal response (upper panel). This concentration caused a 14-fold stimulation of CTGF mRNA expression. 10 nM dexamethasone caused an 8-fold induction, whereas a concentration of 1 μM had no effect. Because these concentrations correspond to the appropriate pharmacological rank of order of potency, the effect of dexamethasone was a common glucocorticoid, as a novel inducer of CTGF mRNA and protein expression in vitro and in vivo. This effect was independent of TGF-β1, because we could show that dexamethasone represses its mRNA expression. Considering that prolonged administration of anti-inflammatory steroids leads to a delay in wound repair and an increase in local wound complications, as demonstrated by a series of experiments and clinical studies (17, 18), we also examined the effect of dexamethasone on the expression of CTGF during the healing of full-thickness excisional wounds.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine BALB/c 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml, 100 μg/ml). For experiments, cells were grown for 3–4 days to 70% confluence. 1 h after the medium was changed, the cells were treated with dexamethasone or TNF-α. In a combined treatment with dexamethasone and cytokines, cytokines were added at the same time as dexamethasone. Aliquots of cells were harvested before and at different time points after the addition of these reagents. Total RNA was isolated from these cells and used for RNase protection analysis. Each experiment was repeated at least twice.

For fetal bovine serum, penicillin/streptomycin and Dulbecco’s modified Eagle’s medium were purchased from Life Technologies, Inc., growth factors and cytokines were obtained from Boehringer Mannheim, and dexamethasone was from Sigma.

Animal Care—BALB/c mice were obtained from the animal care facility of the Max Planck Institute of Biochemistry. They were housed and fed according to federal guidelines, and all procedures were approved by the local government of Bavaria.

Glucocorticoid Treatment of Mice—For glucocorticoid treatment of mice, two independent experiments were performed. 12 female BALB/c mice (6–12 weeks of age) were injected daily subcutaneously at 9 a.m. and 6 p.m. with 1 mg (experiment 1) or 0.5 mg (experiment 2) dexamethasone in phosphate-buffered saline per kg body weight for either 1 or 3 days. Control animals were injected with phosphate-buffered saline. After 1 or 3 days of treatment, animals were sacrificed at 2 p.m. and tissues from each time point were pooled, frozen in liquid nitrogen, and stored at −80 °C until used for RNA isolation.

For wound healing experiments, 7 female BALB/c mice (6–12 weeks old) were injected daily as described above for 7 days before wounding. Wounds were generated as described below. During the wound healing period, the daily injections were continued until wounds were harvested after 1 and 5 days. Tissues from each time point were combined, frozen in liquid nitrogen, and stored at −80 °C until used for RNA isolation.

Wounding and Preparation of Wound Tissues—Three independent wound healing experiments were performed with female BALB/c mice (8–12 weeks old), as already described (19). For each experiment, 27 animals were anesthetized by intraperitoneal injection of avertin. The hair on the animals’ backs was cut with fine scissors, and the skin was wiped with 70% (v/v) ethanol. Six full-thickness wounds were generated on each animal by excising the skin and the panniculus carnosus. The complete wound, including 2 mm of the margins, was excised at each time point. A similar amount of back skin from three nonwounded animals served as a control. Wounds from animals at each time point were combined, frozen immediately in liquid nitrogen, and stored at −80 °C until used for RNA isolation.

RNA Isolation and RNase Protection Analysis—Total RNA isolation was performed as already described (20). RNase protection assays were carried out according to Werner et al. (19). Briefly, antisense transcripts were synthesized in vitro with T3 or T7 RNA-polymerase and [32P]UTP (800 Ci/mmol) using the templates described below. RNA aliquots of 20 μg were hybridized at 42 °C overnight with 100,000 cpm of the labeled antisense probe. As a loading control, 1-μg aliquots of the same batch of RNAs were loaded onto a 1% agarose gel and stained with ethidium bromide. Nonhybridized RNA was digested at 30 °C for 1 h with RNases A and T1, which were subsequently digested by proteinase K at 42°C for 15 min. Protected fragments were separated on 5% acrylamide, 8 M urea gels and detected by autoradiography.

DNA Templates—DNA templates for RNase protection analysis were generated by polymerase chain reaction using primers corresponding to the published murine sequences. For CTGF, a 170-base pair fragment corresponding to nucleotides 1019–1188 (3) was cloned, and for TGF-β1, the template described previously (21) was used. Furthermore, a 172-base pair fragment of human CTGF corresponding to nucleotides 1012–1183 (2) was used.

Western Blot Analysis of CTGF Protein—Cells were grown in 5 ml of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and penicillin/streptomycin (100 units/ml, 100 μg/ml) per 10-cm Petri dish with or without dexamethasone. 6 h after addition of the glucocorticoid, conditioned medium from 6 Petri dishes was harvested and centrifuged to remove cell debris. Heparin-binding proteins were precipitated from the supernatant with 120 μl of heparin-Sepharose (1.1 slurry; Amer- sham Pharmacia Biotech) overnight at 4 °C. Heparin-Sepharose beads were sedimented by centrifugation and washed three times with 20 ml Tris-HCl, pH 7.4, 300 mM NaCl. Heparin-Sepharose-bound proteins were extracted by a 5-min incubation in Laemmli sample buffer at 95 °C, and the amount representing 1 Petri dish was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, CTGF protein was detected using a rabbit polyclonal antisera directed against a carboxyl-terminal peptide (NH2-CFRSLYRKYMGDMACOOL) and an alkaline phosphatase detection system (Promega). Conditioned media from nontreated and solvent-treated cells were used as negative controls.

RESULTS

FIG. 1. Dexamethasone induces CTGF mRNA expression in cultured 3T3 fibroblasts. Exponentially growing BALB/c 3T3 fibroblasts were treated for 1, 6, and 10 h with 1 μM dexamethasone (Dex). Nontreated cells (control) or solvent-treated (solvent) cells were used as controls. 20 μg of total cellular RNA were analyzed for CTGF transcripts by RNase protection assay. 20 μg of RNA were used as a negative control. The lane labeled probe contained 1000 cpm of unhybridized probe. Exposure time was 18 h at −80 °C. An ethidium bromide stain of 1 μg of the same batch of RNAs is shown below.
Dexamethasone Is a Novel Inducer of CTGF Expression

**Fig. 2.** CTGF mRNA and TGF-β1 mRNA expression are inversely regulated by dexamethasone in exponentially growing cultured 3T3 fibroblasts. Exponentially growing BALB/c 3T3 fibroblasts were treated with different concentrations of dexamethasone as indicated. Nontreated (control) and solvent-treated cells (solvent) were used as controls. 20 μg of total cellular RNA were analyzed for CTGF and TGF-β1 transcripts by RNase protection assay. 20 μg of tRNA were used as a negative control. The lane labeled probe contained 1000 cpm of unhybridized probe. Exposure times were 16 h for CTGF and 40 h for TGF-β1 at −80 °C.

methasone on CTGF mRNA expression is likely to be mediated by the glucocorticoid receptor. The same induction of CTGF mRNA expression was obtained in analogous experiments with human primary fibroblasts, although to a lesser extent (not shown). To exclude the possibility that the high CTGF mRNA levels are the result of increased expression of TGF-β1, the only known inducer of CTGF so far, we assayed the same batch of RNAs for TGF-β1 mRNA expression in an RNase protection analysis with a TGF-β1 specific antisense probe (Fig. 2, lower panel). Dexamethasone concentrations of 10—1000 nM reduced the expression of TGF-β1 mRNA by approximately 80%, whereas 1 nM had no effect on TGF-β1 expression levels. This demonstrates that the induction of CTGF expression by dexamethasone is not mediated by TGF-β1 but rather is a direct effect of the glucocorticoid.

Having demonstrated the induction of CTGF by dexamethasone at the mRNA level, we wanted to find out if this could be verified at the protein level. To address this question, we treated exponentially growing 3T3 fibroblasts as before (Fig. 2) with different concentrations of the glucocorticoid and harvested conditioned medium after 6 h. Heparin-binding proteins were enriched by their capacity to bind to heparin-Sepharose beads and were analyzed by immunoblotting using a CTGF-specific antiserum and an alkaline-phosphatase detection system. The 38-kDa CTGF protein is indicated with an arrow.

**Fig. 3.** Dexamethasone induces CTGF protein expression in exponentially growing 3T3 fibroblasts in a dose-dependent manner. Exponentially growing BALB/c 3T3 fibroblasts were treated with different doses of dexamethasone as indicated and the conditioned medium was harvested after 6 h. Nontreated (control) and solvent-treated cells (solvent) were used as controls. Heparin-binding proteins were enriched by their capacity to bind to heparin-Sepharose beads and were analyzed by immunoblotting using a CTGF-specific antiserum and an alkaline-phosphatase detection system. The 38-kDa CTGF protein is indicated with an arrow.

To investigate whether the results we obtained in vitro with cultured 3T3 fibroblasts had any significance in vivo and thus any relevance for therapeutic applications, BALB/c mice were daily injected with pharmacological doses of dexamethasone. After 1 and 3 days of treatment, we harvested different tissues and isolated total cellular RNA. RNase protection assays confirmed our in vitro studies; CTGF mRNA expression was significantly up-regulated in heart, kidney, and back skin, all of which are organs frequently affected by severe fibrosis, whereas TGF-β1 mRNA expression was down-regulated in these tissues (Fig. 4). The same results were obtained with lung and liver (not shown).

Since impaired wound healing by corticosteroids is a well known phenomenon that may be due to the suppression of the inflammatory phase of wound healing (22, 23), we tested the influence of dexamethasone on the CTGF mRNA expression in a full-thickness excisional wound healing model. As a first step we determined the time course of expression during wound healing in nontreated mice. For this purpose we isolated mRNA from skin as well as from full-thickness wounds at different intervals after injury and performed RNase protection assays (Fig. 5). CTGF mRNA was maximally expressed between 12 h and 1 day after wounding and reached basal levels after 7 days. Interestingly, we recently observed similar kinetics of induction for the CTGF inducer TGF-β1 (21). To study the influence of glucocorticoids on this process, mice were treated with dexamethasone for 7 days prior to wounding, and treatment was continued for the duration of the experiment. Surprisingly, no major differences in CTGF mRNA expression were observed in wounded skin of dexamethasone-treated mice compared with control mice. Fig. 6 shows the CTGF mRNA expression in normal skin and 1 and 5 days after wounding in an RNase protection analysis. Although in normal skin a significant induction of CTGF mRNA expression could be observed as al-
we analyzed the CTGF mRNA expression in exponentially growing 3T3 fibroblasts after treatment with TNF-α. TNF-α down-regulated CTGF mRNA levels by 90% within 8 h and almost completely suppressed CTGF mRNA expression after 24 h (Fig. 7). In an additional experiment we determined if this cytokine might have a modulating effect on the induction of CTGF expression by glucocorticoids. For this purpose, exponentially growing 3T3 fibroblasts were treated simultaneously with dexamethasone and TNF-α or IL-1β for different time periods (Fig. 8). RNase protection analysis revealed that TNF-α almost completely abolished the inducing effect of dexamethasone on the expression of CTGF mRNA, whereas IL-1β had no effect. These data demonstrate that TNF-α can compensate for the stimulatory effect of dexamethasone on CTGF expression.

**DISCUSSION**

Tissue injury initiates a series of tightly regulated processes involving inflammation, the proliferation of fibroblasts, endothelial and epithelial cells, and the deposition of extracellular matrix. If this initially positive process gets out of control, pathological fibrosis can occur in almost any organ or tissue in the body, whereby the most frequently affected sites are liver, kidney, lung, and skin (15, 24). Fibrosis can have severe consequences, ranging from disfigurement to failure of an entire organ and death. Because CTGF is a potent inducer of fibroblast proliferation and deposition of extracellular matrix molecules (1), it seems plausible that aberrant expression of this factor can substantially contribute to the development of fibrotic disease. And indeed, CTGF has been shown to be transiently overexpressed during normal repair processes such as wound healing (Ref. 7, and this study), but permanently overexpressed during pathological fibrosis in almost any organ or tissue in the body, whereby the most frequently affected sites are liver, kidney, lung, and skin (15, 24). Fibrosis can have severe consequences, ranging from disfigurement to failure of an entire organ and death. Because CTGF is a potent inducer of fibroblast proliferation and deposition of extracellular matrix molecules (1), it seems plausible that aberrant expression of this factor can substantially contribute to the development of fibrotic disease. And indeed, CTGF has been shown to be transiently overexpressed during normal repair processes such as wound healing (Ref. 7, and this study), but permanently overexpressed in various fibrotic conditions (11, 13).

Although the development of antifibrotic drugs has become a field of significant interest, there is still a lack of effective therapy. Since fibrosis is often the result of acute or chronic inflammation, anti-inflammatory therapy using glucocorticoids is often applied but is only effective in blocking the progress of the disease in some cases.

In addressing the question of whether glucocorticoids influence the expression of CTGF, we identified dexamethasone as a novel potent inducer of this growth factor on the mRNA and protein level in vivo and in vitro. This effect is not mediated by the CTGF stimulator TGF-β1, since it was down-regulated by...
Dexamethasone Is a Novel Inducer of CTGF Expression

Because large amounts of the CTGF inducer TGF-β1 are released from platelets upon hemorrhage (27). In addition, this kinetics correlates with the time course of induction of TGF-β1 on the mRNA level in the same wound healing model (21). However, a very different kinetics of CTGF mRNA expression had been obtained with a different wound healing model (7), where subcutaneously implanted steel chambers had been used. In this model peak induction of CTGF mRNA expression was observed after 9 days. The fact that a different cell population was assayed in this experimental design provides a possible explanation for the delay. For example, the chamber only contained newly formed granulation tissue, whereas cells situated at the wound edge did not contribute to the tested RNA pool. Our model, on the contrary, assays all cells in the wound bed itself and in the vicinity of the wounds.

Surprisingly, dexamethasone treatment of mice had no significant effect on the CTGF mRNA levels during wound repair, particularly at early stages of the repair process. A substantial increase in CTGF mRNA expression was only observed in nonwounded, but not in wounded skin. Three reasons might account for the similar levels of CTGF mRNA in the wounds of glucocorticoid-treated and nontreated mice. First, dexamethasone could down-regulate the CTGF inducer TGF-β1, and the reduced TGF-β1 levels might in turn compensate for the CTGF mRNA up-regulation by the glucocorticoid. This hypothesis is supported by our finding that dexamethasone reduces TGF-β1 mRNA levels during wound healing (21). However, since platelets are the major source of TGF-β1 protein in a wound, the reduced expression of this factor within the wound is unlikely to cause major changes in CTGF expression. Second, the over-expression of CTGF mRNA during wound healing and the induction by dexamethasone might not be additive, so no further enhancement of the expression level could occur. Third, and most importantly, other factors present in an acute wound, might counteract the induction of CTGF by glucocorticoids. Because serum growth factors such as platelet-derived growth factor, epidermal growth factor and basic fibroblast growth factor, which are also abundantly present in an acute wound, have no effect on the CTGF mRNA expression (7), pro-inflammatory cytokines such as IL-1β and TNF-α might be the most likely candidates. These cytokines are highly expressed during the inflammatory phase of wound healing (28) and in other inflammatory conditions (29).

To test this hypothesis, we analyzed CTGF mRNA expression in fibroblasts after treatment with TNF-α. TNF-α not only suppressed the basal expression of CTGF in exponentially growing fibroblasts but also completely abolished the induction of CTGF mRNA expression by dexamethasone in these cells. One putative mechanism of this inhibition might be the interaction of the AP-1-complex and the activated glucocorticoid receptor, which have been shown to mutually block their trans-activation function (14).

Collectively, these data suggest that dexamethasone up-regulates CTGF expression in various tissues and organs, although this up-regulation is likely to be counteracted by TNF-α in inflamed areas. The actual effect of the dexamethasone-mediated increase in CTGF expression on the extent of fibrosis is as yet unknown. However, because elevated levels of CTGF have been shown to correlate with increased matrix deposition and fibrosis (11, 13) our finding might at least partially explain the phenomenon that glucocorticoid treatment can sometimes even aggravate the course of fibrotic disease.

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