Communication

The Effects of Dexamethasone on in Vitro Collagen Gene Expression

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Glucocorticoids have been shown to be useful in the treatment of certain types of chronic liver disease both by inhibiting fibrosis and by improving liver function. We have previously demonstrated in an in vitro model of hepatic fibrogenesis that dexamethasone inhibits the synthesis of types I and IV collagen. In the present study we have evaluated the level of regulation responsible for the dexamethasone-induced changes in collagen gene expression in a defined in vitro system. Primary cultures of adult rat hepatocytes treated with and without dexamethasone under classical cell culture conditions or using defined media were evaluated for synthesis and abundance of procollagen and β-actin mRNAs. Cells treated with dexamethasone had decreased types I and IV procollagen mRNA steady state levels due to diminished transcription rates of the genes. On the other hand, β-actin mRNA levels were unaffected by dexamethasone. Transient expression experiments were performed to more precisely define the mechanism whereby dexamethasone affects type I procollagen gene transcription. The recombinant plasmid, pAZ1009, containing the mouse a2(I) procollagen gene promoter linked to the chloramphenicol acetyltransferase gene, was transfected into mouse fibroblast cell lines. Cells transfected with the pAZ1009 plasmid in the presence of dexamethasone had a significant decrease in chloramphenicol acetyltransferase activity when compared to cells not exposed to dexamethasone. These data suggest that dexamethasone inhibits collagen synthesis through a direct effect on the collagen gene promoter and appears also to have a post-transcriptional effect on procollagen mRNA content.

Glucocorticoids have been used in the treatment of chronic active hepatitis in order to prevent hepatic fibrosis (1). The beneficial effects of steroids in chronic liver disease have also been attributed to enhanced hepatocyte viability and improved expression of liver-specific function (2–6). The mechanisms by which steroids inhibit collagen synthesis appear to be complex. In addition to their generalized anti-inflammatory action (7) they have been found to decrease the levels of post-translational enzymes associated with collagen synthesis (8, 9). Studies of the molecular effects of dexamethasone on collagen synthesis have shown that dexamethasone decreases type I procollagen mRNA content in chicken (10), rat (11), and human (12) skin fibroblasts. The level of regulation responsible for this inhibition of steady state procollagen mRNA levels by corticosteroids has been a subject of considerable study. Cutroneo and co-workers (10) have suggested that transcriptional regulation in fibroblasts may be responsible for this inhibition while Raghow et al. (11) and Hämäläinen et al. (12) have indicated that a steroid-induced reduction of type I procollagen mRNA stability may be significant.

We have shown that hepatocytes maintained in serum supplemented media (SSM) or in a serum-free hormonally defined media (HDM) had a decrease in type I procollagen mRNA content when grown in the presence of dexamethasone (13). The level of gene regulation responsible for this effect of dexamethasone on procollagen mRNA content was not investigated in that study. Recently, our study of the effects of dexamethasone on an in vivo model of fibrosis revealed transcriptional inhibition of types I and IV procollagen gene expression by dexamethasone (14). However, our inability to control for the anti-inflammatory action of dexamethasone in the in vivo model may have confounded our analysis. Therefore, in the present study we have examined the level of regulation responsible for the dexamethasone-induced changes in gene expression of two cultured cell types implicated in hepatic fibrosis. We have focused specifically on transcriptional regulation of the pro-a2(I) collagen gene using nuclear run-on assays (15, 16) and the chloramphenicol acetyltransferase transient expression vector system described by Gorman et al. (17) to further delineate the effects of steroids on collagen gene expression.

EXPERIMENTAL PROCEDURES

Isolation of Hepatocytes—Male Sprague-Dawley rats were purchased from Marland Farms and maintained under 12-h light/dark cycles with food and water available ad libitum. Rats weighing between 200 and 275 g were used as the source of hepatocytes. Single cell suspensions of hepatocytes were prepared by the procedure of Berry and Friend (18) using the perfusion mixture of Leffert et al. (19).

Culture Conditions—Hepatocytes were cultured in RPMI 1640 (GIBCO) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (and for the first day of culture, 250 μg/ml fungizone). This medium was supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) to produce SSM, or with a defined

1 The abbreviations used are: SSM, serum supplemented media; HDM, serum-free hormonally defined media.

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mixture of hormones, growth factors, and trace elements to produce an HDM. The HDM has been described previously (20). Isolated hepatocytes were plated and cultured for the first 24 h in HDM plus 10% serum. Thereafter, cultures were maintained in either SSM or HDM, and half of the cultures were treated with dexamethasone (1×10^{-7} M). Cultures were fed every 24 h with freshly prepared medium for the duration of the experiment.

NIH-3T3 cells and L-cells, mouse fibroblast cell lines, were maintained in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml fungizone).

RNA Extraction and Hybridization Studies—Cells were initiated by seeding 8×10^6 cells/100-mm tissue culture dish and were grown under the culture conditions specified. In each experiment, cells were pooled from three to five dishes/culture condition (13). Total RNA was extracted using a modification of the procedures of Chirgwin et al. (21), as previously described (22).

Northern and dot blot hybridization studies were performed as previously described (13). For Northern blot studies, 10 μg of total RNA from each of the four conditions were electrophoresed, transferred to GeneScreen (Du Pont-New England Nuclear) as described by the manufacturer, baked, prehybridized, and hybridized with a radiolabeled probe. The cDNA clones were radioactively labeled by primer extension as described by Summers and Mason (23); [32]P dCTP (specific activity of 3000 Ci/mmol, Amersham Corp.) was included in the reaction to obtain specific activity of 2–6×10^{6} cpm/μg. Recombinant plasmids were used as probes in these experiments included: rat pro-α2(1) collagen (24), mouse type IV collagen (25), and chicken β-actin (26). After hybridization the membranes were washed and exposed to Kodak XAR-5 film at −70°C. The autoradiographs were then evaluated by densitometry scanning. For dot blot studies, the RNA was serially diluted, incubated for 5 min at 65°C in 3% formaldehyde, spotted on the GeneScreen matrix, and baked before prehybridization and hybridization with [32]P-labeled probes. The hybridized filters were exposed to Kodak XAR-5 film and developed before densitometry scanning.

In Vitro Nuclear Run-on Analysis—Nuclei were isolated from the cell monolayers described for nuclear run-on assays (16). Hepatocyte cell cultures (three to five 100-mm dishes/assay) were washed twice with 50 ml of ice-cold Hank’s balanced salt solution. Cells were scraped and lysed in a Dounce homogenizer in ice-cold hypotonic solution containing 0.1% Triton X-100, and the RNA labeling, isolation, and hybridization were performed as described previously (15) with minor modifications (16). Briefly, nuclei were suspended at 30°C in a reaction buffer containing 0.5 to 1 mCi of high specific activity [32]P dUTP (Amersham Corp.)/ml. The RNA transcription was allowed to continue for 15 min at 30°C, and then the nuclei were lysed with a buffer followed by proteinase K digestion. The labeled nuclear RNA was then extracted with a phenol/chloroform/isooamy alcohol mixture followed by two RNA precipitations. The RNA was precipitated in 10% trichloroacetic acid between the two ethanol precipitations in order to remove most of the unincorporated UTP. Cells were precipitated in 10% trichloroacetic acid between the two ethanol precipitations in order to remove most of the unincorporated UTP.

Densitometry tracing of autoradiographs from Northern blot hybridization experiments comparing mRNA of cells grown under four different culture conditions

| Condition       | SSM + dex as % of SSM | HDM + dex as % of HDM |
|----------------|------------------------|------------------------|
| Type I collagen | 33.0 ± 6.8*             | 39.8 ± 8.4             |
| Type IV collagen| 36.7 ± 9.3             | 40.7 ± 6.1             |
| β-Actin         | 114 ± 11               | 97.5 ± 12              |

*Data are expressed as mean ± S.E. of three experiments.

Primary liver cell cultures plated onto tissue culture plastic in the presence of serum exhibit decreased levels of liver-specific functions and increased numbers of fibroblasts with time as described in our previous studies (13). The addition of dexamethasone to the culture medium resulted in improved liver-specific functions but did not alter the selection for fibroblasts. On the other hand, the use of serum-free HDM maintained in vivo levels of tissue-specific function, and few mesenchymal-like cells were observed. Once again dexamethasone had no apparent effect on whether hepatocytes or mesenchymal-like cells survived in the cultures. The effects of dexamethasone on the liver-specific functions in cultures under defined conditions will be reported elsewhere.

To evaluate the molecular effects of both the varied media and the administration of dexamethasone on collagen gene expression, we isolated total RNA from hepatocytes grown under four conditions (SSM ± dexamethasone and HDM ± dexamethasone). β-Actin expression was used as a control. The mRNA steady state levels of these genes were then analyzed by both Northern and dot blot hybridization. When hepatocytes grown in either SSM or HDM were exposed to dexamethasone there was a significant decrease in the hybridization signal for types I and IV procollagen mRNAs as shown by densitometry scanning of the blots (Table I). In contrast, β-actin mRNA content was unchanged by the addition of dexamethasone to hepatocytes grown in either SSM or HDM.

To determine what level of gene expression was responsible for the dexamethasone-induced steady state changes, we isolated nuclei from the hepatocytes grown under the four conditions, labeled the RNA transcripts with [32]P dUTP, and hybridized these transcripts to specific cDNAs bound to nitrocellulose filters. Fig. 1 is an autoradiograph of one of three such experiments. It shows that the decrease in steady state levels of both procollagen mRNAs in the presence of dexamethasone is associated with decreased transcription of the collagen genes in cells grown either in SSM or HDM. These results are confirmed by densitometry of three sets of experiments (Table II). However, the change in transcription was not always of sufficient magnitude to account for the marked decrease in the cytoplasmic abundance of that mRNA. Therefore, both transcriptional and post-transcriptional mechanisms appear to affect collagen synthesis. There was no apparent effect of dexamethasone on β-actin gene transcription.

To confirm the effect of dexamethasone on type I procollagen gene transcription as well as to more precisely define the mechanism by which dexamethasone inhibits transcription of this gene, transient expression vector experiments

| Mechanism of dexamethasone's effect on collagen gene expression | Transcriptional | Post-transcriptional |
|---------------------------------------------------------------|-----------------|---------------------|
| Effect on type I procollagen gene transcription               | Decreased       | Inhibited (not shown) |
| Effect on type IV procollagen gene transcription              | Decreased       | Inhibited (not shown) |

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with the pro-α2(I) collagen gene promoter were performed. The recombinant plasmid, pAZ1009, which contains the mouse α2(I) procollagen gene promoter linked to the structural CAT gene and the SV40 enhancer (27), was transfected into two mouse fibroblast cell lines (NIH-3T3 and L-cells). The resultant chimeric catenase transferase expression was measured in cells grown in the presence or absence of 10−6 M dexamethasone. As shown in Fig. 2, NIH-3T3 cells transfected with the pAZ1009 plasmid in the presence of dexamethasone had a significant decrease in chloramphenicol acetyltransferase activity when compared to cells not exposed to dexamethasone. No dexamethasone effect was noted in the positive control, pSV2CAT, which contains the SV40 enhancer and promoter, or the negative control, pAαCAT, both of which lack the collagen gene promoter. The results of six such experiments (two in NIH-3T3 cells and four in L-cells) are shown in Table III. These data indicate a marked decrease in chloramphenicol acetyltransferase activity in the cells treated with dexamethasone. When these experiments were repeated in the presence of 10−7 M dexamethasone, similar results were obtained.

DISCUSSION

Corticosteroids have a significant role in the treatment of many pathological states involving almost every organ system. Therapy with these agents has been shown to be beneficial in certain forms of chronic fibrotic liver disease due in part to their inhibition of collagen synthesis (1). In this study, we employed liver cells cultured under defined conditions to investigate the molecular mechanisms which may be responsible for these beneficial effects in vivo. We determined that dexamethasone showed both transcriptional and post-transcriptional regulation in its inhibition of the expression of types I and IV collagen in the primary liver cell cultures.

Corticosteroids have been demonstrated to inhibit collagen synthesis in many experimental models (7-14), and a variety of mechanisms have been proposed to explain this inhibition of collagen synthesis; e.g. they have an anti-inflammatory action (7), and they tend to decrease the levels of post-transcriptional enzymes associated with collagen synthesis (8, 9). In addition to these other mechanisms of action, steroids have been shown to decrease procollagen mRNA steady state levels in a series of recent studies (10-12). These studies have shown different levels of gene regulation to be responsible for these steady state changes in fibroblast cultures. Raghow et al. (11) and Hämäläinen et al. (12) have shown that steroid treatment of rat and human skin fibroblasts leads to a decrease of type I procollagen mRNA half-life. However, Cockayne et al. (10) showed transcriptional regulation rather than a post-transcriptional effect of steroids in chicken dermal
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fibroblasts. These differences can be partially explained by variations in the systems examined, in the cell type studied, and in the dose and duration of dexamethasone used.

Our data support the interpretation that there is a direct effect of dexamethasone on type I and type IV procollagen gene transcription as one of several causes of steroid-induced inhibition of collagen synthesis. In addition, dexamethasone appeared to show a post-transcriptional component in its regulation of total procollagen mRNA abundance as the decrease in transcription was generally less than the observed decrease in steady state mRNA levels. This transcriptional effect of dexamethasone on the collagen genes in vitro is consistent with our previous study of the effects of dexamethasone on hepatic fibrogenesis in the in vivo model system, murine schistosomiasis (14). In that study we also used the nuclear run-on assay to determine that the decreased hepatic collagen content in mice infected with schistosomiasis and treated with dexamethasone was associated with decreased type I and IV collagen gene transcription (14). Walsh et al. (29) have also shown that dexamethasone decreases α1(I) and α2(I) procollagen gene transcription in both neonatal rat liver and small intestine.

Further support for a transcriptional effect of dexamethasone on collagen gene expression is provided by our experiments employing the chloramphenicol acetyltransferase transient expression vector, PAZ1009. In these experiments, transcription of the CAT gene is driven by the mouse α2(I) collagen gene promoter, and we have found a marked decrease in chloramphenicol acetyltransferase activity when the cells transfected with this construct were treated with dexamethasone. Therefore, dexamethasone appears to be exerting a direct effect on the collagen gene promoter in order to cause this decrease in chloramphenicol acetyltransferase activity. Furthermore, these experimental results suggest a possible mechanism by which steroids may inhibit the transcription of type I collagen: a steroid-receptor complex may bind to an upstream region of the gene and inhibit its expression. Drouin et al. (30) have shown that glucocorticoids inhibit the transcription of the pro-opiomelanocortin gene and have suggested that this action may be associated with the glucocorticoid receptor binding to the steroid core enhancer sequence. It is possible that steroids might similarly interact with a glucocorticoid-like responsive element and inhibit transcription of the collagen genes. Deletional mutant studies are currently underway in order to ascertain which region(s) of the mouse pro-α2(I) collagen gene promoter may be responsible for the observed inhibition of transcription by dexamethasone.

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