Suppression of Stellate Cell Type I Collagen Gene Expression Involves AP-2 Transmodulation of Nuclear Factor-1-dependent Gene Transcription

(Received for publication, January 22, 1996, and in revised form, May 23, 1996)

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The regulation of collagen gene expression was studied in culture-activated rat hepatic stellate cells, the fibrogenic effector cell involved in hepatic fibrogenesis. Treatment of cells with a 5-lipoxygenase-specific inhibitor caused a reduction in α(I) collagen mRNA transcript abundance, which suggested that leukotriene production was involved in maintaining the activated cell's high level of collagen mRNA production. The underlying mechanism involved a decrease in collagen gene transcription. Suppression of gene transcription was localized to an nuclear factor-1 (NF-1) binding domain in the proximal promoter and an AP-2 binding domain adjacent to it. Gel retardation assays demonstrated that an increase in AP-2 binding adjacent to the NF-1 site was likely to be the transmodulator responsible for the suppression of the NF-1-dependent gene expression. The data suggest that post-translational alterations in AP-2 activity are responsible for this unappreciated mechanism of regulating the collagen gene.

Collagen gene expression is regulated transcriptionally and post-transcriptionally during development and pathologic wound healing conditions typified by hepatic fibrosis and cirrhosis (1–6). In this disease, the hepatic stellate cell (also referred to as Ito cell, lipocyte, or fat-storing cell) transforms into a myofibroblast-like cell and overproduces predominantly type I collagen, as well as numerous other matrix proteins (7). This process is not limited to the liver as parallel pathways exist in most solid organs (e.g. kidney, lung, heart) during pathologic fibrotic states (1, 3–6). A better understanding of the aberrant mechanisms responsible for the overexpression of the collagen gene should lead to the development of therapeutic approaches to limit the disease process.

Recent studies utilizing transgenic models involving α(I) collagen minigenes have indicated that the proximal promoter (400 base pairs) contains most of the regulatory regions (including NF-1 and Sp-1 sites in footprints 1 and 2) required for accurate expression of the hepatic stellate cell collagen gene at base line and following short term injury (1). However, other tissues clearly need additional regions of the upstream promoter sequence (UPS) and/or the 1st intron emphasizing the complex cell-specific transcription factors, which are likely to exist (1, 3–6).

Recent studies in our laboratory have found that the lipoxygenase pathway is critically involved in stellate cell transformation that occurs in vitro and serves as a paradigm of hepatic fibrogenesis in vivo (8). Transformation is associated with both an increase in the abundance of collagen mRNA as well as increased cell proliferation (1, 7). Specific inhibitors of the 5-lipoxygenase (5LO) pathway can serve as useful probes to dissect this transformation process. These inhibitors abolish the stellate cell proliferative response to platelet-derived growth factor, the most potent stellate cell mitogen (8). The effect occurs distal to mitogen-activated protein kinase (MAPK) with suppression of immediate early gene transcription (8). Since MAPK translocates to the nucleus and can phosphorylate and modify numerous potential transcription factors, this suggests that 5LO inhibitors alter transcription factor activity. Since stellate cell transformation is associated with alterations in transcription factor activity, we hypothesized that 5LO inhibitors could alter type I collagen transcript abundance as well and thereby serve as a useful tool to further dissect the factors that regulate this pathologic process.

In the present study, treatment of cultured stellate cells with a specific 5-lipoxygenase inhibitor resulted in a marked decrease in the abundance of the type I collagen mRNA transcript. We addressed the possibility that the decrease in collagen mRNA steady state levels involved a decrease in collagen gene transcription. This was evaluated through the use of a chloramphenicol acetyltransferase (CAT) reporter gene linked to the UPS and the 1st intron of the rat α(I) collagen gene. The 5LO inhibitor-sensitive region was localized to the previously mentioned proximal region of the UPS and specifically the most proximal NF-1 site was found to be required for the effect. This binding domain was further dissected through the use of a series of electrophoretic mobility shift assays utilizing nuclear extracts from stellate cells ± 5LO inhibitor treatment. Through the use of oligonucleotide competition binding assays, it was found that the 5LO inhibitor treatment alters the binding complex within the footprint 1 domain. The alteration in binding was largely attributable to an increase in AP-2 binding at a site adjacent to the NF-1 site. Mutation of this AP-2 site in the CAT reporter gene abolished the 5LO inhibitory effect, suggesting that the increased AP-2 binding was required for the 5LO inhibitory effect. Since enhanced AP-2 binding has been shown to transmodulate and repress NF-1-dependent growth hormone and luteinizing hormone receptor gene transcription, the data suggest a novel mechanism of regulation of collagen gene transcription (9, 10). The 5LO inhibitor drug decreases type I collagen mRNA transcript abundance in part by depressing
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EXPERIMENTAL PROCEDURES

Chemicals—The 5LO-specific inhibitor, ICI 230487 (kindly provided by Zeneca) was prepared as a a 1000 × stock solution in ethanol just prior to use. The final concentration used was 1 µM, and this reflects the optimal selective concentration used in other studies (8). The 5LO inhibitor was added to tissue culture medium directly and then 8–10 h later the medium was replaced with fresh medium ≥ the 5LO inhibitor. Preliminary studies suggested this method optimized the relative stability of the compound under these culture conditions. Control cultures treated with equivalent volumes of ethanol vehicle (final concentration, 0.1%) were indistinguishable from untreated cells.

Cell Culture—Hepatic stellate cells were isolated from Sprague-Dawley male rats by previously described methods and subcultured on tissue culture flasks precoated with type I collagen or uncoated when transfections were performed (11). Experimental manipulations were performed with cells at passage 2–6 maintained on 75-cm² precoated plates.

RNA Measurements—The relative transcript abundance of rat α(I) collagen and glyceraldehyde dehydrogenase mRNA was assessed by Northern blotting using the Chomczynski method of RNA extraction as described previously (12). Representative gels are shown for each measurement. All experiments were repeated three times with similar results.

Transfection Studies—Stellite cells were transfected using the LipfectAMINE reagent and cell extract handling, extraction, quantitation, and CAT measurements were performed as described previously (11). The plasmids used for transfection included the intcolCAT plasmids (−3.6/1.6 or −1.7/1.6) which contain either −3.6 or −1.7 kb of the 5′-UBS of the rat α(I) collagen linked to the 1st exon and 1.6 kb of the 1st intron and the CAT reporter gene (6). The −1.3/1.6 intcolCAT plasmid was a derivative of the −3.6/1.6 intcolCAT plasmid produced by digestion with NheI/Tth1111 restriction endonucleases. Ends were then blunted and ligated with T4 DNA ligase. Mutagenesis at codons −101 and −100 from G-G to T-T in the −3.6/1.6 intcolCAT plasmid was performed using the overlap extension polymerase chain reaction technique to produce −3.6/NF-1 mut/1.6) plasmid (13). Mutagenesis at codons −89 to −87 from G-G to T-T and at codons −93 to −91 from G-C-C to A-T-T in the −3.6/1.6 intcolCAT plasmid was performed with the same technique to produce the −3.6/AP-2 mut/1.6) plasmid. The mutated plasmids were sequenced to confirm the positions of the mutations.

Transfection efficacy was monitored by parallel transfection with a plasmid containing a β-galactosidase plasmid, as described previously (11).

Gel Retardation Assays—Nuclear extracts (10 µg/lane) from hepatic stellate cells were incubated with radiolabeled double-stranded oligonucleotides (Life Technologies, Inc.) and electrophoresed on a native gel, as described (14). Competition binding assays were performed using varying amounts of unlabeled double-stranded oligonucleotides. In some experiments, extracts were preincubated with equivalent amounts of either polyclonal α-NF-1 or polyclonal α-AP-2 (Santa Cruz) antisera or nonimmune rabbit serum prior to incubation with the radiolabeled oligonucleotide (footprint 1).

Western Blotting—Nuclear extracts were resolved on SDS-PAGE and transferred as described previously (14). Probing with polyclonal α-Sp-1 or α-AP-2 antisera (Santa Cruz) was done following the manufacturer’s recommendations. The antigen was subsequently identified using peroxidase-conjugated anti-rabbit secondary antibodies followed by the enhanced chemiluminescence Amersham method. Alternatively, the antigen was obtained by performing multiple gel retardation assays, combining several retarded bands via electrophoresis (Bio-Rad), concentrating the sample with a Centricon (Amicon) concentrator and then resolving by SDS-PAGE and transferblotting.

Statistical Analysis—Differences between means were evaluated using Student’s t tests (p < 0.05 significance cutoff) using the Statworks program on a Macintosh 6100 computer.

RESULTS AND DISCUSSION

Lipoxygenase Inhibition Reduces Type I Collagen mRNA Transcript Abundance—Previous studies of stellite cells found that during culture in medium containing reduced concentrations (i.e. 0.4%) of serum, the cells continue to produce significant quantities of leukotrienes (leukotriene C_4 >> leukotriene B_4) as well as collagen protein (8). All studies were performed in this reduced serum-containing medium to minimize the potential contributions of serum stimulants of collagen production. It was found that treatment of cells with the lipoxygenase-specific inhibitor, ICI 230487, led to a decrease in the abundance of the type I collagen mRNA transcript (Fig. 1). Laser densitometric scanning and quantitation of type I collagen mRNA transcript abundance after normalization for RNA loading (using the glyceraldehyde dehydrogenase RNA band, as shown) revealed a 3.4-fold decrease in transcript abundance: control cells, type I collagen mRNA abundance (collagen/glyceraldehyde dehydrogenase) = 1.25 ± 0.22 versus 5LO inhibitor-treated cells; type I collagen mRNA abundance (collagen/glyceraldehyde dehydrogenase) = 0.36 ± 0.19 (mean ± S.D.; n = 6, p < 0.001). Preliminary studies found that lipoxygenase inhibition induced by other inhibitors (i.e. caffeic acid or nordihydroguaiaretic acid) proportionally reduced the mass of total collagen produced as well (data not shown). Therefore, the observed suppression is likely a consequence of the reduction in leukotriene production and not a nonspecific effect of the ICI compound.

Lipoxygenase Inhibition Reduces NF-1-dependent Collagen Gene Transcription—To explore the possibility that the 5LO inhibitor effect on collagen transcript abundance might involve changes in gene transcription, a series of transient transfection assays were performed. These studies utilized the intcolCAT reporters, which contain varying lengths of the 5′-UBS of the α(I) collagen gene contiguous with the 1st exon and 1.6 kb of the 1st
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### Table I

| Plasmid                       | Control          | 5LO-inhibitor-treated | p value (n) | Fold Decrease |
|-------------------------------|------------------|-----------------------|-------------|---------------|
| −3.6/1.6                      | 2.6 ± 0.28       | 0.97 ± 0.03           | <0.01 (2)   | 2.7           |
| −1.7/1.6                      | 1.6 ± 0.6        | 0.88 ± 0.28           | <0.02 (4)   | 1.9           |
| −1.3/1.6                      | 1.6 ± 0.36       | 0.69 ± 0.29           | <0.03 (2)   | 2.3           |
| −3.6/NF-1 mut/1.6             | 1.0 ± 0.09       | 1.1 ± 0.1             | NS (2)      |               |
| −3.6/AP-2 mut/1.6             | 1.0 ± 0.12       | 1.04 ± 0.13           | NS (4)      |               |

* CAT/mg of protein (normalized for transfection efficacy).  
  "Not significant.

FP-1 site number: −107 to −86:

| FP-1 wild type |
|----------------|
| NF1 region     |
| AP-2 region    |
| NF1 mutated region |
| AP-2 mutated region |
| NF-1 consensus |
| AP-2 consensus |

**Oligonucleotides derived from FP-1**

| Oligonucleotide                   |
|----------------------------------|
| FP-1 wild type                   |
| NF1 region                       |
| AP-2 region                      |
| NF1 mutated region               |
| AP-2 mutated region              |
| NF-1 consensus                   |
| AP-2 consensus                   |

* NF-1 was from adenovirus origin of replication.  
  a From Santa Cruz Biotechnology, Inc.

intron linked to the CAT reporter gene. Following transfection, the cells were handled as described above ± 5LO inhibitor × 24 h. As shown in Fig. 2 and Table I, the ICI drug caused a reduction in intColCAT expression when either −3.6 kb (−3.6/1.6 plasmid), −1.7 kb (−1.7/1.6 plasmid), or −1.3 kb (−1.3/1.6 plasmid) of the UPS was present. These data suggested that the 5LO inhibitor responsive element could reside in the more proximal region of the promoter. Since the NF-1 site contains in the −400-base pair region of the proximal promoter has been implicated as one of the major regulators of collagen gene transcription, we evaluated the capacity of the 5LO inhibitor to suppress CAT expression using a reporter that contained a specific mutation in the NF-1 binding site of this region (termed footprint 1) (1, 3, 15). By using this mutated reporter (−3.6/NF-1 mut/1.6), it was found that the 5LO inhibitor effect was completely abolished. These results suggest that the 5LO inhibitor effect on collagen transcript abundance is partially due to suppression of NF-1-dependent gene transcription.

Lipoxygenase Inhibition Alters Footprint 1 Binding—Since changes in transcription factor activity may be related to changes in transcription factor binding or abundance, stellate cell nuclear extracts were analyzed utilizing electrophoretic mobility shift assays and the radiolabeled footprint 1 (FP-1) region (see Table II), which was implicated as a key region involved in the 5LO inhibitor effect on gene transcription. When the FP-1 gel shift was compared between untreated cells and cells treated with the ICI 5LO inhibitor, it was found that the treatment paradoxically increased the specific binding. This is shown in adjacent lanes in Fig. 3 (e.g. compare Fig. 3A, left panel, lane 2 (ICI 5LO inhibitor-treated) versus lane 3 (untreated) or Fig. 3B, left panel, lane 2 (ICI 5LO inhibitor-treated) versus lane 3 (untreated)). It should be noted that the major band in the gel shift is indicated by a single arrow. An additional broader band with a slower electrophoretic mobility appeared in the gels as well. The binding specificities of this upper band and its transcription factor composition (see below) appeared to resemble the major band, although the presence of an additional transcription factor cannot be excluded. However, the major difference in footprint 1 binding induced by the 5LO inhibitor can be explained by changes in the composition of the band with the faster electrophoretic mobility (see below).

Previous studies have obtained slightly different gel shift patterns using footprint 1 (1–3). This is likely due to cell-specific differences in the relative composition of nuclear binding factors, because the extracts had been derived either from unrelated cells or stellate cells that were not culture-activated (1–3). Nevertheless, as demonstrated below, the NF-1 transcription factor is shown to play a major role in the footprint 1 gel shift in the current stellate cell study as well as the previously mentioned stellate cell study (1).

To examine the transcription factor composition of the footprint 1 gel shift, competition assays were performed utilizing increasing concentrations of excess unlabeled NF-1 oligonucleotide or a mutated NF-1 oligonucleotide (see Fig. 3A). As shown in Table II, the NF-1 oligonucleotide was derived directly from the footprint 1 sequence. When extracts from untreated cells were used (see Fig. 3A, left panel, lanes 7–10), the gel shift was abolished by competition with 50-fold excess of the unlabeled NF-1 oligonucleotide. In contrast, the mutated NF-1 oligonucleotide (NF-1 mut sequence shown in Table II) did not reduce the gel shift band intensity (Fig. 3A, left panel, lanes 3–6), confirming that the competition was specific for the NF-1 domain. When extracts from the 5LO inhibitor-treated cells were similarly analyzed (see Fig. 3A, right panel, lanes 7–10), competition with the NF-1 oligonucleotide reduced the gel shifts, but failed to completely eliminate the gel shift band (despite using 200 × excess oligonucleotide). The mutated NF-1 oligonucleotide had no effect on the gel shift, as expected (Fig. 3A, right panel, lanes 3–6). These results suggest that the footprint 1 gel shift in untreated cells is a consequence of transcription factor binding to the NF-1 domain within the footprint 1 sequence. In contrast, the footprint 1 gel shift in the 5LO inhibitor-treated cells is partially due to the NF-1 domain, but is also the result of transcription factor binding to another region in the footprint 1 sequence. Examination of the footprint 1 sequence (see Table II) reveals an AP-2 sequence overlapping with the contiguous NF-1 sequence. This AP-2 region was then used as the unlabeled competitor in gel shift assays with the radiolabeled footprint 1. As shown in Fig. 3B, there was a marked difference between control untreated cells (left panel, lanes 3–12) versus 5LO inhibitor-treated cells (right panel, lanes 3–10). For control untreated cells, neither excess AP-2 nor excess mutated AP-2 (AP-2 mut sequence shown in Table II) oligonucleotide caused any decrease in the gel shift pattern. However, in 5LO inhibitor-treated cells, excess AP-2 oligonucleotide markedly decreased the gel shift band (see Fig. 3B, right panel).
FIG. 3. 5-Lipoxygenase inhibitor treatment alters FP-1 binding complex. Stellate cell nuclear extracts (treated in culture ± ICI 5LO inhibitor × 24 h) were incubated with radiolabeled footprint 1 oligonucleotide ± increasing concentrations of either the NF-1 domain in footprint 1 (or a mutated NF-1 oligonucleotide) (A) or the AP-2 domain in footprint 1 (or a mutated AP-2 oligonucleotide) (B). The complexes were then resolved via electrophoretic mobility shift assay. The major specific retarded band is indicated by the arrow on the left of the gel. A. NF-1 oligonucleotide competition differentially alters the FP-1 complex in control versus 5LO inhibitor-treated cells. Left panel: lane 1, no extract; lane 2, 5LO inhibitor-treated cell extract; lane 3, control untreated cell extract; lanes 4–6, control untreated cell extract + excess (10 ×, 50 ×, 100 ×) unlabeled mutated NF-1 oligonucleotide (NF-1 mut); lane 7, control untreated cell extract; lanes 8–10, control untreated cell extract + excess (10 ×, 50 ×, 100 ×) unlabeled NF-1 oligonucleotide (NF-1 mut); lane 11, 5LO inhibitor-treated cell extract; lanes 12–14, control untreated cell extract + excess (10 ×, 50 ×, 100 ×) unlabeled mutated NF-1 oligonucleotide (NF-1 mut); lane 15, control untreated cell extract. B. AP-2 oligonucleotide competition differentially alters the FP-1 complex in control versus 5LO inhibitor-treated cells. Left panel: lane 1, no extract; lane 2, 5LO inhibitor-treated cell extract; lane 3, control untreated cell extract; lanes 4–6, control untreated cell extract + excess (10 ×, 50 ×, 100 ×, 200 ×) unlabeled mutated AP-2 oligonucleotide (AP-2 mut); lane 7, control untreated cell extract; lanes 8–10, control untreated cell extract + excess (10 ×, 50 ×, 100 ×, 200 ×) unlabeled AP-2 oligonucleotide. Right panel, lane 7 versus lanes 8–10). The mutated AP-2 oligonucleotide had no effect on the gel shift band (Fig. 3B, right panel, lane 3 versus lanes 4–6), confirming that the competition was specific for the AP-2 domain. These results with the NF-1 and AP-2 competition gel shifts collectively suggest that the retarded band in the footprint 1 gel shift in control untreated stellate cells is due to binding to the NF-1 domain. In contrast, the band in the footprint 1 gel shift in 5LO inhibitor-treated cells is the result of simultaneous binding of NF-1 and AP-2 to their respective overlapping domains within the footprint 1 sequence. To confirm that the transcription factor composition of footprint 1 is predominantly NF-1 in control cells versus NF-1 + AP-2 in 5LO inhibitor-treated cells, an additional series of footprint 1 gel shifts were performed with the respective extracts pretreated with antibodies to NF-1 or AP-2.

As shown in Fig. 4A, α-NF-1 or α-AP-2 pretreatment abolished the band in the footprint 1 gel shift in 5LO inhibitor-treated cells. Pretreatment with nonimmune serum (NI) had no effect on the gel shift. When the same antibody pretreatments were used on control cell extracts, only the α-NF-1 reduced the footprint 1 gel shift (Fig. 4B). These results reinforce the conclusion that the footprint 1 gel shift is predominantly due to NF-1 under control conditions, whereas 5LO inhibitor treatment causes both NF-1 and AP-2 to bind at adjacent overlapping sites in footprint 1. When the footprint 1 gel shift band (as indicated by the arrow in Figs. 3 and 4) in 5LO inhibitor-treated cells was eluted and used in a Western blot assay (see Fig. 5, lanes 5 and 6), the AP-2 protein was identified. This provides further validity to the observation that the AP-2 protein was present in the gel shift.

To further pursue the significance and the mechanism of the change in the footprint 1 gel shift in 5LO inhibitor-treated cells, several complementary approaches were taken. The AP-2 consensus binding site in footprint 1 was mutagenized in situ in
the intcolCAT reporter (termed −3.6(AP-2 mut)/1.6) and used in additional transfection assays. As indicated in Fig. 2 and Table I, this mutation eliminated the response to the 5LO inhibitor. This suggests that the footprint 1 AP-2 site and the associated AP-2 binding are required for the inhibition of intcolCAT expression, which occurs during 5LO inhibitor treatment. The change in AP-2 activity may be due to a generalized change in the relative abundance or activity of the regulatory transcription factors. When nuclear extracts in control versus 5LO inhibitor-treated cells were used in Western blot assays, there was no change in the abundance of the AP-2 protein (Fig. 5, lanes 3 and 4). Comparable Sp-1 abundance is shown for comparison (Fig. 5, lanes 1 and 2). To evaluate the critical NF-1 transcription factor, a consensus NF-1 binding site unrelated to the NF-1 binding domain in footprint 1 was radiolabeled and used in a gel shift assay. This consensus NF-1 site does not contain any potential AP-2 binding site. As shown in Fig. 6 (lanes 3 and 4), ICI 5LO inhibitor-treated cell extracts contained a markedly enhanced AP-2 gel shift versus control untreated cells. This suggests that the observed change in the composition of the transcription factors in footprint 1 is due in part to a generalized increase in the relative binding activity of the AP-2 transcription factor. Furthermore, the comparable abundance of the AP-2 transcription factor in treated versus untreated cells demonstrated in the Western blot suggests that a post-translational mechanism is likely to be responsible for the change in AP-2 binding activity. Future studies will be needed to define the actual modifications in the AP-2 protein or the AP-2 binding complex.

In summary, the current work suggests that 5LO inhibitor treatment results in an increase in AP-2 binding activity. The enhanced AP-2 binding changes the composition of the footprint 1 transcription factor complex. Simultaneous AP-2 and NF-1 binding occurs at their respective overlapping binding domains. This in turn results in a decrease in collagen gene transcription. This work suggests that AP-2 transmodulation of NF-1-dependent collagen gene transcription represents a potential mechanism for regulating collagen gene transcription.

Acknowledgments—We thank J. Mullen and J. Vande Vusse for technical assistance and D. Rowe and D. Breault for plasmids used in transfection.

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