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Regulation of Angiogenin Expression in Human HepG2 Hepatoma Cells by Mediators of the Acute-Phase Response

Sigitas J. Verselis,[1] Karen A. Olson,* and James W. Fett*,[2]
*Center for Biochemical and Biophysical Sciences and Medicine and *Department of Pathology, Harvard Medical School, 250 Longwood Avenue, Boston, Massachusetts 02115*

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Angiogenin is a potent inducer of neovascularization *in vivo.* However, like other angiogenic molecules, its specific physiologic roles and mechanisms regulating its expression remain to be elucidated. Angiogenin is a liver-derived component of normal serum whose concentration can increase in various disease states. This suggests that it might participate in the acute-phase response. In an initial study we showed that angiogenin protein and mRNA levels transiently increased in mice following an acute inflammatory stimulus. We now report that IL-6, a major inducer of acute-phase proteins, stimulates the synthesis and secretion of angiogenin protein in human HepG2 cells within 24 hr following treatment, an effect enhanced by dexamethasone. IL-6 also increases the amount of angiogenin mRNA without altering its half-life. This increase, suppressible by cycloheximide, peaks at 12 hr following stimulation and returns to basal levels by 48 hr. IL-1 alone slightly decreases the basal production of angiogenin protein and mRNA, but essentially abolishes the response to IL-6 in the absence or presence of dexamethasone. This antagonistic effect by IL-1 on IL-6 activity is not a result of changes in mRNA stability nor is it dependent on new protein synthesis. Thus, the combined effects of IL-6, IL-1, glucocorticoids, and perhaps other related factors may specifically control angiogenin expression. Since angiogenin is regulated in a manner similar to that of acute phase proteins both *in vitro* and *in vivo,* it may play a role in the host response to injury. © 1999 Academic Press

Angiogenesis, the creation of a new vascular network, is essential to the normal physiologic processes of embryologic development, reproduction and wound repair (1). In healthy individuals angiogenesis is under the tight control of both pro- and antiangiogenic molecular mediators (2). One of these proangiogenic regulators is angiogenin, a 14.1 kDa protein that induces blood vessel formation *in vivo* in the chick chorioallantoic membrane and rabbit cornea and meniscus (3, 4). *In vitro* angiogenin induces endothelial cell proliferation (5), supports endothelial and tumor cell adhesion (6, 7) and undergoes nuclear translocation (8). Angiogenin was originally isolated from medium conditioned by human colon carcinoma cells (1) and antagonists of its functions exhibit potent antitumor activity against this and other types of human tumors implanted into athymic mice (9–11). Moreover, angiogenin is a component of normal human and bovine plasma (12, 13) and bovine milk (14), and in rodents is predominantly synthesized in the adult liver (15, 16).

We observed previously that while angiogenin serum concentrations of healthy volunteers fall within a narrow range (274-496 ng/ml), increases up to three-fold above the mean could be detected in hospitalized patients.[3] These fluctuations in plasma concentrations are characteristic of a group of liver-derived proteins known as acute phase proteins (APPs) (17). APPs, whose synthesis is the result of the host response to injury, infection, neoplasia or immunological perturbations, act in concert to restore the preexisting physiologic state. Since angiogenesis is a major component of the tissue remodeling which may occur following an acute insult, investigations were initiated to determine

[1] Present address: Dana Farber Cancer Institute, 44 Binney Street, J223, Boston, MA 02115.
[2] To whom correspondence should be addressed at: Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Seeley G. Mudd Building, 250 Longwood Avenue, Boston, MA 02115. Fax: (617) 566-3137. E-mail: james_fett@hms.harvard.edu.

Abbreviations: APP(s), acute phase protein(s); BSA, bovine serum albumin; CHX, cycloheximide; DXM, dexamethasone; IFN-γ, interferon-γ; IL, interleukin; PMSF, phenylmethylsulfonyl fluoride; RE(s), responsive element(s); TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

[3] K. A. Olson, K. H. Falchuk, and J. W. Fett, unpublished results.

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the potential participation of angiogenin in this process. In an initial study we demonstrated that angiogenin mRNA levels did indeed rise following injection of mice with an inducer of acute inflammation (16). This response was rapid, transient and specifically localized to the liver. Associated with this induction was an increase and subsequent fall in the concentration of serum angiogenin, behavior characteristic of APPs.

The regulation of APP synthesis has also been extensively studied *in vitro* using both primary and transformed liver cells. Two classes of APPs have been defined based on their regulation by specific cytokines and cofactors (18). The expression of human class 1 proteins such as C-reactive protein, α_1-glycoprotein and serum amyloid A is increased by interleukin (IL)-1 and tumor necrosis factor-α (TNF-α). In contrast, the synthesis of class 2 APPs, which include human fibrinogen, haptoglobin and α1-proteinase inhibitor, is stimulated by members of the IL-6 family of cytokines (19). IL-6-type cytokines also synergize with IL-1 to stimulate class 1 proteins while IL-1 inhibits the IL-6-induced production of class 2 APPs. Glucocorticoids can augment the effects of IL-1 and IL-6 toward both classes of proteins. Finally, interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) are capable of regulating protein synthesis in cultured primary human hepatocytes and hepatomas, but their actions are limited to only a small subset of APPs (20, 21).

This report extends our previous *in vivo* study and examines the effects of several cytokines on the production of angiogenin by HepG2 cells. We find that the pattern of responses is consistent with its regulation as a class 2 APP. Thus, angiogenin may play a role in host defense as it relates to angiogenesis-associated tissue repair.

MATERIALS AND METHODS

Cytokines and reagents. Natural human TNF-α (10^8 units/mg) was from Endogen; human recombinant IFN-γ (2.5×10^7 units/mg) was from Genzyme; porcine platelet TGF-β1 and human recombinant IL-1β ($\geq 5 \times 10^8$ units/mg) were from R&D Systems; human recombinant IL-6 ($>2 \times 10^8$ units/mg) and fatty acid-free bovine serum albumin (BSA) were from Boehringer Mannheim. Seakem GTG agarose was obtained from FMC; [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]dATP (6000 Ci/mmol), and L-[^{35}S]methionine (>800 Ci/mmole) were from NEN Life Science Products; protamine sulfate, deoxycholate, formalin-fixed *Staphylococcus aureus*, phenylmethylsulfonyl fluoride (PMSF), dexamethasone (DXM), novobiocin and cycloheximide (CHX) were from Sigma.

Cell line and culture conditions. Human hepatoma HepG2 cells (American Type Culture Collection no. HB-8065) were maintained in complete medium consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and gentamicin. For experiments, HepG2 cells were grown in complete medium in either 12-well cluster plates (Corning Costar) for protein determinations and metabolic labeling experiments or in 6-well plates for RNA isolation.

Angiogenin protein secretion. HepG2 cells grown to confluence were washed with DMEM and incubated in serum-free medium (DMEM/0.1% BSA) containing the various cytokines with or without DXM. Culture supernatants were harvested at the times indicated and stored at −20°C for subsequent determination of angiogenin concentrations. The cells were trypsinized, counted and assessed for viability by trypan blue exclusion. Angiogenin protein concentrations were normalized to cell number and all experiments were performed in triplicate.

Inhibition RIA. Angiogenin concentrations were determined by an inhibition RIA (22) with modifications. Samples as well as dilutions of a human angiogenin standard in DMEM/0.1% BSA (60 μl/well) were placed into 96-well tissue culture plates (Corning Costar). Iodinated angiogenin (60,000 cpm/10 μl in PBS/0.25% protamine sulfate; 60 μl/well) followed by rabbit anti-human angiogenin antiserum prepared in the Center (1:20,000 dilution in PBS/0.25% protamine sulfate; 120 μl/well) were then added to the standards and samples. After a 2 hr incubation at room temperature, a 90 μl aliquot from each well was transferred in duplicate to a 96-well RIA plate (Dynatech) precoated and blocked with affinity purified goat anti-rabbit IgG (100 μl/well at 10 μg/ml; Organon Teknika) and PBS/0.5% protamine sulfate (150 μl/well), respectively. Following an overnight incubation at room temperature, the wells were washed with PBS/0.05% Tween-20, cut out and counted in an ICN Micromedic 4/600 gamma counter. The data were analyzed by four parameter logistics using the RIA.AID program (Robert Maciel, Inc.).

Metabolic labeling and immunoprecipitation of angiogenin. Angiogenin and fibrinogen, as a positive control, were biosynthetically labeled and immunoprecipitated according to the method of Perlmutter *et al.* (23) with modifications. HepG2 cells were grown to confluence and washed with DMEM. IL-6 (500 units/ml) was then added to appropriate wells in DMEM/0.1% BSA. After a 24 hr incubation, plates were washed first with Hanks' buffered salt solution and then with methionine-free DMEM/0.1% BSA. Subsequently, monolayers were pulsed for 4 hr with L-[^{35}S]methionine in methionine-deficient medium (100 μCi/ml) and washed with methionine-containing DMEM/0.1% BSA and PBS. Cells were then solubilized in PBS containing 1% Triton X-100, 0.5% deoxycholate, 10 mM EDTA and 2 mM PMSF and subjected to two freeze–thaw cycles. Cell lysates were centrifuged for 2 hr at 40,000 x g and stored at −70°C. For immunoprecipitation, 10 μg of the mouse anti-human angiogenin monoclonal antibody 26-2F (22) or 20 μg of the IgG fraction of goat anti-human fibrinogen (Organon Teknika) in 100 μl of 1% Triton X-100, 0.5% SDS, 5 mg/ml BSA and 1 mM PMSF was added to 100 μl of cell lysate and incubated overnight at 4°C. Formalin-fixed *S. aureus* (100 μl) was then added to each sample. After 1 hr at 4°C the immunoprecipitate was recovered by centrifugation, washed with PBS, boiled for 5 min in sample buffer and subjected to SDS/10% PAGE under reducing conditions. Following electrophoresis, the gel was fixed, impregnated for 15 min with Enlightening (Dupont) and dried for fluorography on Kodak Xomat-AR film.

Northern blot analysis and half-life determinations. Confluent HepG2 cultures were washed extensively with DMEM and incubated overnight in DMEM/0.1% BSA. Plates were washed again with DMEM and fresh DMEM/0.1% BSA containing the mediators to be tested was added to individual wells for the times indicated. Total cellular RNA was isolated from HepG2 cells by acid guanidinium thiocyanate/phenol/chloroform extraction (24). RNA was separated by electrophoresis, transferred onto nylon membranes and hybridized as described (16). A 511 bp DNA probe specific for human angiogenin was used (25) which was labeled by the random hexamer method using [α-^{32}P]dCTP and the Multiprime DNA Labeling System (Amersham). Following exposure to Kodak Xomat-AR film at −70°C, relative hybridization intensities were determined using a scanning densitometer (E-C Apparatus Corp.) equipped with a Waters 740 Data Module (Millipore). As a control for equal loading,

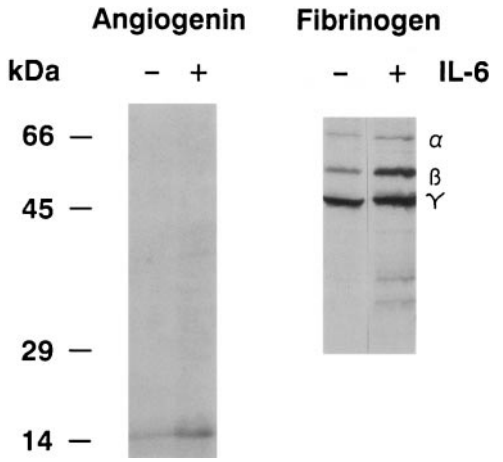

FIG. 1. Effect of IL-6 on accumulation of metabolically labeled angiogenin. HepG2 cells were incubated for 24 hr in the presence or absence of IL-6 (500 units/ml), then pulse labeled for 4 hr with L-[^{35}S]methionine. Angiogenin and fibrinogen, as a positive control, were immunoprecipitated and visualized by fluorography as described in "Materials and Methods." In addition to angiogenin, the synthesis of all three subunits of fibrinogen [α (66 kDa), β (52 kDa) and γ (46.5 kDa)] is induced by IL-6.

membranes were stripped and rehybridized with a human β-actin oligonucleotide probe (40-mer, synthesized by Dr. W. Dafeldecker) labeled with [γ-^{32}P]dATP using a 5′ DNA terminus labeling kit (Life Technologies).

To determine mRNA half-lives, cells were treated with mediators for 24 hr and then incubated with 0.5 mM novobiocin to arrest transcription. Total RNA was isolated after the indicated time intervals and transferred to nylon membranes using a Minifold II Slot-blotter (Schleicher & Schuell) according to Sambrook *et al.* (26). Hybridization and development of the slot blots were as described above for Northern blots.

RESULTS

Effect of IL-6 on angiogenin protein synthesis. IL-6 modulates the synthesis of the broadest range of APPs and was, therefore, tested for its capacity to regulate angiogenin synthesis in HepG2 cells. Human IL-6 induces a dose-dependent increase in the rate of angiogenin secreted, causing a ~2-fold stimulation above basal levels (22 ± 2 ng/10^6 cells/48 hr) at concentrations of IL-6 from 500 to 2000 units/ml (data not shown). Immunoprecipitable, metabolically labeled [^{35}S]angiogenin protein accumulates in IL-6-treated cells demonstrating that the cytokine increases its *de novo* synthesis as well as that of fibrinogen, a known APP (Fig. 1). DXM in combination with IL-1, TNF-α or IL-6 maximally stimulates the expression of many APPs *in vitro* and *in vivo* (27). Likewise, DXM (1 μM) enhances the IL-6 induction of angiogenin secretion to levels greater than 3-fold above baseline, while treatment of HepG2 cells with DXM alone does not affect constitutive angiogenin synthesis (Table I).

The time course for secretion of angiogenin in the presence and absence of IL-6 is shown in Fig. 2. Increased concentrations of angiogenin are evident as early as 12 hr following IL-6 stimulation, with the difference in concentrations between treated and untreated cells reaching a maximum after 24 hr. The rate of secretion is 2.5-fold higher during the first 24 hr

TABLE I

Secretion of Angiogenin in Response to Various Cytokines

Cytokine	Concentration[a]	Angiogenin secreted[b] (% control)
IL-1	0.1	108 ± 10
	1	89 ± 8
	10	86 ± 6
	100	60 ± 14
	1000	71 ± 10
DXM	1 μM	99 ± 4
IL-6	500	217 ± 33
IL-6 + DXM	500 + 1 μM	323 ± 10
IL-6 + IL-1	500 + 1000	116 ± 14
TNF-α	1	106 ± 6
	10	95 ± 10
	100	106 ± 6
	500	98 ± 14
IFN-γ	10	99 ± 17
	100	107 ± 27
	1000	125 ± 19
	10000	114 ± 13
TGF-β	5 ng/ml	111 ± 10
	200 ng/ml	108 ± 11

Note. Confluent HepG2 cultures in triplicate were incubated in the absence or presence of various amounts of cytokines in DMEM/0.1% BSA, added either individually or in combination. After 48 hr, supernatants were harvested and assayed for angiogenin by RIA.

[a] Units/ml unless otherwise specified.

[b] Angiogenin concentrations were normalized to HepG2 cell number and expressed as a percentage of angiogenin secreted by uninduced cells ± S.D.

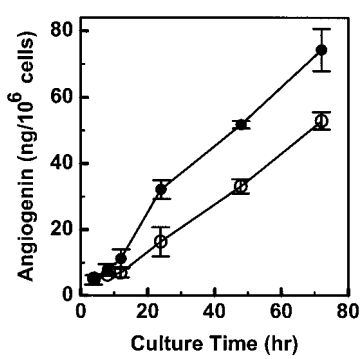

FIG. 2. Time course of angiogenin secretion in response to IL-6. HepG2 cells in triplicate were incubated in the presence (●) or absence (○) of IL-6 (500 units/ml). Culture supernatants were harvested at the times indicated and the angiogenin concentrations were determined by RIA and normalized to cell number. Each time point represents the mean ± S.D. of three separate experiments.

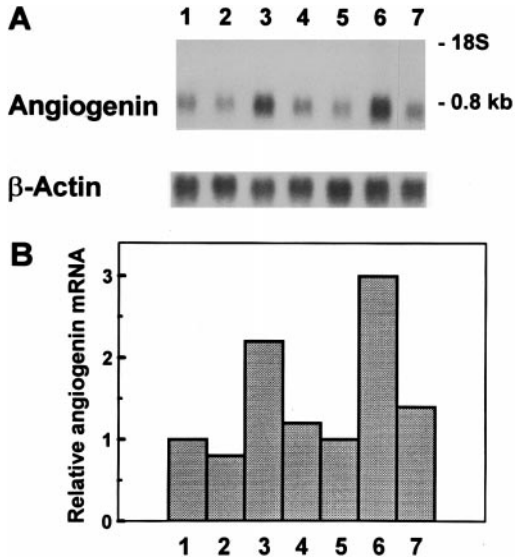

FIG. 3. Effect of IL-6, IL-1 and DXM on the accumulation of angiogenin mRNA. A Northern blot was prepared with RNA isolated from HepG2 cells treated for 12 hr with medium alone (lane 1), IL-1 (1000 units/ml) (lane 2), IL-6 (500 units/ml) (lane 3), IL-6 + IL-1 (lane 4), IL-1 + DXM (1 μM) (lane 5), IL-6 + DXM (lane 6) or IL-6 + IL-1 + DXM (lane 7). The autoradiogram of the blot, hybridized with angiogenin and β-actin probes, is shown (*A*). In (*B*) the angiogenin signals from the autoradiogram were quantitated by scanning densitometry and expressed relative to the signal measured for medium alone (lane 1). Similar results were obtained in five separate experiments. DXM did not affect basal angiogenin mRNA synthesis (data not shown).

following IL-6 stimulation compared to the rate for unstimulated cells (1.42 versus 0.58 ng/10^6 cells/hr, respectively). After 24 hr, the rates are comparable for both IL-6 treated and untreated cells (0.88 and 0.76 ng/10^6 cells/hr, respectively). Thus, IL-6 induces a transient increase in angiogenin secretion during the first 24 hr following stimulation.

Effects of IL-1 and other cytokines on angiogenin protein secretion. At several concentrations TNF-α, IFN-γ and TGF-β1 do not affect angiogenin secretion appreciably (Table I). IL-1, however, downregulates the basal secretion of angiogenin by ~30% in a concentration-dependent manner. Moreover, IL-1 virtually abolishes the IL-6 induction of angiogenin secretion (Table I). These inhibitory effects of IL-1 were not caused by a decrease in cell viability, which was monitored by trypan blue exclusion.

Effects of IL-6, IL-1 and DXM on angiogenin mRNA. A single 0.8 kb angiogenin mRNA species is observed in unstimulated and IL-6-treated HepG2 cells by Northern blot analysis (Fig. 3). As is the case for stimulation of angiogenin protein, IL-6 at 500 units/ml induces a ~2-fold increase in the steady-state level of angiogenin mRNA above unstimulated amounts. This IL-6 induction of angiogenin mRNA is prevented by CHX (data not shown), indicating that ongoing protein synthesis is required. DXM alone has little or no effect on the amount of angiogenin mRNA, but together with IL-6 causes a greater than 3-fold increase over the uninduced state (Fig. 3). Angiogenin mRNA induction by IL-6 is evident at 4 hr post stimulation, reaches a maximum after 12 hr and returns to basal levels by 48 hr (Fig. 4).

Since IL-1 depresses both basal and IL-6-induced angiogenin protein secretion (Table I), its effect on angiogenin mRNA was examined. Following a 12 hr treatment with IL-1 alone, there is a slight yet reproducible decrease in mRNA accumulation compared to untreated cells (Fig. 3). However, IL-1 essentially eliminates the IL-6 ± DXM-induced increases in angiogenin mRNA. Thus, the IL-1 antagonistic activity on both constitutive and IL-6-stimulated angiogenin expression is regulated at the pretranslational level. Unlike the IL-6 stimulatory effect, inhibition of IL-1 on basal and IL-6-induced angiogenin mRNA levels are unaffected by CHX treatment (data not shown) and hence not dependent on new protein synthesis.

Angiogenin mRNA half-life determinations. As both IL-6 and IL-1 appear to regulate angiogenin ex-

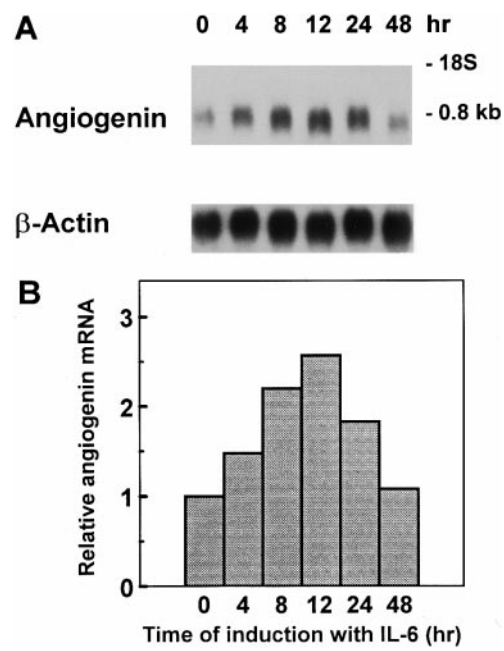

FIG. 4. Time course of accumulation of angiogenin mRNA in response to IL-6. A Northern blot was prepared with total RNA isolated from HepG2 cells treated for the designated times with IL-6 (500 units/ml). The autoradiogram of the blot, hybridized with angiogenin and β-actin probes, is shown (*A*). In (*B*) the angiogenin signals from the autoradiogram were measured by scanning densitometry and expressed relative to the signal from uninduced cells (lane 1). Similar results were obtained in three separate experiments.

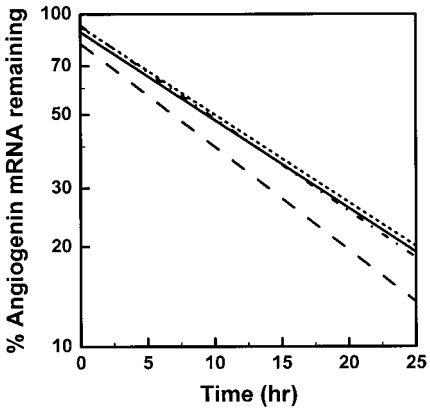

FIG. 5. Effects of IL-6 and IL-1 on the half-life of angiogenin mRNA. HepG2 cells were first treated for 24 hr with IL-6 (500 units/ml, – – –), IL-1 (1000 units/ml, — —), IL-6 + IL-1 (——) or medium alone (– — –). Cells were then incubated with novobiocin (0.5 mM). Total RNA was isolated at 0, 1, 3, 6, 12 and 24 hr after novobiocin addition and slot blotted as described in "Materials and Methods." The angiogenin signals were measured by scanning densitometry and expressed as a percentage of the signal at the time of novobiocin addition (0 hr). Lines derived from linear regression analysis of the data points (representing the mean from at least two separate experiments) are shown. Half-lives are defined as the times at which 50% of the mRNA remain.

pression pretranslationally, changes in the stability of mRNA, which may contribute to the effects observed, were examined. The rate of degradation of angiogenin mRNA was determined after treatment of cells with different cytokines followed by the addition of the transcription inhibitor novobiocin (28). Novobiocin was used at a concentration of 0.5 mM which inhibits at least 80% of the ^{3}H-uridine incorporation by HepG2 cells over 24 hr without decreasing cell viability (data not shown). The degradation of angiogenin mRNA was followed for 24 hr after novobiocin treatment (Fig. 5). The half-lives for mRNA from unstimulated, IL-6-treated- and IL-6 + IL-1-treated cells are all similar: 9.7 hr, 10.1 hr and 9.3 hr, respectively. Therefore, neither the IL-6-induced mRNA accumulation nor the IL-1 inhibitory effect on IL-6 activity can be attributed to changes in mRNA stability. However, IL-1 treatment increases mRNA degradation ($t_{½}$ = 6.7 hr) and this may contribute to the decreases observed in protein and mRNA expression caused by IL-1 alone.

DISCUSSION

The fluctuations in serum angiogenin levels that occur in hospitalized patients as compared to their normal counterparts led us to consider whether angiogenin could belong to the group of liver-derived proteins whose synthesis is affected during the host response to infection or injury. These so-called APPs function to contain and eliminate invading pathogens, repair tissue damage and in general return the host to its normal physiologic state (17). To test whether angiogenin could be regulated in a manner inherent to APPs, we performed initial *in vivo* experiments in mice (16). Following injection of thioglycollate, mice entered into acute inflammation as reflected in the rapid increase and subsequent fall in levels of serum amyloid A component, the major APP of mice. Concurrently, serum angiogenin protein concentrations transiently rose as a consequence of upregulation of angiogenin mRNA in mouse liver. This ~3-fold induction in serum levels of angiogenin is typically observed for a subset of APPs that includes fibrinogen, haptoglobin and α_1-acid glycoprotein (29).

Different cytokines, alone and in combination, stimulate the expression of APPs both *in vivo* and *in vitro*, which serves to subdivide these proteins into two classes (18). Class 1 APPs are induced mainly by IL-1 and combinations of IL-1 with IL-6 and glucocorticoids. In contrast, the set of proteins in class 2 is primarily up-regulated by IL-6-type cytokines alone and in combination with glucocorticoids. In the study described here, IL-6 stimulates a ~2-fold increase in the synthesis of angiogenin protein in HepG2 cells, while DXM in combination with IL-6 induces a greater than 3-fold increase in angiogenin production above basal levels. Likewise, IL-6 increases the production of the class 2 APPs fibrinogen and α_1-antichymotrypsin 2.5- and 5-fold, respectively, in HepG2 cells (30). Following IL-6 stimulation of HepG2 cells, the rate of angiogenin secretion increases 2.5-fold during the first 24 hr but returns to uninduced levels thereafter, indicating that IL-6 elicits early, transient events. Furthermore, IL-6 most likely regulates angiogenin synthesis pretranslationally since angiogenin mRNA accumulation increases following stimulation, with elevations clearly evident 4 hr after induction.

The effects of cytokines on APP expression are mediated primarily at the level of transcription (31,32). Following binding to a membrane receptor, IL-6-type cytokines transduce signals from the cell surface to the nucleus via two primary pathways. One involves phosphorylation and activation of nuclear factor NF-IL6, a member of the CCAAT/enhancer binding protein (C/EBP) family, by Ras-dependent MAP kinases. In the second pathway Janus kinases phosphorylate the signal transducer and activator of transcription (STAT3) which subsequently translocates to the nucleus (33). There it forms a transcription complex with IL-6 responsive elements (REs), a process inhibitable by CHX and thus dependent on new protein synthesis (34). Similarly, as noted above, CHX inhibits the IL-6-induced accumulation of angiogenin mRNA.

Gene activation by each of these signaling pathways is mediated by two specific types of IL-6-dependent REs, which have been identified within the promoter

regions of APP genes (35). Type 1 IL-6REs, recognized by C/EBP-like factors, contain the consensus sequence 5′-T(T/G)NNGNAA(T/G)-3′ present in genes of both class 1 and 2 APPs (33). The sequence 5′-TTTTGTAAT-3′ in the angiogenin gene beginning at nucleotide -188 relative to the transcription start site (36) matches this consensus sequence exactly. Additionally, in the human hemopexin gene a C/EBP-like nuclear factor binds to the type 1 IL-6RE sequence 5′-GTGATGTAAT-3′ (37), where the guanine residues underlined represent nucleotides essential for DNA sequence recognition. A similar sequence beginning at nucleotide -61 (5′-GTGAGGTTAAT-3′), in which the essential G nucleotides are conserved, is present in the angiogenin promoter region. A separate sequence motif 5′-CTGGGA-3′, recognized by STAT3 and also present in both classes of APP genes, characterizes type 2 IL-6REs (35). Two identical sequences beginning at nucleotides -337 and -470 are also present in the angiogenin gene.

Glucocorticoid responsiveness is mediated through the consensus sequence 5′-GGTACANNNTGTTCT-3′ (38). This sequence is not present in the promoter region of the angiogenin gene even though DXM potentiates IL-6-stimulated angiogenin production. However, several regions both upstream and downstream from the angiogenin promoter contain sequences resembling this motif, such as 5′-GGTGATGCTGTTCT-3′ at nucleotide +48. These may serve as enhancer elements for glucocorticoids. Even if these aforementioned sequences are not functional binding sites, potentiation by glucocorticoids of IL-6-mediated events is still possible because of the known upregulation of IL-6 receptor subunit mRNA by DXM in HepG2 cells (39).

Combinations of two or more cytokines result in different effects on the expression of individual APPs, thus providing the basis for specific regulation of particular APP responses (30). IL-1 dramatically antagonizes the increased expression of angiogenin mRNA and protein induced by IL-6, although IL-1 alone depresses basal angiogenin levels only slightly. Similar inhibition of basal and IL-6-induced protein and mRNA synthesis by IL-1 has been reported for human fibrinogen and C1 inhibitor, and typifies regulation of class 2 APPs (30, 40). IL-1 increases the rate of degradation of angiogenin mRNA, which may account in part for the suppression of basal angiogenin production. In contrast, IL-1 in combination with IL-6 does not affect the half-life of angiogenin mRNA, implying that inhibition is regulated at the transcriptional level. IL-1 may induce a repressor that interferes with the binding of a transcription complex to an IL-6RE or, alternatively, an IL-1-induced repressor may bind to a gene-specific negative element or silencer DNA sequence (41). Recently, a splice variant of STAT3 was cloned from an eosinophil cDNA library that lacks an internal domain of 50 bp near the C-terminus and acts as a negative transcriptional regulator (42). This variant, STAT3β, is tyrosine phosphorylated and binds as a homo- or heterodimer together with STAT3 to the IL-6/IFNγRE in the intercellular adhesion molecule 1 (ICAM-1) promoter. The STAT3/STAT3β heterodimer, however, fails to transcriptionally activate the ICAM-1 promoter. Furthermore, the IL-1- dependent inhibitory activity is not suppressed by CHX, analogous to what is observed with angiogenin, suggesting that a putative latent repressor may preexist and may be activated by IL-1 posttranslationally.

Other cytokines known to modulate APP expression such as TNF-α, INF-γ and TGF-β do not appear to affect angiogenin protein production in HepG2 cells. It is possible, however, that they may regulate angiogenin in other types of cultured cells or in an *in vivo* setting. The effects of other members of the IL-6 family of cytokines, e.g., leukemia inhibitory factor, IL-11, oncostatin M, ciliary neurotrophic factor and cardiotrophin (19), on angiogenin synthesis have not as yet been examined. However, they would be expected to also induce angiogenin production since all share with IL-6 a common receptor (43), which, when activated, leads to similar effects on APP expression (44).

To summarize, angiogenin is regulated *in vitro* by mechanisms common for many APPs with a pattern of responsiveness reminiscent of class 2 proteins. Angiogenin also contains 5′ recognition sequences that characterize both type 1 and type 2 IL-6REs, although functionality of these regions remains to be determined. These observations, coupled with the *in vivo* data presented previously (16), suggest that angiogenin plays a role in physiologic responses to trauma or infection. Indeed, tissue repair is critically dependent upon the ingrowth of new capillaries into an injured site (1). These vessels supply the nutrients and appropriate auxiliary cells central to the healing and tissue remodeling which accompanies acute phase responses. Thus, APPs that promote angiogenesis are expected to be early and important participants in these processes. As an example, fibrin, a cleavage product of the APP fibrinogen, provides an insoluble matrix that supports the growth of vascular and other cell types leading to angiogenesis and eventual synthesis of mature connective tissue (45). Additionally, haptoglobin, which displays many functions, is also capable of inducing angiogenesis (46). Angiogenin can now be added to this subgroup of angiogenesis-stimulating APPs. It therefore may not be coincidental that all three of these proteins are representative of class 2 APPs, inducible by IL-6, whose serum concentrations rapidly rise by 2- to 4-fold subsequent to tissue injury (16, 29). Several prior studies have indeed indicated that IL-6 can promote angiogenesis (47). More recently, IL-6 was shown

to stimulate the expression of vascular endothelial growth factor by cells in culture, suggesting that IL-6 may function as an indirect angiogenic factor (48). The capacity of IL-6 to also induce the synthesis of haptoglobin (49) and angiogenin, as this report demonstrates, lends further support to this contention.

In conclusion, a role for angiogenin in acute inflammation and wound healing is proposed based on both *in vivo* and *in vitro* studies. As such it may contribute to the angiogenesis-related events inherent to these processes and could be of potential use as a monitor of the acute phase reaction.

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