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ITIH4 Serum Concentration Increases during Acute-Phase Processes in Human Patients and Is Up-Regulated by Interleukin-6 in Hepatocarcinoma HepG2 Cells

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The serum concentration of the inter-α trypsin inhibitor heavy chain 4 protein (ITIH4) increases (from 1.4–3 times) in male patients suffering of different acute-phase processes (myocardial infarction, unstable angina or programmed surgery). The concentration of C-reactive protein (CRP) in these samples ranged from 15 μg/ml to 133 μg/ml. Using the hepatocarcinoma HepG2 cell line we have observed up-regulation of ITIH4 mRNA expression upon dose-response treatments with interleukin-6 (IL-6). This effect correlates with the increase of radiolabeled ITIH4 in the cellular media of ^{35}S-labeled HepG2 cells treated with the cytokine. A similar effect was observed for haptoglobin mRNA, used as a control for acute-phase protein expression. IL-1β, although up-regulating the expression of α_1-acid glycoprotein in these cells, did not induce any effect in the expression of ITIH4. No changes were observed after TNF-α treatments. The results presented here indicate that ITIH4 is a type II acute-phase protein in humans. © 1999 Academic Press

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In previous studies from our laboratory [1, 2] it was observed a new plasma protein in pigs under acute inflammation. This protein was denominated Major Acute-phase Protein (Pig-MAP) and its concentration may increase more than 30 times after turpentine injection or under bacterial infection [2, 3]. A new human protein of 120k relative molecular mass denominated serum glycoprotein 120 (sgp120) [4], plasma kallikrein-sensitive glycoprotein (PK-120) [5] or inter-α-trypsin inhibitor (IαI) family heavy chain human related protein (IHRP) [6], was isolated and cloned. Pig-MAP and this human protein are homologous and new members of the IαI heavy chain family. Recently, it has been proposed inter-α-trypsin inhibitor heavy chain 4 (ITIH4) to designate this protein [7].

The IαI family in mammals refers to a group of related plasma protease inhibitors. These proteins are comprised of various multi-polypeptide molecules showing different assemblies from a group of four distinct polypeptides including three related heavy (H) chains H1, H2 and H3, and a light chain called bikunin. Two Kunitz-type protease inhibitor domains are present in the bikunin chain which are bound to the heavy chains through glycosaminoglycan bridges [7, 8, 9]. The functions of IαI family members are still unclear, although some of them appear to be involved in stabilization of extracellular matrix [8, 9] by the property of the heavy chains to bind hyaluronan [9, 10, 11].

The *ITIH4* gene has been mapped to homologous locations on human and mouse chromosomes [12, 13, 14]. The hepatic *ITIH4* cDNA has been also cloned in pigs [15] and in the rat [16]. The two thirds from the N-terminus end of the protein exhibit a high sequence similarity with the H1, H2 and H3 polypeptides, whereas the sequence of the C-terminus third of H4 differs from the sequence of the other three polypeptides. In this C-terminal region H4 lacks the signal sequence for bikunin assembling through the glycosaminoglycan bridges [5, 6].

Since ITIH4 is a strong acute phase protein (APP) in pig [1, 2, 3] and in the rat [2, 16, 17] it would be of relevance to investigate if this protein also behaves as an APP in humans. Although, no changes in the human ITIH4 mRNA level were observed in hepatic bi-

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TABLE 1

Concentration of ITIH4 in Male Patients Suffering of Acute-Phase Processes ((1) Unstable Angina, (2) Myocardial Infarction, or (3) Programmed Surgery)

		ITIH4 (mg/ml)			CRP (μg/ml)	
#	Age	Hospital arrival	Acute phase	Concentration increase (N° times)	Hospital arrival	Acute phase
1	64	0.57	0.97	1.7	<3	115
1	58	0.36	0.60	1.7	<3	34
1	58	0.54	0.79	1.5	<3	39
2	71	0.43	0.63	1.5	<3	15
2	71	0.54	1.02	1.9	7	133
2	72	0.48	0.90	1.9	<3	35
2	70	0.30	0.91	3.0	<3	55
3	36	0.31	0.43	1.4	<3	74
3	59	0.34	0.52	1.5	15	85

Note. Acute-phase sera were collected after 48–72 h in patients of groups 1 and 2, or 4 days after surgery in patients of group 3.

opsies from some patients with moderate acute phase [16], in the present work we demonstrated that: (i) the serum concentration of ITIH4 increases up to 3-times in acute phase patients, and (ii) the mRNA of ITIH4 was up-regulated by human interleukin-6 (IL-6) in the hepatocarcinoma HepG2 cells.

MATERIALS AND METHODS

Cell culture and treatment with cytokines. HepG2 cells, seeded in 40 mm of diameter Petri dishes, were grown to subconfluence in DMEM suplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under 5% CO_2 at 37°C. Then, cells (10^6/dish) were incubated four different times with fresh medium, containing 1 μM dexamethasone and different amounts of recombinant human cytokines (National Institute for Biological Standards and Control, Hertfordshire, U.K.).

RT-PCR analysis. Total RNA from HepG2 cell cultures was purified by one step method using *Ultraspec-II RNA* isolation system (Bioteck, Houston, Texas, USA). Specific mRNA were then analysed by reverse transcription (RT) coupled to PCR amplification (RT-PCR) [18]. RT was carried out at 37°C for 1 h using 5 μg of total RNA samples and *Ready to go* T-primed first strand kit (Pharmacia Biotech, Barcelona, Spain). PCR was performed using the following custom synthesised specific primers (Pharmacia Biotech): 5′GTGACTGGCCAGTATGAGAG 3′ (5′upstream, covering nt 2434–2453) and 5′CTCTGGTGGCAGAGTGGTC 3′ (3′downstream, covering nt 2836–2854) for amplification of a 421 bp fragment of PK-120 (ITIH4) cDNA, according to [5]; 5′GTCATCCATGACAACTTTGG 3′ (5′ upstream, covering nt 520–539) and 5′CCACCTTCTTGATGTCATC 3′ (3′downstream, covering nt 799–817) for the glyceraldehyde-3-phosphate deshydrogenase (GAPDH) cDNA fragment (298 bp) [19]; and 5′CCTTCTGTGCTGGCATGTC 3′ (5′upstream, covering nt 1039–1057) and 5′CAGCTATGGTCTTCTGAACC 3′ (3′downstream, covering nt 1219–1239) for the beta chain haptoglobin cDNA fragment (201 bp) [20]. The reaction mixture (50 μl) contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 pH 8.8, 0.2 mM of each dNTP, 0.5 μM of the 5′ upstream and 3′ downstream primers, one unit of *Dynazyme* II DNA polymerase (Finnzymes Oy, Espoo, Finland) and the cDNA corresponding to 40 ng of total RNA in the case of ITIH4 and 2 ng of RNA for the amplification of haptoglobin. GAPDH, used as a control, was amplified in the same cDNA samples. Amplification consisted in 34 cycles (Perkin Elmer Cetus DNA thermal cycler) of a denaturalisation step at 95°C for 1 min followed by annealing of primers for 1 min at 62°C for the ITIH4, or 64°C for the haptoglobin cDNA fragment, respectively, and chain extension at 72°C for 1 min. Finally, after an additional extension step at 72°C for 10 min, PCR products were analyzed by ethidium bromide (EtBr)/2% low melting point agarose gel electrophoresis [21]. In some experiments, these PCR amplified ITIH4 and haptoglobin cDNAs fragments were extracted from gels by a phenol-chloroform procedure [21] and sequenced in both strands, with the specific primers used for PCR amplification.

Antibodies to human ITIH4. A preparation of human ITIH4 [2] was used as immunogen to raise antibodies in rabbits. Specific antibodies against ITIH4 were isolated by affinity chromatography using glutaraldehyde-insolubilized pooled human sera as immunoadsorbent [2].

^{35}S-labeling of HepG2 cells and ITIH4 immunoprecipitation. HepG2 cells (10^6/dish) were incubated in a methionine- and cysteine-free medium containing 30 μCi (1 ml/dish) of *PRO-MIX [^{35}S] in vitro* cell labeling mix (Amersham, Madrid), for 24 h with 500 U/ml of cytokines and 1 μM dexamethasone. The culture media were precleared by incubating overnight at 4°C with 50 μl/sample of protein A-Sepharose beads (Sigma, Madrid), and then the supernatants immunoprecipitated with antibodies to ITIH4 (30 μg/sample) and protein A-Sepharose beads (50 μl/sample), as previously described [22]. The precipitates were electroblotted to nitrocellulose membranes at 20 V, for 1 h in a semi-dry transfer cell (Bio-Rad) and then autoradiographyed in hiperfilm-β-MAX (Amersham). Human serum was used as a control in ITIH4 immunoprecipitation and the protein was detected by Western-blot using specific antibodies and second alkaline phosphatase-conjugated sheep anti rabbit IgG [2].

ELISA for ITIH4 determinations. The concentration of ITIH4 in human sera was measured with a sandwich-type ELISA. Two different preparations of antibodies were used: (i) high affinity antibodies (eluted from immunoadsorbent with a glycine pH 2.8 buffer [2] containing 10% dioxane) as capture antibodies, and (ii) antibodies of lower affinity (isolated only with the pH 2.8 glycine buffer [2]), labeled with horseradish peroxidase (HP) [23], to detect the immunocomplexes. Briefly, microtitre polyestirene plates were coated with 100 μl/well of a PBS solution containing 10 μg/ml of high affinity antibodies. After overnight incubation, wells were incubated with 300 μl of PBS containing 3% (w/v) skimmed milk. After washing, 100 μl of samples (human sera, 1:1000 dilution) were added and the plate was incubated for 45 min at room temperature. Wells were washed with 0.05% Tween-20 in PBS and then 100 μl of 2 μg/ml of anti ITIH4 antibodies HP-labeled (in PBS containing 0.1% skimmed milk) were added. The plate was incubated for 30 minutes, and after

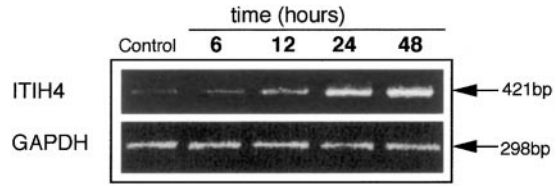

FIG. 1. Time-course analysis by RT-PCR of ITIH4 mRNA expression in response to rh IL-6. HepG2 cells were incubated with 500 U/ml rh IL-6 for 0, 6 h, 12 h, 24 h, and 48 h. Two cDNA fragments corresponding to ITIH4 (421 bp) and GAPDH (298 bp) were amplified in the same samples.

washing, the peroxidase activity was revealed with K-Blue substrate (Neogen Corporation, Lexington, KY). The reaction was stopped with 1 M H_2SO_4 and absorbance read at λ450 nm.

A pool of different human plasma from healthy individuals was used as secondary standard. The concentration of the protein in this standard (0.3 mg/ml) was determined using ITIH4 previously purified and quantified in the laboratory [2].

Sera taken routinely from patients suffering of acute myocardial infart, unstable angina or programmed surgery were obtained from the University Hospital "Lozano Blesa" (Zaragoza, Spain).

Immunochemical determination of haptoglobin, α_1-acid glycoprotein and C-reactive protein (CRP). Haptoglobin and α_1-acid glycoprotein were quantified in the culture media by rocket immunoelectrophoresis using specific antisera (Sigma). CRP was determined by nephelometry using a BNAII Behring nephelometer, in the conditions suggested by the manufacturers (Behring, Marburg, Germany).

RESULTS

ITIH4 increases in the sera of patients with acute phase processes. We have determined ITIH4 in sera from nine male patients suffering of unstable angina, myocardial damage or programmed surgery, at the hospital arrival and some days after (Table 1). All of them shown important increases in the plasma concentration of CRP, indicating acute phase processes. The concentration of ITIH4 also increases during the same period. This increase ranged from 1.4 to 3-times the values at the hospital arrival.

ITIH4 mRNA expression in HepG2 cells. Using designed primers, a unique cDNA fragment of 421 bp was amplified by RT-PCR in HepG2 cells (Fig. 1). This fragment shown a sequence identical to the 3′ terminal region of PK-120 (ITIH4) cDNA (nt 2434–2854) [5] (data not shown). Treatment of HepG2 cells with an optimal doses of rh IL-6 (500 U/ml, see below) induced a remarkable increase of the ITIH4 mRNA levels which reached a maximal plateau at 24–48 h (Fig.1). In the same samples, no significant changes in the expression of GAPDH mRNA were detected. A clear dose-response induction in the expresion of the ITIH4 mRNA was observed when cells were incubated for 24 h with different amounts of rh IL-6 (from 0 to 500 U/ml) (Fig. 2). It is well known that human haptoglobin is an IL-6 dependent acute phase protein [24, 25, 26]. In samples of the same total RNA preparations, we observed increase for human haptoglobin mRNA after IL-6 treatment, as indicated by the 201 bp amplified fragment (Fig. 2). The sequence of this fragment corresponds to that published for human haptoglobin β chain cDNA (nt 1039–1239) [20] (data not shown).

IL-1β and TNF-α did not induced increase in the ITIH4 mRNA levels at any of the doses essayed (Fig. 3, upper and lower panels, respectively).

Haptoglobin, α_1-acid glycoprotein and ITIH4 secretion by HepG2 cells. Figure 4 (A and B panels) shows the doses dependent concentration of haptoglobin and α_1-acid glycoprotein secreted to the medium by HepG2 cells after 24 h treatments with rh IL-6 or IL-1β. At doses of 500 U/ml of rh IL-6 the concentration of haptoglobin in the medium greatly increased and reached values at least 7 times higher than in media from untreated cells. No increase in that concentration was observed in response to IL-1β (Fig. 4A). It has been described that α_1-acid glycoprotein expression is mainly up-regulated in human hepatic cells by IL-1 [27]. We observed that in the cells media the concentration of α_1-acid glycoprotein increase more than 4 times under IL-1 induction and around 2 times with IL-6 at doses of 500 U/ml for both cytokines (Fig. 4B).

Quantification in the medium of ITIH4 was not possible because the fetal calf serum contains high amounts of the homologous protein (unpublished results) which interfer in the conventional immunochemical analysis used. As an alternative, cells were labeled with ^{35}S-aminoacids, the protein in the media was immunoprecipitated with specific anti-ITIH4 antibodies, and the precipitates analysed by autoradiography. Figure 4C shows the effect of rh IL-6 at the optimal doses of the cytokine. Whereas in the medium of control cells (lane 1), the protein was barely detectable, a strong radioactive band was observed in the medium of cytokine treated cells (lane 2). This band shows an electrophoretic mobility identical to that of the native ITIH4 protein immunoprecipitated from a human serum sample, runned simultaneously in the same conditions and revealed by Western blot (Fig. 4C lane 3).

The results presented here indicated that IL-6 greatly up-regulates the expression of ITIH4 in HepG2

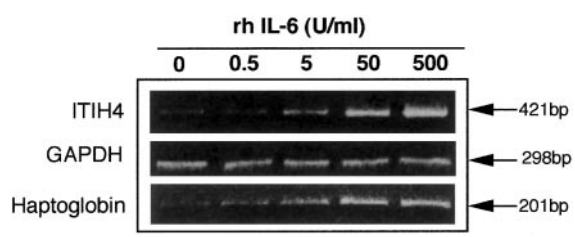

FIG. 2. Dose-response analysis by RT-PCR of ITIH4 and haptoglobin mRNA expression in response to rh IL-6. HepG2 cells were incubated for 24 h with rh IL-6 at 0, 0.5, 5, 50, and 500 U/ml. cDNA fragments for ITIH4 (421 bp), GAPDH (298 bp), and haptoglobin (201 bp) were amplified in the same samples.

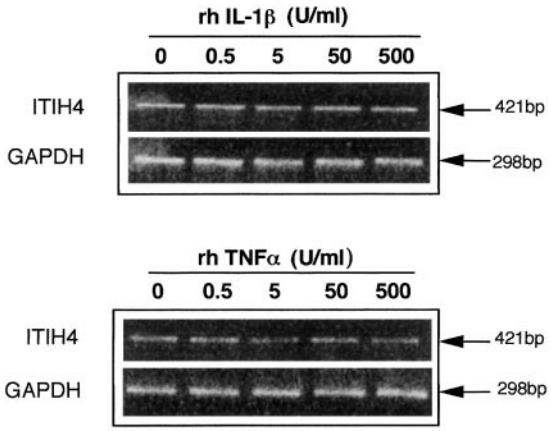

FIG. 3. Dose-response analysis by RT-PCR of ITIH4 mRNA expression in response to rh IL-1β and rh TNF-α. HepG2 cells were incubated for 24 h with rh IL-1β (upper panel) or rh TNF-α (lower panel) with 0, 0.5, 5, 50, and 500 U/ml of each cytokine. cDNA fragments for ITIH4 and GAPDH were amplified as in Fig. 1.

cells, as occurred with haptoglobin. Although IL-1 could regulate some APP genes in HepG2 cells, the expression of ITIH4 is independent of this cytokine, as well as of TNF-α.

DISCUSSION

The physiological function for the recently discovered plasma protein ITIH4 is not known. This protein was initially classified, under the name of sgp120, as a complement regulatory protein [4]. Because its sensitivity to kallikrein degradation, it was later considered as a novel kallikrein substrate being named as PK-120 [5]. Recently, endometrial gene expression of ITIH4 was detected during the oestrous cycle and early pregnancy in pigs [28]. For this, it has been suggested an ITIH4 protective role of the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium.

It is known that ITIH4 is an important APP in pigs [1, 2, 3] and rats [2, 16, 17], so it would be relevant to know if the expression of this protein can be regulated in humans during the acute phase response. In this work, we have analyzed the concentration of this protein in the serum of patients suffering from different acute phase processes. Increases in the serum levels of the protein (ranging from 1.4–3 times the initial values), were observed during the outcome of the disease indicating that ITIH4 is behaving as an APP in humans as occurred for CRP (Table 1). Myocardial damage due to anoxia causes cell necrosis, initiating a systemic inflammatory response, observable about 20 h after, with an increase of several positive APP [29]. A strong acute phase response is well documented in patients with unstable angina accompained with elevated levels of IL-6 [30, 31].

The increases in ITIH4 concentration after surgery confirm the role of the protein as APP. The intensity of the response of the human protein during the acute phase was lower than that observed in pigs with acute inflammation induced after small-bowel autotransplantation or turpentine injection, where we have observed increases of the protein up to 30 times the initial values [2]. However, the increases that human ITIH4 exhibited are in the same range than those reported for haptoglobin [25] In a previous study, Soury *et al.* [16] did not found increase in mRNA for ITIH4 in hepatic biopsies from some patients with only

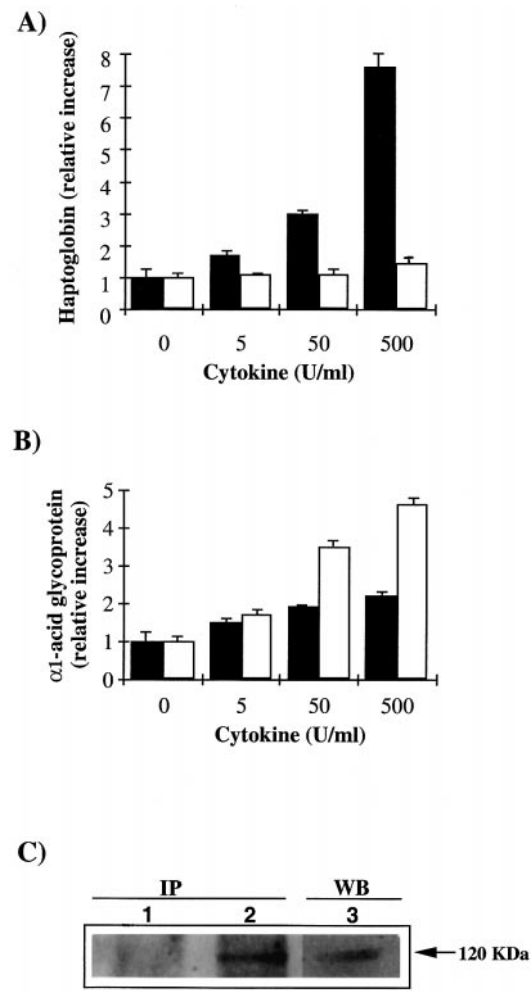

FIG. 4. Secretion of proteins by cytokine treated HepG2 cells. Haptoglobin (A) and α_1-acid glycoprotein (B) were quantified by rocket immunoelectrophoresis in the media from cells incubated 24 h with rh IL-6 (black) and rh IL-1β (white), at the indicated concentrations. Data, average $\pm$ S.D. from two independent experiments performed per triplicate, are expressed as relative increase respect to untreated cells. ITIH4 (C) was immunoprecipitated (IP) in culture media from ^{35}S-labeled HepG2 cells, untreated (lane 1) or treated with 500 U/ml of rh IL-6 (lane 2), and revealed by autoradiography after electroblotting. Lane 3 shows a parallel Western blot (WB) of immunoprecipitated ITIH4 from human serum. The arrow indicates the Mr of the protein.

moderated levels of CRP. Likely, the samples were taken before the induction of the ITIH4 expression would be significant.

HepG2 hepatocarcinoma cells have been previously used as an appropiate *in vitro* cell model for the study of the induction of APP [27, 32], as well as in the study of the expression of H1 to H3 members of the IαI family [33]. We have used the HepG2 cell line to study the expression of ITIH4 mRNA and the corresponding protein secretion in response to rh IL-6, rh IL-1β and rh TNF-α, main regulators of most APP genes [25, 26]. When these cells were treated with different cytokines, they properly responded to these stimuli. Thus, haptoglobin mRNA and the protein secreted were up-regulated in response to rh IL-6 treatments, whereas the secretion of α_1-acid glycoprotein increased mainly in response to rh IL-1β, as previously described [27, 32].

Using RT-PCR amplification, we have observed that HepG2 cells express a form of ITIH4 mRNA. The sequence of the 421 bp cDNA fragment obtained, showed total identity with the sequence previously reported for ITIH4 (data not shown) [5, 6]. Dose-response treatment with IL-6 exhibit a high increase in the expression of ITIH4 mRNA. This increase responded to a real induction by cytokines since in the same samples the GAPDH messenger was practically unaffected. The induction of the ITIH4 mRNA was accompained by an increase in the secretion of the mature protein in the cellular media (Fig. 4C). It has been described [16] that, in human, two forms of mRNA for ITIH4 could exist in the liver as result of the alternative splicing of exons 15 and 16 from a single copy *ITIH4* gene. In HepG2 cells, we have obtained only one amplified cDNA fragment (421 bp), localized in the 3′ terminal region which exhibits no significant homology with any IαI heavy chain cDNAs [5, 6]. This region was different for that amplified by Soury *et al.* [16].

The proteins of the IαI family are differentially regulated during the acute phase response. The H1, H2 and bikunin chains are down-regulated whereas the H3 chain is a positive APP in patients with acute inflammation and in the human hepatocarcinoma Hep 3B line after IL-6 treatment [34]. Increase in H1 and H3 mRNA expression together with a parallel decrease in the H2 messenger was also observed after IL-6 stimulation of HepG2 cells [33]. Our results shown that the expression of ITIH4 mRNA and the secretion of the protein greatly increase in response to rh IL-6 in HepG2 cells. From this and the *in vivo* results we can conclude that ITIH4 is a type II [35] acute phase protein produced by cells of hepatic origin.

Further studies using more controlled acute phase conditions will be performed in humans to gain more insight into the significance of this protein during the human acute phase response.

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