

C1 Esterase Inhibitor Gene Expression in Rat Kupffer Cells, Peritoneal Macrophages and Blood Monocytes: Modulation by Interferon γ

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Summary

Kupffer cells (KC) represent the main part of the tissue macrophages. Beside phagocytosis of particulate material, involvement of KC in immunological and inflammatory reactions has been supposed. As C1 esterase inhibitor (C1-INH) is a serine protease inhibitor involved in such processes, the aim of this work was to study C1-INH synthesis in KC and, by comparison, in peritoneal macrophages (PM) and blood monocytes (MC) of the rat. C1-INH synthesis was studied on the protein level by biosynthetic labeling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, and on the RNA level by Northern blotting of total RNA or by in situ hybridization. KC were found to express C1-INH gene spontaneously. C1-INH synthesis represents $1.3 \pm 0.2\%$ of total protein synthesis at day 1 of culture and the absolute amount each cell synthesis remains constant during the whole time in culture. Transcripts of C1-INH were detected both in freshly isolated and in cultured KC. In contrast, spontaneous C1-INH gene expression was not detectable in freshly isolated PM, but only in cultured PM. In MC, C1-INH was not detectable at any time, whatever. Treatment of the cells with interferon γ increased C1-INH synthesis in KC and in PM and caused an induction of C1-INH synthesis in MC. The results suggest that constitutive C1-INH synthesis is a functional marker for mature tissue macrophages.

Kupffer cells (KC)¹ account for about 10% of all cells of the liver and for about 80% of the tissue macrophages (1). Their main function is supposed to be phagocytosis of particulate material (2). KC, however, produce a variety of lysosomal enzymes and mediators (2, 3) and take part in detoxifications and metabolism of lipids and glycoproteins and probably in mediation of immune response.

C1 esterase inhibitor (C1-INH) is a single chain glycoprotein containing about 35% carbohydrates with a molecular mass of about 105 kD (human) (4). It belongs to the family of serine protease inhibitors (serpins) and was first identified as a member of the proteins of the classical pathway of the complement system, proving to be the only known inhib-

itor of activated C1r and C1s (5). Furthermore, C1-INH has been shown to inactivate kallikrein and the coagulation factors XIa, XIIa, and plasmin (6–8). The hepatocyte is thought to be the major source of plasma C1-INH (9). However, C1-INH is also produced by extrahepatic cells (10, 11). Contrary reports regard C1-INH synthesis by human monocytes (12, 13). In this work we demonstrate that isolated rat KC constitutively synthesize C1-INH, whereas peritoneal macrophages (PM) synthesize only trace amounts. In contrast, we found no constitutive synthesis in rat blood monocytes (MC). IFN- γ , an important mediator of inflammation, upregulates C1-INH synthesis in both macrophage populations and it induces C1-INH gene expression in MC.

Materials and Methods

Animals. We used Wistar rats (2-mo-old, body weight 150–170 g) kept under standard conditions with free access to food and water.

Reagents. Purified C1-INH from human plasma and C1-INH antiserum (tested for cross-reactivity with rat C1-INH) were purchased from Paesel & Lorei (Frankfurt, FRG); rat-IFN- γ was from Holland Biotechnology (Leiden, The Netherlands); anti-desmin (murine IgG1) was purchased from Dako, (Copenhagen, Denmark);

¹ Abbreviations used in this paper: C1-INH, C1 esterase inhibitor; KC, Kupffer cells; MC, blood monocyte; PM, peritoneal macrophage.

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anti- α -SM-actin (murine IgG2a) was from Sigma, (Munich, FRG); the murine mAbs ED1 (IgG1) and ED2 (IgG2a) were a gift of Dr. Christine D. Dijkstra, (Department of Histology, University of Amsterdam, The Netherlands).

Cell Isolation. KC were isolated according to Knook and Sleyster (14) as described elsewhere (15). Briefly, the liver was digested with pronase/collagenase solutions. Nonparenchymal cells were separated by density gradient centrifugation and the KC population was purified in a centrifuge (J2-21, JE-6B rotor; Beckman Instruments, Inc., Fullerton, CA) by counterflow elutriation. The obtained KC were resuspended in culture medium (M199, 15% FCS, 100 U penicillin/ml, 100 μ g streptomycin/ml) and plated out in 24-well plates (500,000 cells/well). After 2 h, the cultures were washed to eliminate nonadherent cells.

The peritoneal cavity of a rat was rinsed with 50 ml HBSS without Ca^{2+} and Mg^{2+} , the obtained cells were sedimentated, washed several times, and resuspended in culture medium (RPMI, 15% FCS, 100 U penicillin/ml, and 100 μ g streptomycin/ml). They were plated out (10^6 cells/well) and washed after 2 h.

Heparinized rat blood mixed with RPMI was laid on Histopaque 1077 (Sigma) and centrifuged at 1,000 *g* for 20 min. The PBMC were washed and resuspended in culture medium (same as for KC). One half of the cells was kept on ice, the other half was plated onto 24-well plates (10^6 cells/well). After 2 h, plated cells were washed and the ice-kept cells were seeded onto the same wells used for the first plating. After 2 h, cultures were washed again.

Rat hepatocytes were isolated according to Seglen (16) as described (17).

Cell Culture. Cells were kept in culture at 37°C in a 5% CO_2 /95% atmosphere and 100% humidity.

DNA Quantification. Freshly isolated cells were resuspended in PBS containing 0.5% Triton X-100. Cultured cells were detached from the plates with PBS/0.5% Triton X-100. DNA was precipitated with 7 M perchloric acid at 0°C, washed, resolved in 1 M perchloric acid at +70°C, and photometrically measured at 600 nm wave length after incubation with a colorimetric reagent (18, 19).

Immunocytochemistry. Cells were fixed with methanol (5 min) and acetone (10 s) at -20°C, washed with PBS containing 0.1% BSA, and covered with FCS (30 min). They were then incubated with the mAbs ED1 or ED2 (45 min) at 37°C, washed, and again incubated with peroxidase-labeled Ig against murine Ig. This antiserum was preabsorbed with rat serum to avoid nonspecific binding. Cells were washed and incubated with PBS containing diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01%) for 10 min, washed, and counterstained in Meyer's haemalaun. As control, irrelevant mAbs of the same Ig subclasses (against desmin or against α -smooth muscle actin) were used instead of the first antibody.

Biosynthetic Labeling, Immunoprecipitation, and SDS-PAGE. Biosynthetic labeling of newly synthesized proteins and immunoprecipitation were performed as described (20). SDS-PAGE under reducing conditions was performed according to the method of Laemmli (21) as described (20).

RNA Extraction and Northern Blot Analysis. RNA was extracted by the guanidine isothiocyanate method of Chirgwin et al. (22) and analyzed as previously described (20).

In Situ Hybridization. Preparation of cells, liver sections, RNA probes, and hybridization was performed according to standard methods (23). C1-INH-specific cDNA (24), in particular, was digested with PstI and EcoRI, a fragment of 211 bases was subcloned in Bluescript SK plasmid DNA (Stratagene, Heidelberg, FRG) and was transcribed into ^{35}S -labeled sense or antisense RNA probes. Equal amounts of counts were added to the cells or to the liver sections.

Results

Characterization of Isolated Cells

KC. Phase-contrast microscopy (Fig. 1 A) did not show the presence or growth of contaminating cells. Immunocytochemical studies gave negative results for albumin, desmin, and α -smooth muscle actin (data not shown) and all cells were positive when stained with the mAbs ED1 or ED2 (Fig. 1, B and C). DNA measurement revealed that nearly all plated cells adhered, whereas the amount of DNA in the cell cultures continuously decreased. In fact, at day 5, a 50% reduction of the amount of DNA as compared with day 1 was found. Total protein synthesis of KC was determined by TCA precipitation of the biosynthetically labeled proteins and β -ray counting. A twofold increase of the secreted proteins per unit of time was observed during culture (5 d). As the number of cells simultaneously decreased to about 50%, it can be concluded that the amount of the proteins secreted by one cell increased four times. These results were not affected by the density of the plated cells (2.5×10^5 , 5×10^5 , and 10^6 cells per well were tested).

PM and MC. PM were kept in culture up to 4 d (Fig. 1 D). Immunocytochemical studies showed that all cells were positive for ED1 (Fig. 1 E), whereas about 60% were ED2⁺ (Fig. 1 F). Protein synthesis capacity was about 30% of that of KC. In MC cultures (Fig. 1 G), all cells were positive if stained with the ED1-monoclonal (Fig. 1 H), but completely ED2⁻ (Fig. 1 K).

Synthesis of C1-INH by Rat KC

Biosynthetic Labeling. As shown in Fig. 2, a protein of a molecular mass of about 75 kD from the cell lysates and a 86-kD protein from the supernatants of KC was immunoprecipitated by a monospecific antiserum against C1-INH. A small amount of the mature (86 kD) protein was also found in the cell lysates. To identify the protein as C1-INH, we preincubated the antiserum with purified plasma C1-INH and subsequently with the radioactive sample. As shown in Fig. 2, preincubation of the antiserum strongly reduced the precipitate.

N-glycosylation of the protein was studied by incubating KC with tunicamycin (25). A similar experiment was performed on isolated hepatocytes. The molecular mass of the intra- as well as the extracellular form is reduced in both cell types, but the difference between the intra- and extracellular protein was not affected (Fig. 3). This demonstrates that glycosylation of C1-INH is complex (26).

To show that the newly synthesized protein is also physiologically secreted, we performed pulse-chase experiments. As shown in Fig. 4, nearly all of the synthesized protein is secreted after 120 min and found in the supernatant.

Next, we examined C1-INH synthesis and secretion by raising time in culture (Fig. 5) and found that from day 1 to day 5 C1-INH synthesis is diminished to about 25%, provided that an equal amount of total protein is used for immunoprecipitation. As the total protein synthesis increased

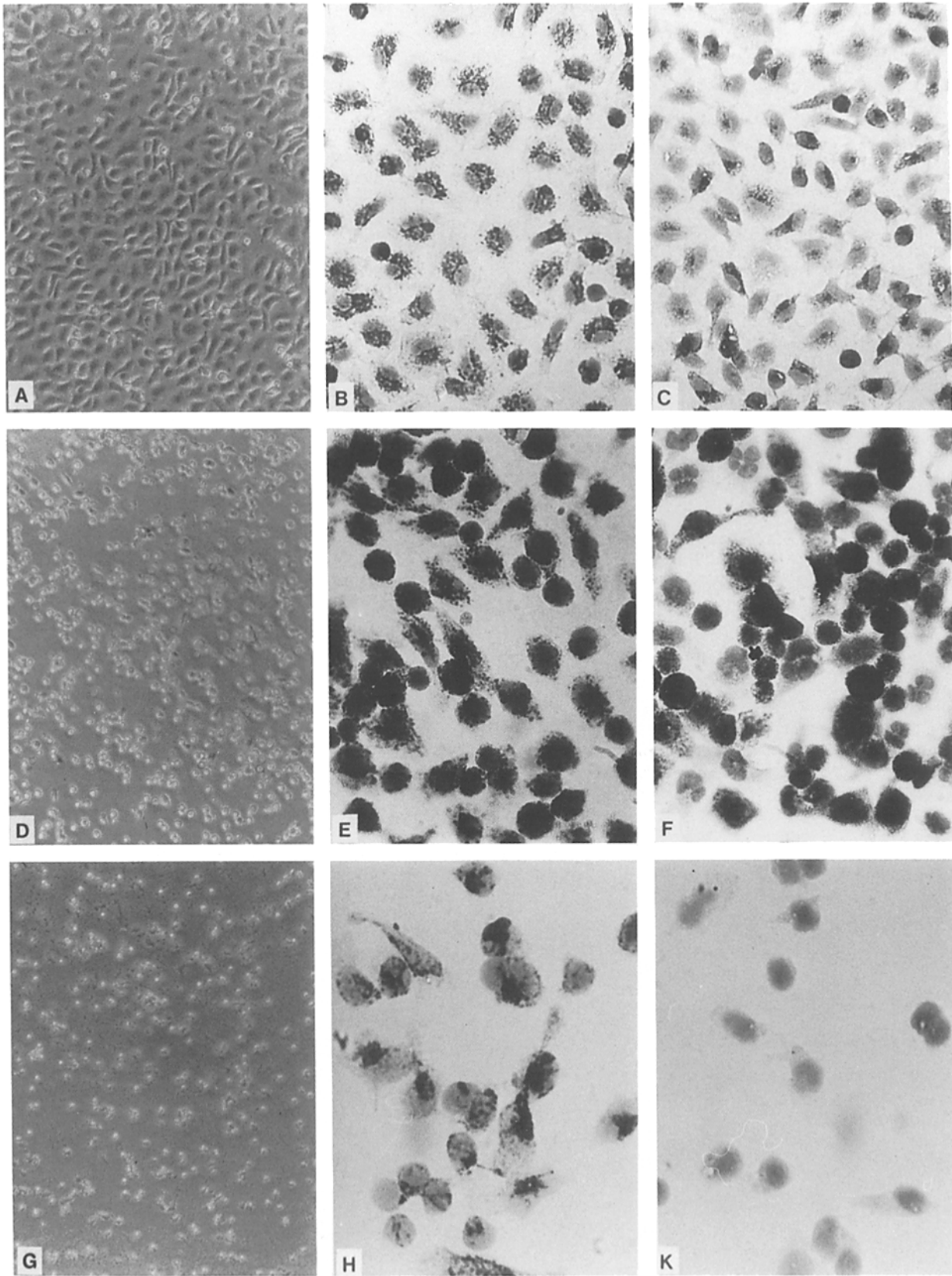


Figure 1. Phase-contrast microscopical pictures of rat KC cultures 2 d after isolation (*A*) and 1-d-old cultures of PM (*D*) and blood MC of the rat (*G*). Immunocytochemical studies of these cultures with mAbs against monocyte-macrophage antigens, detected by a peroxidase-labeled second antibody. In controls, the first antibody was replaced by an irrelevant monoclonal (not shown): ED1 in KC (*B*), PM (*E*), and MC (*H*); ED2 in KC (*C*), PM (*F*), and MC (*K*). (*A*, *D*, and *G*) $\times 100$; (*B* and *C*) $\times 250$; (*E*, *F*, *H*, and *K*) $\times 400$.

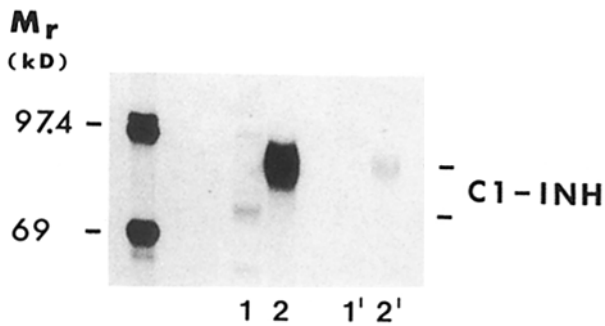


Figure 2. Blocking experiment of the C1-INH antibody. Immunoprecipitates of endogenously labeled C1-INH from cell lysates (1,1') and supernatants (2,2') of KC cultures, analyzed by SDS-PAGE. After preincubation of the antibody with purified plasma C1-INH for 8 h at +4°C, immunoprecipitation was carried out as usual (1',2'). Precipitations shown in lanes 1 and 2 were performed without antigen-blocking using the same amount of radioactivity.

fourfold (see above), cells synthesize a constant amount of C1-INH.

To quantify the amount of C1-INH, the bands were cut from gels, dissolved in H₂O₂ and counted in a β -counter. C1-INH synthesis represents about $1.3 \pm 0.2\%$ of secreted proteins in rat KC at day 1 of culture.

In hepatocytes, share of C1-INH in total secreted proteins was about 20-fold lower. As hepatocyte total protein synthesis rate is higher than that of KC (10-fold as determined by TCA precipitation), the absolute amount of C1-INH synthesized by KC should be about two times higher than that synthesized by isolated hepatocytes.

Northern Blot Analysis and In Situ Hybridization. Northern blot analysis of total RNA from KC at days 1, 3, and 5 of culture showed that the amount of detectable C1-INH-specific

mRNA remains quite constant (Fig. 6). In situ hybridization demonstrated that all the freshly isolated cells and all the cells present in the cultures contained C1-INH-specific transcripts. As shown in Fig. 7, A and C, the hybridization with the sense RNA probe resulted in an equal distribution of background grains. Using the antisense RNA probe (Fig. 7, B and D) an abundant amount of radioactive grains were found on the nucleus and in the cytoplasm. In situ hybridization on rat liver sections, however, was characterized by an equal distribution of grains in the parenchyma and in the sinusoid (data not shown).

Influence of the Isolation Procedure on C1-INH Synthesis in KC. As C1-INH gene expression in freshly isolated KC could be induced by isolation procedure, the cells were isolated by low temperature (+4°C) to prevent any possible activation. Furthermore, KC were also isolated from supernatants after differential centrifugation of a cell suspension obtained from a collagenase-digested liver (procedure usually performed for preparation of hepatocytes). However, in both experiments we achieved results similar to those shown in Fig. 6 (data not shown).

Synthesis of C1-INH by PM and MC

C1-INH was immunoprecipitated from supernatants and cell lysates of PM pulsed at days 1 and 4 of culture, respectively. In comparison to KC, however, the amount of the protein synthesized by PM was much lower. Furthermore, Northern blot analysis of total RNA from freshly isolated PM did not detect C1-INH-specific transcripts. In contrast, C1-INH-specific mRNA was clearly detectable in total RNA from freshly isolated KC as shown in Fig. 8.

Blood MC at the first, third, and eighth day of culture did not constitutively synthesize detectable amounts of C1-INH (data not shown).

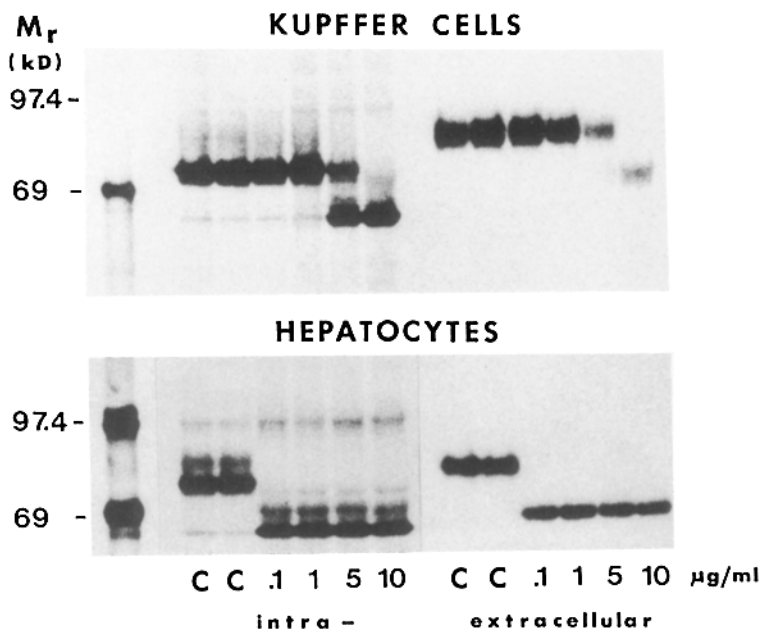


Figure 3. Synthesis and secretion of C1-INH by rat KC and hepatocytes. Effect of tunicamycin on N-glycosylation of the intra- and extracellular protein. SDS-PAGE analysis of the immunoprecipitates of endogenously labeled C1-INH. KC and hepatocytes, both at day 1 of culture, were incubated with tunicamycin (0.1–10 $\mu\text{g/ml}$ medium, C without the antibiotic) for 4 h, then cultures were washed and pulsed for 2 h (600 $\mu\text{Ci/ml}$) in the presence of tunicamycin.

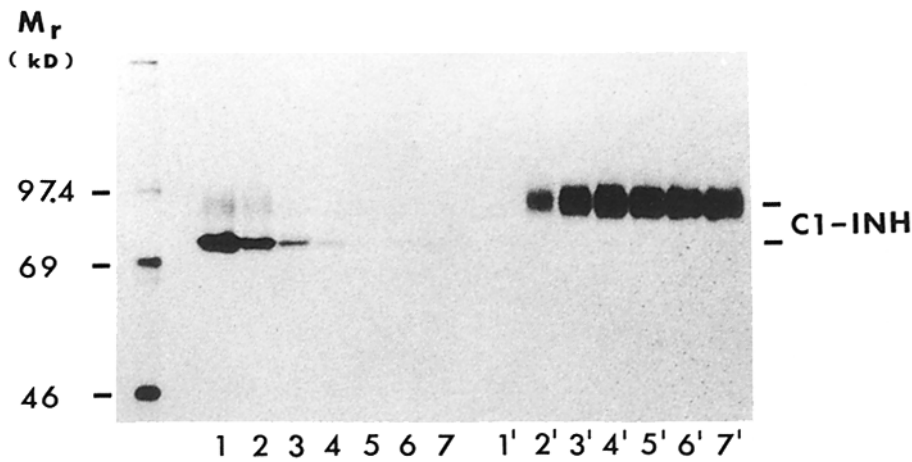


Figure 4. Synthesis and secretion of C1-INH by KC as determined by the pulse-chase experiment. SDS-PAGE analysis of endogenously labeled proteins from cell lysates (1-7) and supernatants (1'-7'). KC cultures were pulsed with [³⁵S]methionine for 1 h, washed with M 199 and incubated with an excess of cold methionine for the following periods of time: 0 min (1 and 1'); 30 min (2 and 2'); 60 min (3 and 3'); 120 min (4 and 4'); 240 min (5 and 5'); 360 min (6 and 6'); and 720 min (7 and 7'). The autoradiogram was obtained after 36 h.

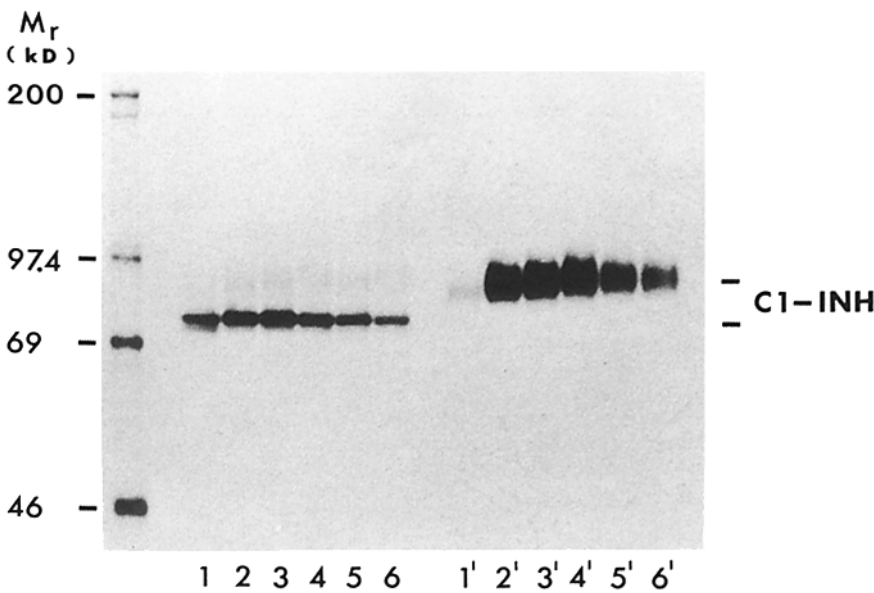


Figure 5. Synthesis and secretion of C1-INH by rat KC after different periods of time in culture. SDS-PAGE analysis of endogenously labeled proteins from cell lysates (1-6) and supernatants (1'-6'). KC were pulsed for 2 h with DMEM without methionine containing [³⁵S]methionine (600 μ Ci/ml). For immunoprecipitation an equal amount of protein was used. Labeling was performed at the following periods of time after isolation: freshly isolated cells in suspension (1 and 1'); 1 d after isolation (2 and 2'); 2 d (3 and 3'); 3 d (4 and 4'); 4 d (5 and 5'); and 5 d (6 and 6'). The autoradiogram was obtained 24 h after exposure.

Modulation of C1-INH Gene Expression in KC, PM, and MC by IFN- γ

The cultures were treated with different concentrations of rat rIFN- γ (10^{-10} – 10^{-4} U/ml). After a treatment of 14 h, the cultures were washed and pulsed with [³⁵S]methionine. The immunoprecipitates of C1-INH from supernatants are shown in Fig. 9. Clear spontaneous synthesis of C1-INH is found in KC whereas trace amounts are produced by PM and no C1-INH synthesis is detectable in MC. IFN- γ induced synthesis of C1-INH in MC and increased C1-INH in KC and, more effectively, in PM.

Discussion

In this work we demonstrate that isolated rat KC constitutively synthesize and secrete the protease inhibitor C1-INH,

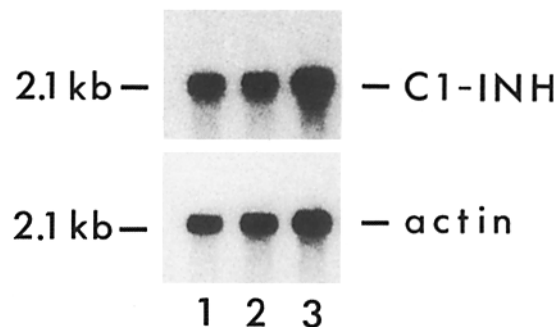


Figure 6. C1-INH gene expression in rat KC. Northern blot analysis of total RNA extracted from KC cultures at day 1 (1), day 3 (2), and day 5 (3) of culture. Total RNA was separated on agarose gel, blotted and hybridized with ³²P-labeled cDNA specific for C1-INH and actin, respectively. The autoradiogram was obtained after 24 h (C1-INH) and 8 h (actin).

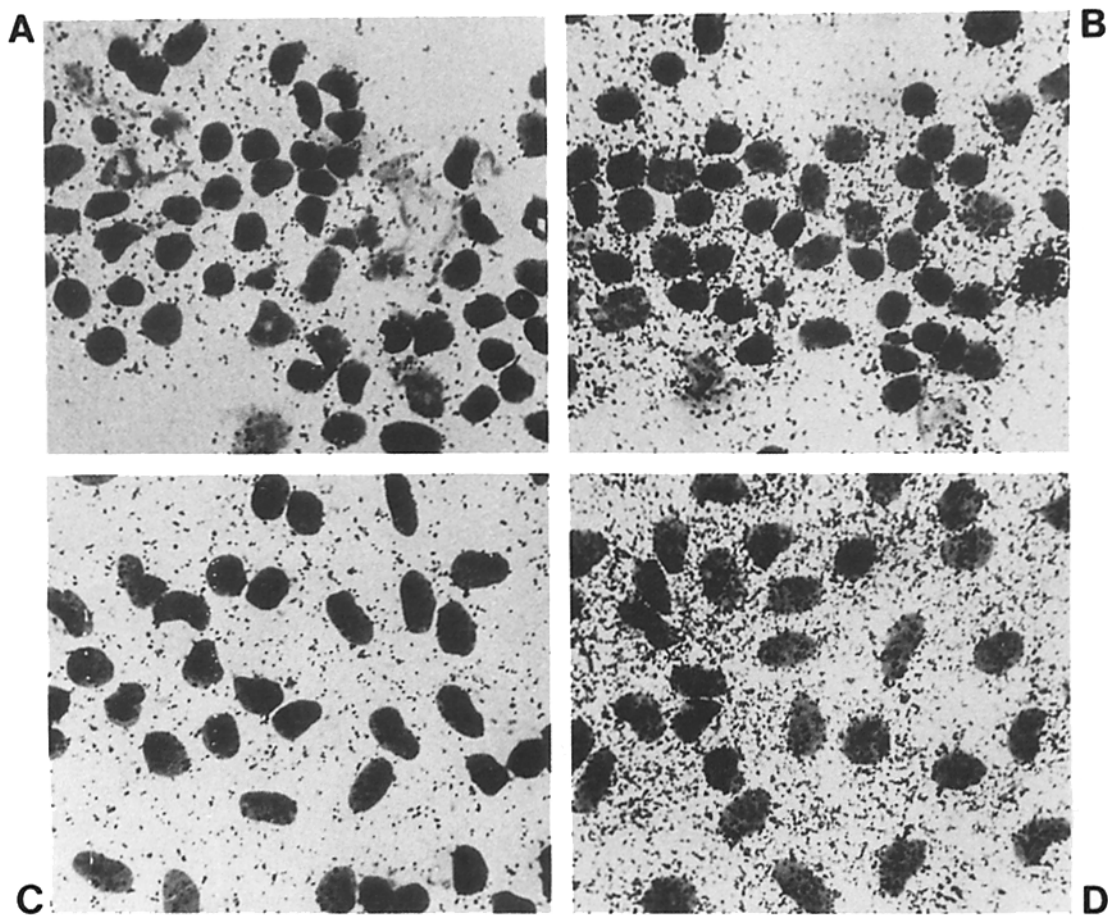


Figure 7. In situ hybridization of freshly isolated (A and B) and 3-d-old cultures (C and D) of rat KC. Freshly isolated cells, centrifuged onto slides, and cultures on cell culture slides were fixed with 4% paraformaldehyde, pretreated and hybridized with the ³⁵S-labeled sense (A and C) or antisense (B and D) RNA probes obtained by transcription of a fragment (211 bases) of the C1-INH-specific cDNA. After hybridization, cells were treated with RNase, washed, coated with Kodak NTB-2 emulsion, developed, fixed, and counterstained. ×400.

whereas PM synthesize only trace amounts. C1-INH is not constitutively synthesized by blood MC. IFN- γ upregulates C1-INH gene expression in all three cell populations.

Because of the different isolation methods used, it is arguable that C1-INH gene expression in freshly isolated KC is due to the isolation procedure. To answer this question, we minimized biological activity of KC by isolating them consistently at +4°C. Furthermore, isolation of KC without pronase treatment of livers entailed reduced yield of phagocytosable material during such procedure and the lack of stimulation of the macrophages. The amount of C1-INH immunoprecipitated from KC isolated in this manner was comparable to the amount of C1-INH produced by cells isolated by standard methods. Another argument disproving an influence of the isolation procedure on C1-INH synthesis is given by the fact that the amount of C1-INH synthesized did not decrease during time in culture, as could be expected if C1-INH synthesis were due to activation of KC.

Our data, which state a lack of a spontaneous synthesis of C1-INH in blood MC, stand at least in part in apparent contrast to the data found in the literature, which are obtained from human cells. Yeung-Laiwah et al. (13) reported

about C1-INH appearing soon after isolation in supernatants of cultured human blood MC. Lotz and Zuraw (12) detected trace amounts of C1-INH in human blood MC only after prolonged culture. Although isolation procedures were similar

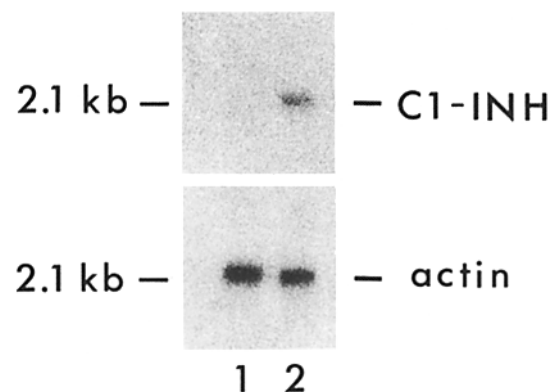


Figure 8. C1-INH gene expression in freshly isolated rat KC and PM. Total RNA was extracted from freshly isolated PM (1) and freshly isolated KC (2), and analyzed by Northern blotting. Hybridization was performed using ³²P-labeled cDNA specific for C1-INH and actin, respectively.

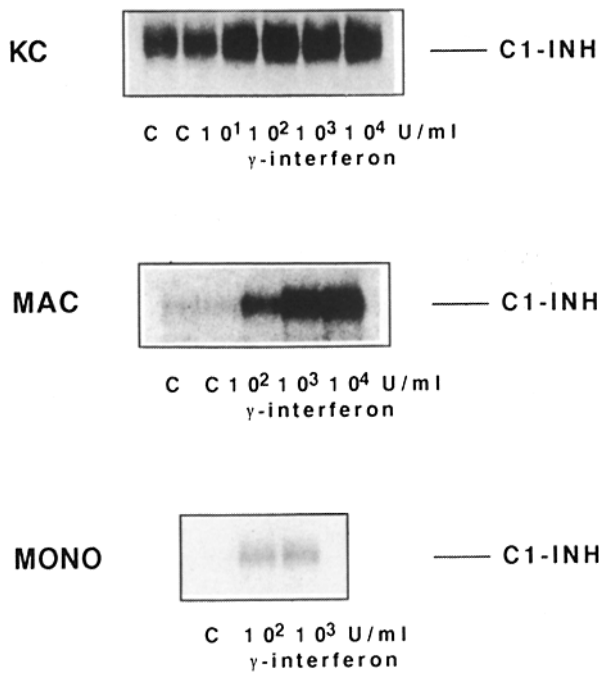


Figure 9. Modulation of C1-INH gene expression by IFN- γ in rat Kupffer cells (KC), peritoneal macrophages (MAC), and blood monocytes (MONO). SDS-PAGE analysis of immunoprecipitates of C1-INH from supernatants. Cultures were incubated with the described concentrations of rec rat IFN- γ (C without) at day 1 of culture. After 14 h, cells were washed and labeled with [35 S]methionine for 2 h.

in both reports, we suppose that conditions of isolation and culture of these cells are critical. First, the presence of contaminating cells could be a source of C1-INH. Although adherent cells other than MC are not expected in preparations of blood cells, we observed the growth of fibroblast-like cells in some of our experiments already after 7 d of culture (Armbrust, T., S. Schwögler, and G. Ramadori, unpublished observations). Thus, characterization of cultures seems to be essential for such a purpose. Second, induction of C1-INH gene in MC by an activating agent such as IFN- γ makes us believe that further ways of induction cannot be excluded, so that a report about a spontaneous C1-INH synthesis by MC has to be viewed critically.

C1-INH was synthesized by PM to a much lesser extent compared with that produced by KC. To some degree, this may be due to the fact that the PM population contained about 40% ED2⁻ cells (MC). In spite of this, the synthesis of C1-INH by ED2⁺ PM is not nearly as high as by (ED2⁺)

KC, as the complete PM population did not, by far, synthesize 60% of the amount of C1-INH produced by KC.

Thus, we could notice gradual differences—phenotypical and functional—between these cell types. The difference observed in C1-INH gene expression could be caused by the differentiation process of blood MC to tissue macrophages. Whereas the latter fail to express C1-INH gene, PM synthesize small amounts of C1-INH, and KC, being fully matured tissue macrophages (100% ED2 positivity), constitutively express C1-INH gene.

The differentiation process of blood MC to tissue macrophages is not understood in detail. Studies have revealed differences between these cells regarding morphology (27), surface glycoproteins, functional aspects (28, 29) and protein synthesis. Concerning the latter, most attention was paid to the proteins of the complement system. It was shown that tissue macrophages have a higher capacity for synthesis of C2, C4, and factor B than MC (30, 31). The results of this study give rise to the suggestion that C1-INH is another useful marker protein of the differentiation process.

However, any differences between MC and tissue macrophages are supposed to be caused by the differentiation. Otherwise, the tissue macrophages are a heterogeneous family of cells with typical differences between the single populations. It is not known, how the common precursor cell, the monocyte, becomes just a KC when it enters the liver, but it is very likely that there is an important influence by the local environment, and that the differences among the populations of tissue macrophages reflect the differences of their function. Thus, C1-INH seems to be important for the function of KC and for the homeostasis in the liver, as it is to be supposed that C1-INH synthesized by KC is active locally.

It must be considered that this protein is also of importance in acutely or chronically damaged liver. Although immigrating MC, which fail to express C1-INH gene, seem to be the main mononuclear cells of inflammatory infiltrates (32), C1-INH, which is synthesized by the resident macrophages, is involved in the modulation of the inflammatory process by its interaction with several systems participating at inflammation. Its importance to these inflammatory processes is confirmed by the fact that it is upregulated by IFN- γ , which is an important mediator of inflammation, itself. It is of further interest to study these infiltrating cells. They might be useful to study the inflammatory process in the liver as well as the differentiation process of blood MC to tissue macrophages.

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