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BIOSYNTHESIS OF FUNCTIONALLY ACTIVE HEPARIN COFACTOR II BY A HUMAN HEPATOMA-DERIVED CELL LINE

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Human plasma heparin cofactor II (HCII) inhibits thrombin by rapidly forming a stable, equimolar complex in the presence of heparin or dermatan sulfate. Cultured human hepatoma-derived cells (PLC/PRF-5) secreted (≈200 ng/ml in 3 days) a protein of MW = 72 kD that was immunoisolated and immunoblotted with anti-HCII, co-migrated on SDS-PAGE with human plasma HCII, and formed covalent complexes with thrombin (MW = 101 kD) in the presence but not absence of heparin or dermatan sulfate; these complexes co-migrated with those obtained by incubating thrombin with human plasma under the same conditions. HCII was not detectable (< 0.13 ng/ml) in post-culture medium from cultured human umbilical vein endothelial cells or human foreskin fibroblasts

Human plasma contains two heparin-dependent inhibitors of thrombin, antithrombin III (ATIII) (1,2) and heparin cofactor II (HCII) (3-8). Both inhibit thrombin by forming stable 1:1 complexes with thrombin that can be distinguished by SDS-PAGE (5,6). ATIII inhibits plasmin and all the proteases of the intrinsic coagulation system, whereas HCII inhibits only thrombin (6-10). HCII also weakly inhibits the chymotrypsin-like enzyme leukocyte cathepsin G (9). Dermatan sulfate accelerates inhibition of thrombin by HCII but has no effect on the activity of ATIII (11,12). ATIII and HCII also have different molecular weights and N-terminal amino acid sequences (6,7,13) and lack antigenic cross-reactivity (5,8). Hepatocytes and hepatoma cells synthesize ATIII (14,15). In this paper, we show that human hepatoma cells synthesize and secrete functionally active HCII.

Abbreviations: ATIII, antithrombin III; HCII, heparin cofactor II; MEM, Minimal essential medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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EXPERIMENTAL METHODS

Materials [^{125}I]-protein A, [^{35}S]-methionine, [^{14}C]-labeled molecular weight standards, and ENHANCE^{TM} were from New England Nuclear; nitrocellulose BA 85 was from Schleicher and Schuell; and William's medium E, MEM, methionine-free MEM, medium 199, fetal calf serum, L-glutamine, penicillin, streptomycin, and trypsin-EDTA were from Flow. T-75 tissue culture flasks were from Corning. Cycloheximide, porcine intestinal mucosa heparin, and dermatan sulfate were from Sigma. Purified human thrombin was obtained from John Fenton, II. Anti-ovalbumin was from Cappel Laboratories, and protein A-Sepharose 4B-CL was from Pharmacia. D-Phe-Pro-ArgCH_2Cl was obtained from Calbiochem.

Cell Culture Human hepatoma cells (PLC/PRF-5) were obtained from Dr. Gretchen Darlington and cultured in William's medium E containing 10% fetal calf serum, L-glutamine (1.6 mM), penicillin (80 U/ml), and streptomycin (80 μg/ml). Hepatoma cells were passaged with 0.5% trypsin - 0.02% EDTA and cultured in T-75 flasks (16-18). Human endothelial cells were derived from umbilical cord veins and cultured in medium 199, 20% human serum, endothelial cell growth factor (20 μg/ml), and heparin (90 μg/ml) as previously described (19-21). Human foreskin fibroblasts were cultured in MEM containing 20% fetal calf serum. For experiments involving immuno-isolation and electrophoretic immunoblotting, hepatoma cells were incubated for 3 days in serum-free medium and endothelial cells and fibroblasts were incubated for 3 days in medium containing 10% rabbit serum. For experiments involving labeling with [^{35}S]-methionine, hepatoma cells were washed, detached by scraping, pelleted by centrifugation, pre-incubated for 30 min with serum-free, methionine-free MEM, re-washed, and labeled in serum-free, methionine-free MEM with 0.5 mCi/ml [^{35}S]-methionine for either 30 min or 2 hr.

Purification of HCII and anti-HCII HCII was purified to homogeneity from human fresh-frozen plasma as previously described (6). Rabbits were immunized by subcutaneous injection with 50-100 μg of purified HCII emulsified in complete Freund's adjuvant. The animals were boosted twice at 3-week intervals with 40-50 μg of HCII in incomplete Freund's adjuvant. The animals were bled 2 weeks after the final injection, and serum was prepared in the usual manner. The antiserum was judged monospecific for HCII by electrophoretic immunoblotting against plasma and purified HCII (Fig. 1).

Immunoisolation of hepatoma HCII HCII was isolated from serum-free post-culture medium by batch immunoadsorption with rabbit anti-HCII coupled to protein A-Sepharose 4B-CL ("immunoisolation") as follows: samples of medium were made 1% in SDS and boiled for 5 min; the samples were diluted to 0.3% SDS with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and Triton X-100 added to final concentration of 1.7%. The rest of the immuno-isolation was carried out as previously described except that the protein A-Sepharose 4B-CL beads were eluted with 2% SDS (22). In experiments using [^{35}S]-methionine, HCII was immunoisolated by the technique of Firestone (23). In both cases, control immunoisolations were performed using anti-ovalbumin.

Gel electrophoresis, autoradiography, and electrophoretic immunoblotting Samples were reduced with dithiothreitol and SDS-PAGE performed in 7.5% polyacrylamide slab gels containing 0.1% (v/v) SDS (24). The following [^{14}C]-labeled molecular weight standards were used: myosin, 200,000; phosphorylase b, 94,000; human serum albumin 68,000; and ovalbumin 45,000. [^{35}S]-labeled samples were treated with ENHANCE^{TM} and autoradiography performed at -70°C. Electrophoretic transfer of proteins from SDS slab gels to nitrocellulose membranes and immunoblotting with [^{125}I]-protein A was performed by the method of Burnette (25).

RESULTS

Specificity of anti-HCII. By electrophoretic immunoblotting, both purified HCII and human plasma contained only one species of MW = 72 kD that reacted with the anti-HCII antiserum (Fig 1, lanes a,b) thus demonstrating the

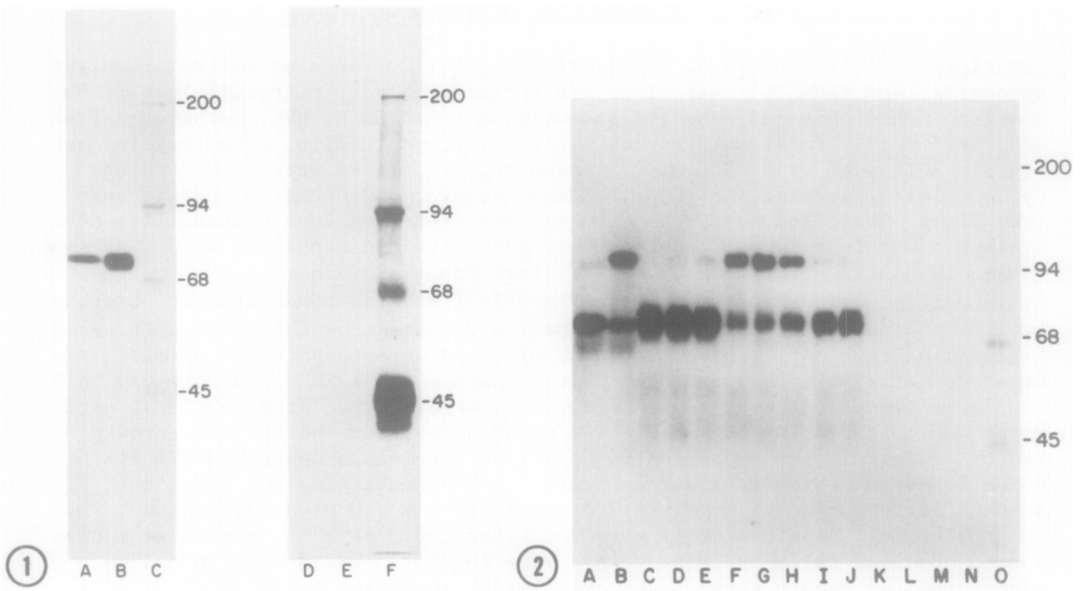

Figure 1 Specificity of anti-HCII. Citrated human plasma diluted 1:50 (10 μl)(lanes a,e) and purified HCII (20 ng)(lanes b,d) were analyzed by SDS - 7.5% polyacrylamide slab gel electrophoresis (SDS-PAGE) after reduction with dithiothreitol. The proteins were electroblotted from the gel onto nitrocellulose paper and reacted sequentially with rabbit anti-HCII (lanes a-c) or anti-ovalbumin (lanes d-f) and then [^{125}I]-protein A. [^{14}C]-labeled molecular weight standards were run in lanes c and f. An autoradiograph of the immunoblot is shown.

Figure 2 Synthesis of functionally active HCII by cultured hepatoma cells. Hepatoma cells were cultured for 3 days in serum-free medium. Samples of human plasma and hepatoma cell post-culture medium were reacted with thrombin in the presence and absence of dermatan sulfate. Plasma (lanes a,b) and immunoisolates prepared from the post-culture medium using rabbit anti-HCII (lanes c-j) or anti-ovalbumin (lanes k-n) coupled to protein A-Sepharose 4B were analyzed by SDS-PAGE and electrophoretic immunoblotting with anti-HCII as described in Figure 1. Human plasma was diluted 1:50. Samples of post-culture medium were reacted for 3 min with varying concentrations of dermatan sulfate and then for 15 min with thrombin (1 U/ml). Autoradiograph of: human plasma (lane a); human plasma, heparin (10 U/ml), and thrombin (lane b); post-culture medium (lanes c,n); post-culture medium and dermatan sulfate (100 μg/ml) (lane d) and post-culture medium and thrombin (lane e). Lanes f-n contained post-culture medium, thrombin, and the following concentrations of dermatan sulfate: (100 νg/ml) (lanes f,k); (10 μg/ml) (lanes g,l); (1 μg/ml) (lanes h,m); (0.1 μg/ml) (lane i); and (0.01 μg/ml) (lane j). Lane o contained [^{14}C]-molecular weight markers.

monospecificity ot the anti-HCII antiserum. Control experiments performed with anti-ovalbumin were negative (lanes d,e).

Synthesis of functionally active HCII by hepatoma cells. To determine if human hepatoma cells synthesize functionally active HCII, the cells were cultured in serum-free medium for three days and the HCII in the post-culture medium analyzed as described in Fig. 1. Post-culture medium contained a band of MW ~ 72 kD (Fig. 2, lane c) which co-migrated with a band detected in human

plasma with the same antiserum (Fig. 2, lane a). The concentration of HCII in hepatoma post-culture medium was estimated to be 200 ng/ml. Control experiments in which the immunoisolation step was performed with anti-ovalbumin instead of anti-HCII (Fig. 2, lane n) detected no HCII. Neither pre-culture medium nor medium from cells cultured with cycloheximide (10 μg/ml) contained any HCII. No HCII was detected in post-culture medium from human umbilical vein endothelial cells or foreskin fibroblasts (lower limit of detection = 0.13 ng HCII/ml) (data not shown).

Formation of complexes between hepatoma-derived HCII and thrombin in the presence of dermatan sulfate. Human plasma HCII (but not AT III) forms covalently-linked complexes with thrombin in the presence of dermatan sulfate (11). When human hepatoma cell post-culture medium was incubated with thrombin and dermatan sulfate, HCII in the post-culture medium formed complexes with thrombin (Fig. 2, lane f) which had a MW = 101 kD and co-migrated with thrombin-HCII complexes formed with plasma HCII in the presence of dermatan sulfate (Fig. 2, lane b). No complexes were formed by the HCII in the post-culture medium in the absence of dermatan sulfate (Fig. 2, lane e). Complex formation was dependent on the dermatan sulfate concentration and decreased markedly below 1 μg/ml (Fig. 2, lanes f-j). Control experiments in which the immunoisolation step was performed with anti-ovalbumin instead of anti-HCII (Fig. 2, lanes k-n) detected no protein bands migrating with either marker HCII or thrombin-HCII complexes.

Hepatoma cells incorporate [^{35}S]-methionine into functionally active HCII Hepatoma cells were labeled with [^{35}S]-methionine and post-culture medium analyzed for HCII to demonstrate de novo synthesis. Post-culture medium (Fig. 3, lane 2) contained one band of MW = 75 kD. Pre-treatment of the cells with cycloheximide 10 μg/ml abolished HCII production (data not shown). Labeled HCII formed complexes with thrombin (MW = 102 kD) only in the presence of heparin (Fig. 3, lanes 3-5). Thrombin inactivated with D-Phe-Pro-ArgCH_2Cl (PPACK), a selective inhibitor of thrombin (26), did not form complexes (Fig. 3, lane 6). Control experiments in which immuno-isolations were performed

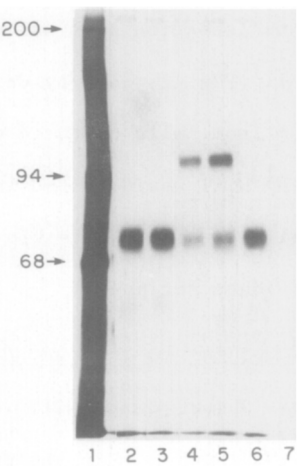

Figure 3 Synthesis of [^{35}S]-methionine-labeled HCII by cultured human hepatoma cells. Hepatoma cells were labeled for 2 hr with [^{35}S]-methionine. Labeled post-culture medium in lanes 2-6 was treated as described below and the HCII in the samples immunoisolated with anti-HCII and analyzed by SDS-PAGE and autoradiography. Autoradiograph of: (lane 1) [^{14}C]-molecular weight markers; (lane 2) post-culture medium; (lane 3) post-culture medium and thrombin (1 U/ml); (lane 4) post-culture medium, thrombin (1 U/ml), and heparin (10 U/ml); (lane 5) post-culture medium, thrombin (10 U/ml), and heparin (10 U/ml); and (lane 6) post-culture medium, thrombin (1 U/ml) inactivated with PPACK, and heparin (10 U/ml). A control study was performed on post-culture medium by immunoisolation with anti-ovalbumin (lane 7).

with anti-ovalbumin were negative (Fig. 3, lane 7). Pulse chase experiments demonstrated that HCII was first detectable in post-culture medium 45 min after beginning labeling (data not shown).

DISCUSSION

Our results demonstrate that the human hepatoma line PLC/PRF-5 synthesizes and secretes HCII in culture. The HCII synthesized by the hepatoma cells and plasma HCII are immunologically similar if not identical, have the same molecular weight on SDS-PAGE, and form covalent complexes with thrombin in the presence of either heparin or dermatan sulfate. Similar results (not shown) were obtained using Hep G2, another hepatoma derived cell line. In contrast, neither cultured human umbilical vein endothelial cells nor human foreskin fibroblasts secreted detectable amounts of HCII. These findings strongly suggest that HCII is of hepatic origin.

HCII is present in plasma in relatively large concentrations (80 ± 30 μg/ml) (27). HCII activity is decreased in plasma from many patients with hepatic failure and disseminated intravascular coagulation (27-29). A small number of patients with hepatic failure in the absence of disseminated intravascular coagulation have also been found to have decreased HCII activity (27,28). Our present results suggest that HCII deficiency in these patients may directly result from impaired hepatic synthesis.

The physiologic function of HCII is presently unknown. It can be postulated that HCII is important in the regulation of one or more of the known effects of thrombin, including: conversion of fibrinogen to fibrin monomers (30); activation of coagulation factors V (31,32), VII (33), VIII (34), and protein C (35); stimulation of platelet aggregation and secretion (36); initiation of endothelial cell prostacyclin synthesis (37); and initiation of mitosis in fibroblasts (38). The observation that HCII is activated by dermatan sulfate suggests that HCII may inhibit thrombin in the connective tissues rather than within the blood vessels. In accordance with this hypothesis, we have found that the rate of inhibition of thrombin by purified HCII is increased by cultured human lung fibroblasts but not but human umbilical vein endothelial cells (E.A. McGuire and D.M. Tollefsen, personal communication).

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