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Synthesis of Factor VIII in Human Hepatocytes in Culture

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Key words

Factor VIII – Human hepatocytes – ELISA

Summary

Although several investigators have attempted to identify the site of synthesis of factor VIII (FVIII), the cellular species responsible for maintenance of plasma FVIII has not been clearly defined. Indications point at hepatocytes and certain endothelial cells. The present study investigated the FVIII coagulant antigen (VIII:Ag) of hepatocytes obtained by two-step collagenase digests of human liver pieces. Following Percoll gradient centrifugation, less than 1% of cells harvested were non-parenchymal. Lysates of freshly isolated and purified hepatocytes contained 165–250 mU of VIII:Ag/10^6 cells as defined by a two-site ELISA employing a haemophilic antibody against human FVIII. This material contained a single peak of VIII:Ag polypeptides as jugded from the VIII:Ag ELISA profile of Mono-Q fast protein liquid chromatography fractions. A haemophilic antibody specific for epitopes of the light chain of FVIII, employed in immunoisolation of VIII:Ag in lysate of human hepatocytes, extracted a polypeptide pattern that was studied in a reduced SDS-PAGE electrophoresis gel and compared to that of immunoisolate from normal plasma. After electroblotting onto nitrocellulose and reaction with a monoclonal antibody towards the light chain of FVIII, the appearance of a doublet at 78–79 kDa in both these materials indicated the presence of the light chain of FVIII in human hepatocyte lysate. During culture, human hepatocytes secreted 20–80 mU of VIII:Ag per 1×10^6 cells per 24 hours. Further, a significant secretion of VIII:Ag was found in media of cultured human hepatoma cells, Hep-G2, whereas human blood monocytes and human fibroblasts did not secrete detectable VIII:Ag. In all of these cell cultures, vWf:Ag was indetectable or present as trace. Our results suggest that the human hepatocyte is a production site of FVIII.

Introduction

The plasma factor VIII/von Willebrand factor complex is a non-covalent assembly of two proteins of different composition and function, and derived from cells of different embryonic compartments. The von Willebrand factor (vWf), serving in primary haemostasis, is a large protein constructed of a series of differently sized multimers. vWf originates in endothelial cells (1, 2, 3) and megacaryocytes (4) under control of a gene located on chromosome No. 12 (5, 6). vWf synthesis is defective or lacking in von Willebrand's disease. Factor VIII (procoagulant: VIII:C; procoagulant antigen: VIII:Ag) circulates in plasma as 80 and 92–170 kDa polypeptides (7, 8, 9, 10), however, originally synthesized with an approximate size of ≃280 kDa under genetic control of a gene located on the X-chromosome (11, 12). FVIII functions in the middle part of the intrinsic coagulation cascade as cofactor for the FIXa proteolytic activation of factor X. FVIII is deficient or abnormal in classical haemophilia A. Although seemingly located in the liver (13, 14, 15, 16, 17), the exact cellular production site of FVIII has as yet not been clearly defined. Liver seems an important site of synthesis of FVIII, since plasma VIII:C in a haemophiliac was normalized following a liver transplantation (13). Human liver contains FVIII mRNA (11, 18), and VIII:Ag (18). However, attempts to localize FVIII by immunohistologic procedures on liver tissue sections were discordant. A monoclonal antibody specific for VIII:Ag reacted with liver sinusoidal cells (14, 16) in one study, and with both hepatocyte rough endoplasmatic reticulum and sinusoidal cells in another (15). In guinea pig (17) and human (18) hepatocytes VIII:Ag immune reactive material has been detected. The possibility exists, that certain endothelial cells are involved in synthesis of FVIII. The present investigation demonstrates the presence of VIII:Ag in lysates of isolated human hepatocytes and the increment in medium VIII:Ag during culture of such cells.

Materials and Methods

Left liver tip was excised from necro kidney donors (25–50 g) leaving the capsule intact. This material was immediately perfused with calcium-free Hepes buffer through visible vessels for 15 min, followed by Hepes buffer containing 0.5 mM calcium and 0.05% collagenase for 20 min (19). After opening of Glissons capsule, cellular material was loosened mechanically, suspended in Hepes buffer with 1% bovine albumin at pH 7.4, filtered through 300 μm and 60 μm nylon mesh, and centrifuged at 100 × g for 4 min. After washing twice, hepatocytes were separated from dead cells, debris and minute numbers of blood cells by centrifugation on a linear Percoll gradient (D = 1.096–1.006) at 800 × g for 20 min at 4° C. After two washes in Hepes buffer containing 1% albumin, 90% of cells recovered (50–400 × 10^6) excluded trypane blue. As determined by light microscopic appearance, lacking reactivity with monoclonal antibodies CD 11 (My3) and CD 14 (My4) (19), and functional tests (adherence, latex/iron particle phagocytosis), less than 1% of cells were macrophages.

Elisa Procedures

ELISA (20) was used for determination of VIII:Ag, employing the same antibody reacting with both light and heavy chains of FVIII at both sides of the sandwich [antibody A in (20)]. This assay has a low limit sensitivity of 4–8 mU of VIII:Ag per ml, when 1,000 milliunits is defined as the amount present in 1 ml of the 1st International Standard for Plasma FVIII. Using polyclonal antibodies from Dakopatts (Glostrup, Denmark), α_2-macroglobulin and α_1-antitrypsin were measured by ELISA as previously reported (21, 22), the sensitivities at 2.0 μg/l and 3.3 μg/l, respectively. Similarly, ELISA for albumin was constructed. In these ELISA assays, a normal control serum (OSAU Behring, Marburg, FRG), served as the primary standard. vWf:Ag was quantitated by ELISA (23).

Study of Hepatocyte Lysates

Cells (15 × 10^6) were sonicated in 5 ml of Hanks Balanced Salt Solution and centrifuged at 5,000 × g for 10 min. The supernatant was filtered through 0.22 μm filters (GV, Millipore Corp., Bedford, Mass.)

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and concentrated to 1 ml using a Millipore CX-30 filter. 500 µl of 1:2.5 diluted material in PBS pH 6.8, equivalent to approximately 1.5 U of VIII:Ag, was injected onto a Mono-Q HR 5/5 column (Pharmacia, Uppsala, Sweden). Fast Protein Liquid Chromatography was performed using an electronically controlled dual pump system (P-500, Pharmacia) and 214 nm light absorbancy recording. Buffers (0.02 M Tris, pH 7.5 and 0.02 M Tris with 1 M NaCl, pH 7.5) were mixed through pump delivery to form a linear gradient of NaCl from 0 to 1 M. Flow was 2 ml/min. 100 µl of each fraction (480 µl) was applied to a microtiter plate precoated with a haemophilic antibody specific for both light and heavy chains of FVIII. Using the same antibody at the detection side VIII:Ag was expressed semiquantitatively by the 492 nm absorption as determined by ELISA. For comparison, a highly purified FVIII concentrate (Hemofil-M, a gift of Baxter Travenol Therapeutics), corresponding to approximately 2 U of VIII:C was subjected to chromatography and characterized using the same procedures as for the hepatocyte sonicate.

Immunoisolation Procedures

Immunoisolation of FVIII polypeptides was performed using vinyl sulfone agarose (Mini-Leak, Chem-En-Tek, Copenhagen) coupled with a haemophilic immunoglobulin specific for the light chain of FVIII (3 mg/ml of gel), strictly following manufacturers recommendations. After blocking against unspecific binding with 0.1 M ethanolamin, 2 ml aliquots of gel were incubated for 48 h at 4° C with either 8 ml of hepatocyte lysate supernatant of 20×10^6 cells, or 10 ml of EDTA plasma, in each case brought up to a total volume of 20 ml using Tris 0.15 M with NaCl 0.15 M (TBS) supplemented with (final reaction concentrations): Benzamidine 2 mM, Aprotinin 100 KIE/ml, NaN_3 0.02%, and EDTA 5 mM. After ten washes with TBS containing the same additives as above plus Tween-20 0.1%, the gel was eluted with 2 ml of TBS containing SDS 2%, β-mercaptoethanol 5%, and EDTA 5 mM at 65° C for 20 min. Finally, eluates were concentrated to approximately 200 µl using CX-30 filters.

SDS-PAGE Electrophoresis and Immunoblotting

After heating at 95° C for 5 min, samples of immunoisolated material were electrophoresed in a modified Laemmli system (24) using 7.5% separation gel and 4% stacking gel. Molecular weight markers ranging from 29–205 kDa were from Sigma. Following electrophoresis, the gels were silver stained, or polypeptides were transferred onto a 0.45 µm nitrocellulose filter (Schleicher & Schuell, Dassel, FRG) using a semi-dry electrotransfer method (25) (Jancos, Copenhagen, Denmark).

Detection of Polypeptides on Nitrocellulose

Prior to incubation, non-specific binding was blocked using TBS containing bovine serum albumin at 0.5%. Subsequently, nitrocellulose filters were incubated at room temperature overnight with a murine monoclonal antibody against FVIII (CMD, Bournemuth, UK), diluted 1:500 in TBS containing Tween-20 0.1% (TBS-Tween). After 4 washes in TBS-Tween filters were incubated for 1 h with biotinylated rabbit anti-mouse immunoglobulin (Dakopatts), and, after 4 additional washes, with avidinperoxidase complex (Dakopatts) for further 1 h. After four washes as above, development was performed using the chromogen 3-amino-9-ethylcarbazol in sodium acetate buffer containing substrate hydrogen peroxide. Strips of molecular weight markers were stained with 0.1% Amido Black in 40% methanol, 10% acetic acid.

Table 1 Medium F VIII:Ag during culture of human hepatocytes

Hours	1	2	7	12	36	84
FVIII:Ag mU/ml	49 ± 33	53 ± 11	31 ± 4	7*	37 ± 9.5	30 ± 6.5

Legend: VIII:Ag in media (mean ± SD) of human hepatocytes (0.75×10^6 cells/ml) during culture (n = 4). Medium RPMI-1640 supplemented with Ultroser-G 4% was changed (>75% of volume) at each point of measurement.
*Two cultures were below the measurement range.

Study of Hepatocytes in Culture

Freshly isolated cells were plated at a cell-density of 0.75×10^6 cells/ml in culture trays, Nunc Multidish (Nunc, Roskilde, Denmark), or trays with filter supports (Costar Transwell, Badhoevedorp, The Netherlands). Medium was RPMI-1640 supplemented with either Ultroser-G at 4% (LKB, Villeneuf, France), or Fetal Calf Serum at 5%, in both instances supplemented with: insulin 10^{-7} M (Nordisk Gentofte, Copenhagen, Denmark), dexamethason 10^{-7} M (Merck, Sharp & Dohme, West Point, PA), glucagon (Novo Industry, Bagsvaerd, Denmark), glutamine 3 mM, penicillin 10^6 U/l, streptomycin 100 mg/l, and in one instance epidermal growth factor (EDGF) at 10 ng/ml (Serva, Heidelberg, FRG). Cell-cultures were kept at 37° C in a humidified atmosphere with CO_2 at 5%. Medium was changed as indicated in Table 1 and Fig. 3, substituting >75% of medium at each change. Culture medium was subjected to ELISA quantitation of albumin, VIII:Ag, vWf:Ag, α_1-antitrypsin, and α_2-macroglobulin.

Study of Other Cell Cultures

Hep-G2 cells and human fibroblasts, seeded at cell densities approximating 1×10^6 cells/ml in the same medium as above, were kindly provided by Dr. O. Sonne, Institute of Physiology, University of Aarhus. Cultures of human peripheral monocytes were established as previously described (22), but in here, medium was supplemented with Ultroser-G at 4%, but hormones were not included (insulin, glucagon and dexamethason).

Results

VIII:Ag was recorded in buffers of each step used for isolation and purification of hepatocytes. As determined by ELISA, we found traces of VIII:Ag in the collagenase buffer by the end of perfusion, showing that during this phase, either residual plasma material was present, or that some cells disrupted during this step. Likewise, the albumin containing buffers for wash of bulk cellular material contained VIII:Ag at 30–90 mU of VIII:Ag/ml. However, resuspension buffers used in the steps following the Percoll gradient centrifugation had no VIII:Ag (values below background blind). Supernatants of freshly isolated and purified hepatocytes sonicated in Hanks buffer contained 165–250 mU of VIII:Ag and around 4 mU of vWf:Ag per 10^6 cells (four isolation experiments). When concentrated supernatant of hepatocyte lysate was subjected to Mono-Q chromatography, a single peak of VIII:Ag reactivity appeared (Fig. 1A) with maximum in fraction No. 40. This peak eluted along with (Fig. 1B) the first peak (fraction No. 39) out of two (second peak: fraction No. 52) of VIII:Ag reactivity of the Hemofil-M preparation. As can be seen from both figures, the OD^{214nm} recordings show that practically all non-VIII:Ag peptides were excluded ahead of the VIII:Ag material. Immunoisolated, reduced and silver stained VIII:Ag polypeptides extracted from normal plasma by a light chain specific haemophilic antibody are shown in Fig. 2, lane A, and polypeptides isolated from hepatocyte lysate in lane B. Lanes C (plasma) and D (hepatocyte sonicate) demonstrate the corresponding nitrocellulose blots after reaction with a murine monoclonal antibody against the light chain of FVIII. Lanes A and B contain numerous bands, some of which are similarly sized. In both lanes a 78–79 kDa band of moderate density is found. On the immunoblot, lanes C and D, this band appears as a doublet. A number of bands of lower molecular weight also are reactive with the monoclonal antibody with apparant sizes in the range of 54–73 kDa. Possibly these represent preexisting breakdown products of FVIII in plasma and hepatocyte sonicate, or result from degradation of the light chain of FVIII during immunoisolation.

Serum-Free Cultures

During culture, shape and details of cells were preserved, but some reduction of size was observed. Cytoplasmatic projections

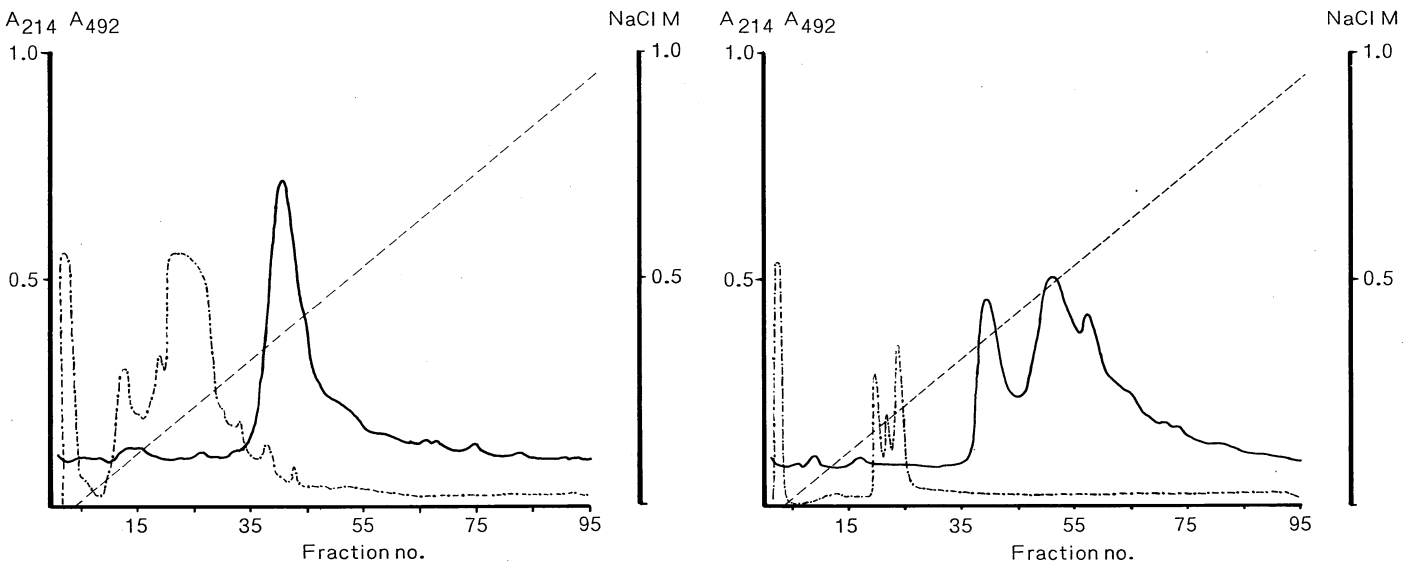

Fig. 1 FVIII:Ag in freshly isolated human hepatocytes. A: 15 × 10^6 hepatocytes (3 × 10^6/ml) were sonicated (30 µ, 60 sec) in Hanks balanced salt solution, centrifuged (5,000 × g, 10 min) and filtered through a Millipore GV filter (pore-size 0.20 µm) for removal of cellular debris. The filtrate was concentrated from 5 ml to 1 ml using a Millipore CX-30 filter and finally was 200 µl subjected to chromatography (Mono-Q HR 5/5). Absorbancy at 214 nm is indicated (–·–·–) and FVIII:Ag ELISA recordings (492 nm) of fraction samples (——). B: Factor VIII concentrate Hemofil-M (Baxter Travenol, Glendale CA), subjected to the same chromatographic procedures and recordings as used in A

developed sporadically only, and cells showed practically no tendency to spontaneous adherence. The VIII:Ag ELISA blind-value was unaffected by Ultroser-G at 4%. In four hepatocyte isolations, cultures showed a significant production of VIII:Ag. During the first 12–24 hours of culture, a high release of VIII:Ag was observed, as can be seen from Table 1 and Fig. 3, showing values of VIII:Ag in the >75% aspirated medium at changes. The inset of Fig. 3 illustrates the corresponding calculated secretions per 24-hours and the accumulated productions of VIII:Ag per 10^6 cells. Maximum secretion of VIII:Ag was 79.3 ± 13.5 mU per 24 h (mean ± SD, n = 3). In culture experiment where cells were seeded in Falcon trays (no culture support), a 24-hour production rate of VIII:Ag per 10^6 cells at 19.7 ± 7.4 mU of VIII:Ag (mean ±SD, n = 24) was found. In all cultures, vWf:Ag was either at, or below the limit of detection (4 mU/ml). Medium albumin production was fairly constant at around 1.3 mg per 0.75 × 10^6 cells/24 h. The maximal 24 h production rate of α_1-antitrypsin was at 570 ± 48 ng (mean ± SD, n = 4) and of α_2-macroglobulin at 51.8 ± 10 ng (mean ± SD, n = 4), which is slightly less that previously found during cultivation of hepatocytes reported elsewhere (21).

Hep-G2 cells were cultivated using the same medium as for hepatocytes. In two subcultures of the same cell-line investigated for two weeks, we found an average 24 hour production of VIII:Ag at 34.1 ± 2.7 mU from close to 10^6 cells. No vWf:Ag could be detected. Media from human fibroblasts and monocyte-macrophages cultivated for 21 and 12 days respectively had no VIII:Ag by ELISA (n = 2, each).

Discussion

This investigation is the first study reporting FVIII synthesis in a human cell. In the past, several investigations have indicated that the liver is a production site of FVIII. The in vivo significance of FVIII synthesis in whole liver was established in a case of liver transplantation to a haemophilia A patient in whom normal VIII:C was recorded after the operation. In vitro, results from two immunolocalization studies of liver tissue sections, employing exactly the same monoclonal antibody against FVIII (14, 15), results were inconsistent, as both showed that VIII:Ag immune reactivity was present in liver endothelial cells, whereas one (15), also detected VIII:Ag material in the hepatocyte endoplasmatic

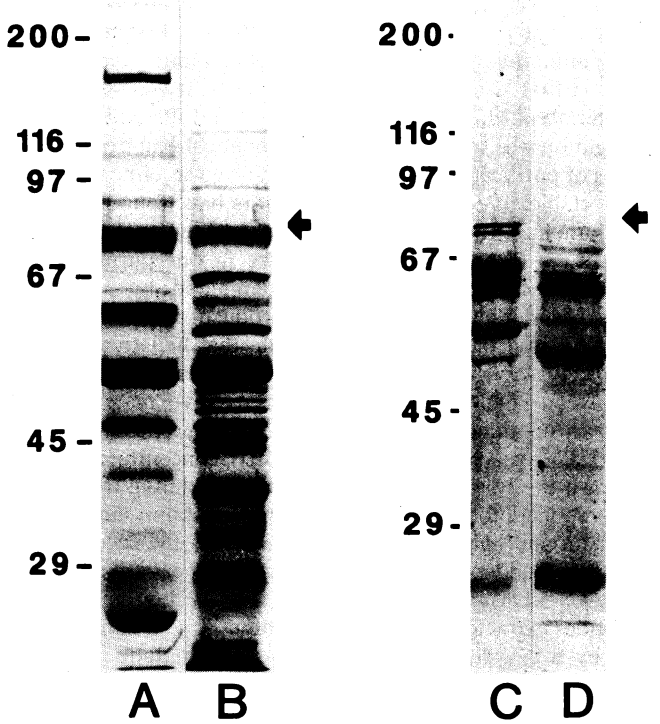

Fig. 2 Silver-stained SDS-PAGE gel of immunoisolated and reduced FVIII polypeptides. Lane A: EDTA-plasma, lane B: Hepatocyte sonicate. C and D are Western blots of same gel as in A+B, reacted with a monoclonal antibody against FVIII. Lane C: EDTA-plasma, lane D: hepatocyte sonicate

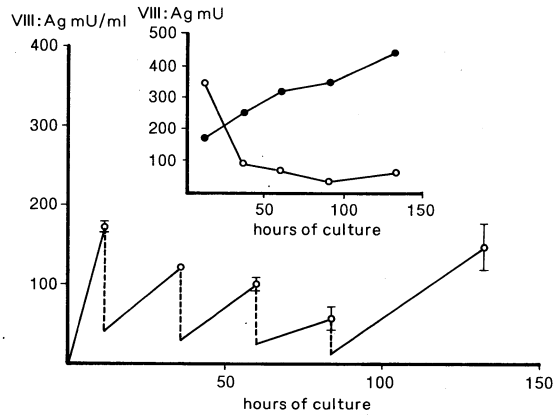

Fig. 3 Factor VIII:Ag ELISA values (mean ± SD) of medium from three cultures of human hepatocytes (0.75×10^6 cells/ml). Cells were cultivated on nylon filter supports (Costar Transwell, Costar Europe, Badhoevedorp, Netherlands) in medium RPMI-1640 supplemented with 4% Ultroser G, insulin 10^{-7} M, glucagon 10^{-7} M, dexametason 10^{-7} M, glutamine 2 mM, penicillin 1×10^6 U/l, streptomycin 100 mg/l, epidermal growth factor (EDGF) 10 ng/l at 37° C in humified atmosphere with 5% CO_2. 75% of the medium was replaced with fresh medium at times indicated. Oblique lines signify the amount of VIII:Ag secreted between changes. The inset shows accumulated FVIII:Ag secretion per 10^6 cells (●) and calculated VIII:Ag production per 10^6 cells/24 h (○). From the same isolation, the supernatant of sonicated cells contained 200 mU of VIII:Ag per 10^6 cells

reticulum. Human liver is not readily accessible for study. Cultures established from unseparated bioptic material will not be able to disclose an exact site of FVIII production, since cultures invariably will contain mixtures of different cells including Kupffer cells, endothelial lining cells, and hepatocytes. We used whole liver tissue pieces subjected to the two-step collagenase perfusion technique, that predominantly releases the hepatocyte fraction (26). By immunologic tests using monoclonal antibodies against Mø cells (CD11, CD14), and functional tests, it was shown that cell material purified by this procedure and Percoll gradient centrifugation was free of significant amounts of Kupffer cells. In all stages of purification and culture, vWf:Ag was either very low or indetectable. As numerous investigators have found evidence that endothelium, including liver sinusoidal lining cells, contain vWf (1, 2, 3, 27), the lack of vWf:Ag in our purified cells, indicate that cultures were not contaminated by significant amounts of endothelial cells. A stable production of albumin, α_1-antitrypsin and α_2-macroglobulin indicate that hepatocytes were metabolically active during culture (28). Concentrated hepatocyte lysate gave a single peak of VIII:Ag reactive material after Mono-Q chromatography, appearing in the same position as the first VIII:Ag peak of an ultra-pure FVIII concentrate. Others have shown (8, 29), that this first FVIII peak eluting from a salt gradient applied to the Mono-Q column contains undegraded heavy chain and light chain of FVIII, whereas the second peak represents varied lengths of partially degraded heavy chain (sized down to 93 kDa) plus light chain of FVIII. Immunoisolated polypeptides extracted from human hepatocyte lysate and normal plasma by a FVIII light-chain specific haemophilic antibody, contained a 78–79 kDa polypeptide band in the reduced and EDTA treated samples. This band appeared as a doublet at 78–79 kDa in the electroblot after reaction with a monoclonal antibody specific against the light chain of human FVIII, indicating that the light chain of FVIII were present in both these materials. However, in the silver stained gel, a number of other and less well defined bands with lower molecular weight were also visible. Some of these, sized at 54–73 kDa, reacted with the monoclonal antibody. These bands could be light chain polypeptides of FVIII degredaded by thrombin or FXa (29, 30).

During culture, hepatocytes secreted VIII:Ag, as judged from the rise in VIII:Ag of fresh medium. Moreover, the accumulated production of VIII:Ag exceeded the measured content of VIII:Ag in sonicate supernatant of cells from the same isolation. An immediate release of VIII:Ag was seen after plating of cells. This may be due to an acute phase reaction. A similar abrupt increment was not seen with α_1-antitrypsin or α_2-macroglobulin. However, some decay of cells cannot be ruled out at this stage of culture. In the following days, cell culture media showed a more stable production of VIII:Ag, as can be seen comparing data from Table 1 with those of Fig. 3 (inset). The highest production rates of VIII:Ag were seen from cells plated on a permeable cell support. An overall production rate ranging from approximately 20 to 80 mU of VIII:Ag/10^6 cells per 24 hours was recorded.

In support of results from cultures of hepatocytes, we also noted a significant production of VIII:Ag material from a neoplastic cell-line of human hepatocytes, Hep-G2, secreting for longer periods of time around 30 mU of VIII:Ag per 24 hours. In these cultures, cell-densities were comparable to those used for plated hepatocytes. In neither media of cultivated hepatocytes nor Hep-G2 was significant amounts of vWf:Ag detected. As liver is the largest gland of the human body, it could be assumed, that hepatocyte produced FVIII is a major determinant of circulating plasma FVIII. Assuming the biological half-life of FVIII at 15 hours, a demanded 24 h production rate at 2,400 U will be able to maintain a plasma level of FVIII at 1 U/ml in a person with a plasma volume of 3,000 ml. If each Mio hepatocytes produce some 30 mU/24 h, then a total number of around, $1{,}000 \times 10^9$ will be be sufficient for this production.

The site of coupling of FVIII onto vWf can only be speculated. It is possible, that FVIII from the hepatocyte is exported to the liver endothelial cell and bound to vWf here before the complex is being released to systemic circulation. This explanation helps understanding previous findings (14, 15), that both vWf and FVIII-immune reactive material could be detected in the endothelial sinusoidal lining cells of human liver. However, cleared and internalized FVIII from plasma should also be considered.

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