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Intracellular Localization of Fibrinogen.* (27635)

MARION I. BARNHART AND GORDON F. ANDERSON

Department of Physiology and Pharmacology, Wayne State University, College of Medicine, Detroit, Mich.

Numerous reports about experimental work on cytotoxic drugs in animals, as well as clinical and pathological facts from human patients suffering liver disease, are the basis for attention to the liver and its role in the synthesis of many blood proteins. Miller and Bale, utilizing perfusion technics and radioactive isotopes, were the first to report that a physiologically normal liver produces plasma albumin and alpha and beta globulins, including fibrinogen(1). The liver is composed of multiple cell types and which cell type synthesizes any one specific plasma protein remains to be determined. We have employed immunochemical(2,3) and cellular fractionation procedures(4,5,6) to gain information on the cellular site of fibrinogen production. This protein was found intracellularly in the microsome and soluble fractions of liver parenchymal cells. The microsomal localization probably indicates the sites of synthesis while the location of fibrinogen in the soluble part of the cell represents storage.

Materials and methods. Specific antifibrinogen sera against either canine or bovine fibrinogen were produced and used to identify the cellular and intracellular localization of fibrinogen. Fibrinogen was purified from fresh plasma by ethanol fractionation followed by glycine, polyvinylpyrrolidinone and tannic acid extraction of impurities(7). Simonetti, Casillas and Pavlovsky(8) reported that tannic acid fractionation permits a complete separation of antihemophilic factor from fibrinogen. Our fibrinogen was 97-100% clottable, easily soluble after freeze drying, migrated as a homogeneous beta globulin on immunoelectrophoresis. The fibrinogen appeared to be a single antigenic component when tested against antiplasma or antifibrinogen in agar gel systems for 10 days. This fibrinogen was free of profibrinolysin, according to immunologic tests and biologic activity studies enduring 3 days after attempts were made to activate with either streptokinase or urokinase. Purified fibrinogen was mixed with $Al(OH)_3$ as adjuvant and given as a single intramuscular injection to rabbits(7). Potent antisera were produced within 10-14 days and checked by immunologic procedures to establish the individual specificity of each serum. Antifibrinogen sera were selected which did not form precipitin bands against either purified prothrombin, profibrinolysin, gamma globulin or albumin. The reactivity of the antisera was lost by adsorption with purified homologous fibrinogen. Adsorption of the antifibrinogen serum with dried whole serum did not appreciably alter the potency, and strong precipitin bands still formed against purified fibrinogen. *Agar gel diffusion systems* as modified by Wilson and Pringle (2,3) provided the immunologic evaluation. Two different kinds of cellular material were

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obtained from canine and bovine livers. *Intact and functional liver* parenchymal cell concentrates free of Kupffer cells were prepared from perfused liver according to St. George(6) with Dowex 50W resin columns. *Sub-cellular fractions of liver* were separated by using the procedures of Schneider and Hogeboom(4) and deDuve and associates(5). Studies were made on the total cell homogenate (TCH), nuclei (N), cytoplasmic extract (CE), heavy mitochondria (HM), light mitochondria (LM), microsomes (M) and soluble (S) fractions. All cell fractions except the soluble fraction probably contained associated ribosomes even after repeated washings. The nuclear fraction was the most heterogeneous. *Cell fractions of other organs.* Total cell homogenates were prepared from canine spleen, kidney and pancreas and from chicken liver. Only the chicken liver preparation provided a valid control since the other cellular homogenates represented too heterogeneous a cell population and contained many blood cells whose association with fibrinogen may be explained on the basis of adsorption. Chicken liver homogenates did not form a precipitin reaction when tested with either of the antifibrinogens. Such negative findings support the view that the precipitin reaction with canine and bovine materials is specific.

Results. Fibrinogen in hepatic parenchymal cells. A concentrate of intact and viable bovine parenchymal cells formed a precipitin band in agar gel when reacted against anti-bovine fibrinogen. This band identified with a plasma constituent which migrated as a beta globulin in immunoelectrophoresis, and was evidently fibrinogen. The precipitin line also cross reacted with purified bovine fibrinogen. When a multi-spectrum anti-bovine serum was used several precipitin bands formed against parenchymal cells. These cells very likely were producing several different plasma proteins. By using highly purified bovine prothrombin and fibrinogen it was possible to determine which band related to prothrombin, and which one was fibrinogen. Fibrinogen appeared to be in low concentration in the parenchymal cell concentrate. By ultrasonic destruction of cell membranes and internal structure more fibrinogen was made available for diffusion, and a heavier precipitin band was formed (Fig. 1). Similar results were obtained using canine parenchymal cell concentrates. Differential cell counts on Leischman stained smears of a typical parenchymal cell fraction showed 96½% parenchymal cells, 3% free parenchymal cell nuclei and 0.5% red blood cells. Neither Kupffer cells, leucocytes nor platelets were observed in counts up to 500 or 600 cells. Thus it seems reasonable that the immunologic reaction represents fibrinogen localization within the parenchymal cells.

Intracellular localization of fibrinogen. To study the intracellular localization of fibrinogen, cell fractions were obtained from both bovine and canine livers and reacted against the homologous anti-fibrinogen serum. Three, 4 and 7 compartment agar diffusion plates were used. Similar results indicative of the intracellular localization of fibrinogen were obtained in both species. Precipitin bands formed with the total cell homogenate, cytoplasmic extract, microsomal and soluble fractions (Fig. 2). These bands represented identical constituents in these cell fractions since they formed bands of complete identity when checked against each other (Fig. 3). Each precipitin band also identified completely with fibrinogen purified from the appropriate species. There does not seem to be an appreciable localization of fibrinogen in either the nuclear or mitochondrial fractions (Fig. 4). Occasionally a faint precipitin band formed with these fractions but repeated washing reduced or eliminated the reaction so that microsomal contamination of the nuclear and mitochondrial fractions probably accounted for the reaction. Similar washing of microsomes did not appreciably alter the precipitin reaction. This makes unlikely the view that adsorption of extracellular fibrinogen during fractionation is an explanation for the microsomal reaction. Cell fractions also were prepared from a severely hypoprothrombinemic dog following coumadin treatment. In this animal, circulating prothrombin was 4 U/ml, the prothrombin time was 3 minutes, while fibrinogen remained normal. As in the normal animals, TCH, CE, M and S fractions all gave distinct precipitin bands when re-

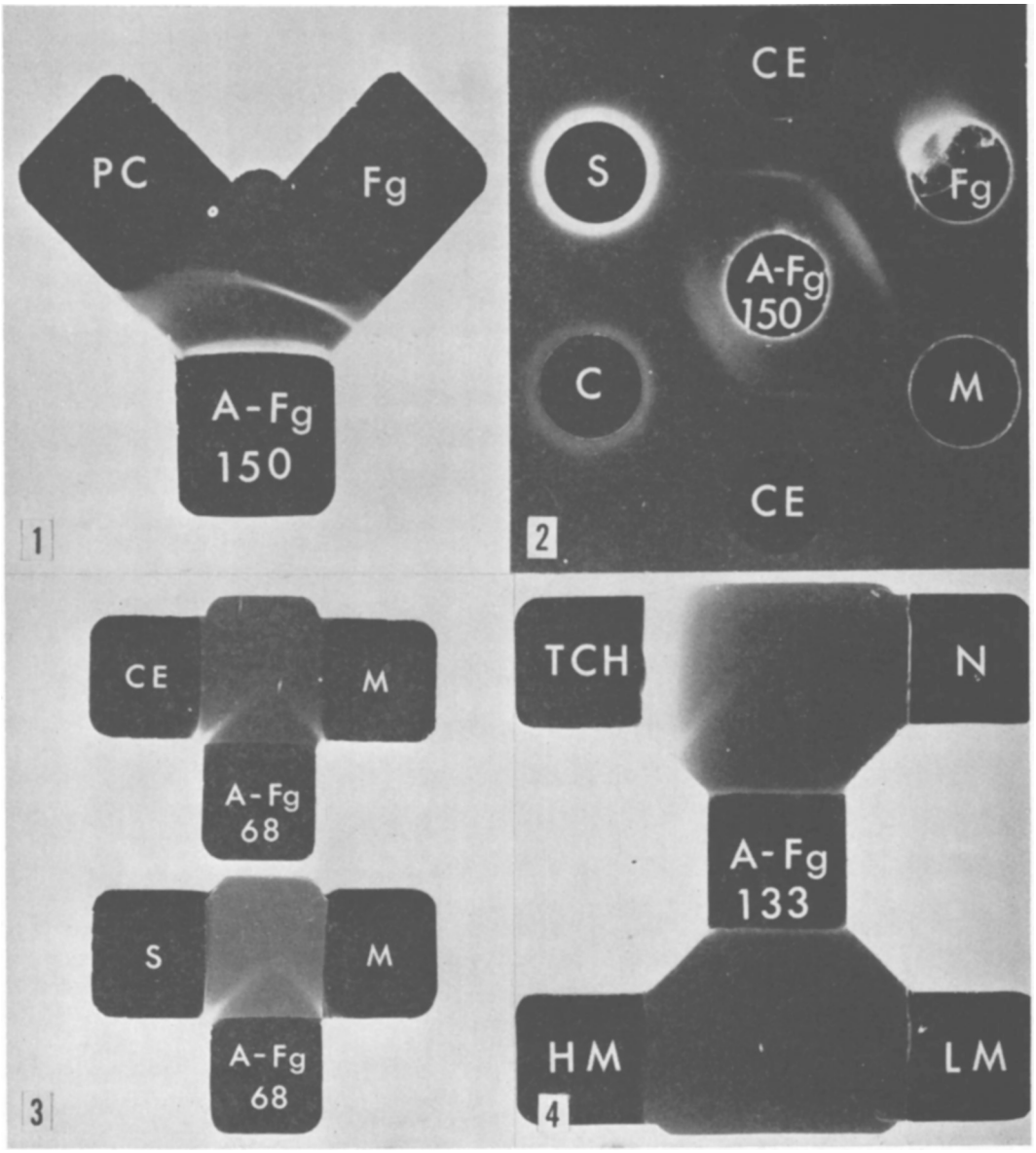

FIG. 1. Identification of fibrinogen within bovine liver parenchymal cells. Note the band of identity between sonically-treated parenchymal cells (PC) and purified bovine fibrinogen (Fg) when reacted against a specific anti-bovine fibrinogen (A-Fg). A 10% PC fraction in 0.25 M sucrose was used.

FIG. 2. Intracellular localization of fibrinogen in bovine liver cell fractions. The precipitin band formed continuously between fibrinogen and the cellular fractions, cytoplasmic extract (CE), soluble (S), microsomes (M), and the specific anti-bovine fibrinogen (A-Fg) and also identified partially with a concentrate (C) of plasma proteins containing fibrinogen. All cell fractions were 10% suspensions in 0.25 M sucrose.

FIG. 3. Intracellular localization of canine fibrinogen to cytoplasmic extract (CE), soluble fraction (S) and microsomes (M). Immunologically identical components (fibrinogen) were found in these cell fractions as indicated by the continuous precipitin bands formed against anti-canine fibrinogen (A-Fg). CE and S were 10% suspensions while M was a 30% suspension in 0.25 M sucrose.

FIG. 4. Absence of fibrinogen in nuclear and mitochondrial fractions of canine liver. Note localization of fibrinogen indicated by the precipitin band formed between total cell homogenate (TCH) and anti-canine fibrinogen (A-Fg). No precipitin bands formed with the nuclear fraction (N) or with heavy mitochondria (HM) or light mitochondria (LM). All fractions were 10% suspensions in 0.25 M sucrose.

acted against anti-dog fibrinogen. When these hypoprothrombinemic fractions were tested against antiprothrombin they gave either a reduced reaction or no precipitin bands formed.

Discussion. Although the liver has long been regarded as the source of most of the plasma proteins with the exception of gamma globulins, definitive studies have been lacking to indicate the cell type concerned with a specific protein synthesis or storage reaction. Only in the case of prothrombin(9,10) has the liver parenchymal cell been identified, using fluorescent antibody technics, as the site of synthesis in man, dog and cattle. There are several reports on albumin synthesis (11-13) using microsomes obtained from all the cell types comprising the liver. Peters (11), with agar gel diffusion studies, found that chicken serum albumin was bound to microsomes and could be released with lipid solubilizing agents. Information concerning fibrinogen synthesis has been conflicting. Perfusion and radio isotope work indicated the hepatic production of fibrinogen(1,14). Gitlin and associates(15) used a fluorescent anti-fibrin stain in a variety of human tissues and were unable to establish a cellular localization for fibrinogen.

The present study provided a direct demonstration of the localization of fibrinogen in hepatic parenchymal cells. Reticulo-endothelial cells did not account for the immunologic reaction with specific anti-fibrinogen serum since the isolated, intact, parenchymal cell concentrate did not contain R. E. cells. Furthermore, fibrinogen was predominantly associated with the microsomal and soluble fractions of liver cell homogenates. This subcellular localization in the microsomes is the basis for our opinion that the site of fibrinogen synthesis is the ribonucleoprotein structures, corresponding morphologically to endoplasmic reticulum and long associated with a variety of radioactive isotope uptake reactions possibly reflective of protein synthesis. On the other hand, the concentration of fibrinogen in the soluble fraction of liver cells may indicate a storage area for fibrinogen until needed in the circulating blood.

Summary. Hepatic parenchymal cells contained fibrinogen when immunologic technics and isolated, functional, parenchymal cell concentrates were applied in this study. With cellular fractions the greatest concentrations of fibrinogen, intracellularly, were in the soluble and microsomal fractions. These data are compatible with the view that fibrinogen is synthesized in the microsomes, released to the soluble part of the parenchymal cell and stored there until additional fibrinogen is required in the blood.

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