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HUMAN PLASMA PROTEIN C IN LIVER DISEASES: A HISTOPATHOLOGICAL STUDY

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ABSTRACT

Protein C is a vitamin K-dependent glycoprotein present in human plasma at a concentration of 4 um/ml. All vitamin K-dependent proteins are procoagulant, whereas Protein C exerts and anticoagulant activity. To detect Protein C in hepatocytes, monoclonal antibodies were used. We found that Protein C is present in liver cells, which probably are the site of its production. Furthermore, this protein is not present in Kupffer cells nor is it found in endothelial cells lining the bile ducts. In several pathological conditions in which intravascular coagulation is present, the serum level is decreased. This can be verified morphologically on tissue sections by immunohistochemical staining. In this study, we were able to demonstrate that in liver disease the amount of Protein C in hepatocytes is reduced depending on the severity of cell damage.

Key Words: Protein C, Anticoagulant Activity, Liver Disease.

INTRODUCTION

PROTEIN C is a vitamin K-dependent glycoprotein [1,2,3] present in the human plasma at a concentration of 4 um/ml[13].

It is composed of two polypeptide chains linked by disulfide bridge(s). The molecule contains five carbohydrate chains linked to the heavy chain (MW = 41,000), and two carbohydrate chains linked to the light chain (MW = 21,000); the carbohydrates include hexose, glucosamine, and neuraminic acid [4,13].

The active form of protein C (activated protein C) inactivates coagulation factors Va and VIII: C by a limited proteolysis [5,6,8]. Protein C is activated by some proteo-

lytic enzymes, such as thrombin and trypsin, and its activation is increased about 20,000 fold by thrombomodulin, produced by endothelial cells.[7-10]

A deficiency of protein C might lead to thrombotic disease [11,12]. In hospitalized patients with intravascular coagulation protein C serum levels are reduced[9].

Since the liver seems to be the source of protein C, this study sought to evaluate probable correlation between liver diseases and protein C detectable in hepatocytes by immunoperoxidase staining procedure.

MATERIALS AND METHODS

In this study cases from the Department of Pathology at Loyola University Medical Center were utilized. The cases considered include several liver diseases: fatty metamorphosis, cirrhosis, cholestasis, and non-specific hepatitis.

Detection of the protein C in paraffin-embedded sections was accomplished by using monoclonal antibodies against human plasma protein C (Mallinckrodt Diagnostic Product Division, Maryland Heights, MO).

The preparation contained 0.5 mg of protein in 0.5 ml of buffer (50 mM boric acid, 0.2 M NaCl, pH 7.5) with no additional preservatives.

This monoclonal antibody was purified from mouse ascites fluid by salt fractionation followed by gel filtration and ion exchange chromatography.

The antibody producing cell line was obtained by the hybridization of a murine myeloma line (sp 2/0-Ag 14) with spleen cells from a BALB/C mouse immunized with human plasma protein C.

The murine immunoglobulin class is IgG1, pI = 7,0 and the specificity is for heavy chain of non-activated human plasma protein C. There is no binding to activated protein C, and the binding of this antibody inhibits activation of human plasma protein C.

IMMUNOPEROXIDASE STAINING PROCEDURE

1. Warm slides at 60°C for 30 minutes.
2. Place slides, without allowing to cool, in two changes of Limonene for 5 minutes each.
3. Rehydrate sections in 100%, 95%, and 70% Ethanol for 2 minutes each.
4. Rinse slides in distilled water.
5. Encircle tissue sections with diamond marker.
6. Place slides in freshly prepared 6% Hydrogen Peroxide (40 ml of PBS and 10 ml of 30% Hydrogen Peroxide) for 11 minutes.
7. Rinse slides with PBS.
8. Wipe excess PBS from around tissue sections.
9. Apply Diluted Primary Antibody (20 ul of antisera in 3 ml of 1% Normal Mouse Serum in PBS) for 30 minutes.
10. Rinse slides in PBS and wipe excess PBS around tissue sections.
11. Apply Diluted Biotinylated Antibody from ABC kit (10 ml of PBS and 1 drop of Concentrated Biotinylated Antibody) for 30 minutes.
12. Rinse slides with PBS and wipe excess PBS from around tissue sections.
13. Apply Diluted ABC Reagent for 45 minutes.

14. Add 10 ml of PBS to Diaminobenzidine (DAB) bottle using a 10 cc syringe and needle, shaking bottle well.
15. Rinse slides with PBS and wipe excess PBS from around tissue sections.
16. Add 0.3 ml of 30% Hydrogen Peroxide to DAB bottle.
17. Apply DAB to tissue sections for 3 to 5 minutes.
18. Rinse slides well in running tap water.
19. Counterstain slides with Modified Harris Hematoxylin, decolorize, and blue using the slide stainer.
20. Dehydrate, clear, and mount using Permount.

RESULTS

Utilizing the immunoperoxidase staining procedure a positive stain visualized with DAB characterizes the presence of intracytoplasmic brown granules. These granules may occupy the entire cytoplasm or may be concentrated in one of the poles of cell and/or in the peri-nuclear area.

Fig. 1. shows a positive reaction for human plasma protein C in normal liver. (P.A.P. $\times 250$) The brown granules are present in the cytoplasm of hepatocytes, demonstrating that protein C is normally present in liver cells, which most likely are the site of its production. The granules are not present in Kupffer cells nor in the lining endothelial cells of bile ducts.

Fatty changes are due to accumulation of neutral triglycerides in the hepatocyte. The cytoplasm of liver cells contains fat vacuoles that displace the nucleus to the periphery.

Fig. 2. (P.A.P. $\times 250$) shows an immunoperoxidase staining for protein C in fatty liver. The reaction is negative in most of the damaged cells. However, a weak positive reaction can be noticed in some hepatocyte, which are in good metabolic condition.

In the case of cholestasis and non-specific hepatitis, the reaction is negative in most of the tissue, but some areas show a positive reaction, and, in some hepatocytes the presence of protein C is clearly evident. (Fig. 3, P.A.P. $\times 250$)

In the case of intracellular cholestasis and mild fatty changes, a strong positive reaction is present in some hepatocytes, whereas some of them show a weak reaction. (Fig. 4, P.A.P. $\times 250$)

In cirrhosis there are marked architectural changes in the liver tissue, and fibrous tissue is formed in reaction to hepatocyte injury and loss. A regenerative activity is also present. The fibrous scars and the regenerative areas determine the formation of nodules, which can vary in size, depending on the cause of cirrhosis (micronodular and/or macronodular).

The immunoperoxidase staining for protein C in cirrhotic liver is negative, with the exception of very few cells in which a weak reaction is present, showing that the hepatocytes are damaged and not capable of producing protein C. (Fig. 5, P.A.P. $\times 250$)

DISCUSSION

Most likely the liver is the site of production of plasma protein C. In several pathological conditions, in which intra-vascular coagulation is present, the serum level of protein C is decreased.

These conditions include: 1. infections such as endocarditis, peritonitis, and mediastinitis; 2. malignancies such as acute promyelocytic leukemia, histiocytic lymphoma, breast cancer, germ cell carcinoma; 3. other conditions: atherosclerosis, acute obstetrical complications, cardiomyopathy[9].

In this study we were able to demonstrate that in several liver diseases (especially alcoholic liver disease), the presence of protein C in hepatocytes (detectable by monoclonal antibodies), is reduced according to the severity of cell damage.

The finding might be due to a cell membrane damage and/or to an alteration in the metabolic activity of the hepatocyte.

Protein C is a glycoprotein, that is for its synthesis the cell utilizes the organelles that are supposed to do this work: ribosomes, rough endoplasmic reticulum, Golgi apparatus, and mitochondria.

The most relevant damage occurring in alcoholic liver disease is detectable in mitochondria (which provide the energy for biosynthesis), that is the abnormal mitochondrial activity might be the cause of a decreased protein C serum level.

However, further investigation is suggested to determine if the rough endoplasmic reticulum (in which protein synthesis occurs), and/or Golgi apparatus (in which carbohydrates are added to the proteins) may be involved.

Ultrastuctural studies of hepatocytes in alcoholic liver disease demonstrated several mitochondrial alterations: swelling, crystalline inclusions, disruption of the outer mitochondrial membrane. The most common mitochondrial change is elongation of the cristae and their aggregation. In some cases a loss of normal mitochondrial matrix granules is observed. Alterations of the rough endoplasmic reticulum are not specific[14].

It is not surprising that severe liver disease is often associated with bleeding tendencies. Although the liver is a major source of coagulation factors, it must be understood that chronic liver failure induces a number of non-hematologic abnormalities that may contribute to bleeding. Portal hypertension with formation of esophageal varices, changes in gastric mucus composition and increased acid secretion which may cause peptic ulceration are important sources of bleeding in severe liver disease. Portal hypertension also causes splenomegaly and concomitant thrombocytopenia that exacerbate mechanical causes of bleeding.

Along with a generalized decrease synthetic rate of all Vitamin K-dependent proteins in liver failure, a marked reduction of the major inhibitors of these factors exist thereby causing an imparied clearance of their activated forms. Overt disseminated intravascular coagulation may occur and with further fall of platelet count, fibrinogen level and increase in fibrin degradation products, exacerbation of bleeding may result. Patients with liver disease have a complex coagulation disorder that may pose considerable problem to the clinician.

As a result of this study we can conclude that in liver diseases where hepatocellular damage is prominent, protein C production is severely restricted. Moreover, the amount of protein C produced is dependent on the extent of cell damage.

Further investigation is warranted to examine protein C production in chronic liver diseases at the ultrastructural level by using more sensitive techniques including Immunoelectron Microscopy.

REFERENCES

Stenflo J., A new vitamin K-dependent protein: purification from bovine plasma and preliminary characterization. J. Biol. Chem. 251: 355, 1976.

Kisiel W. Ericsson L.H., Davie E.W.: Proteolytic activation of protein C from bovine plasma. Biochemistry. 15: 4893, 1976.

Kisiel W., Human plasma protein C: isolation, characterization, and mechanism of activation by alfa-thrombin. J. Clin. Invest. 64:761, 1979.

Walter F.J., Sexton P.W., Esmon C.T. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. Biochem. Biophys. Acta. 571: 333, 1979.

Comp P., Esmon C. Activated protein C inhibits platelet prothrombin-converting activity. Blood. 54: 1272-1281, 1979.

Esmon C.T., Comp P.C., Walter F.J. Functions for protein C, in Suttie J.W. (ed): Vitamin K metabolism and vitamin K dependent proteins. Baltimore, University Park Press, 1980, pp. 72-83, 88-95.

Esmon N.L., Owen W.G., Esmon C.T. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem. 257: 859, 1982.

Marlar R.A., Kleiss, A.J., Griffin J.H. Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. Blood. 59: 1067, 1982.

Griffin, J.H., Moshev D.F., Zimmerman T.S., Kleiss A.T. Protein C, an antithrombotic protein, is reduced in hospitalized patients with intravascular coagulation. Blood. 60: 261, 1982.

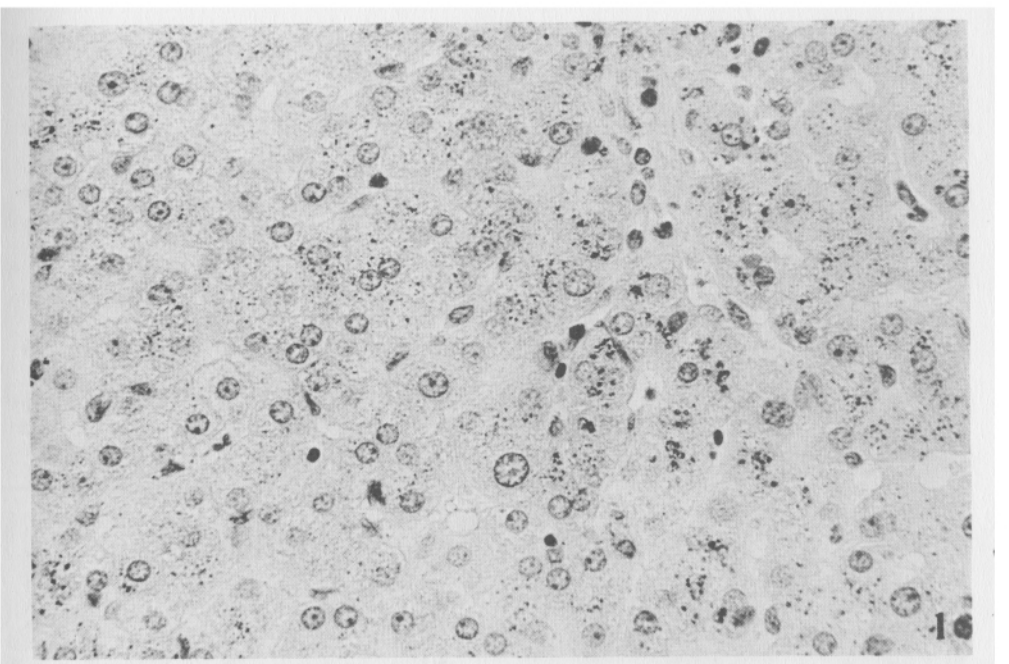
Bertina R.M., Broekmans A.W., van der Linden I.K., Mertens K. Protein C dificiency in a Dutch family with thrombotic disease. Thromb. Haemostas. 49: 1, 1982.

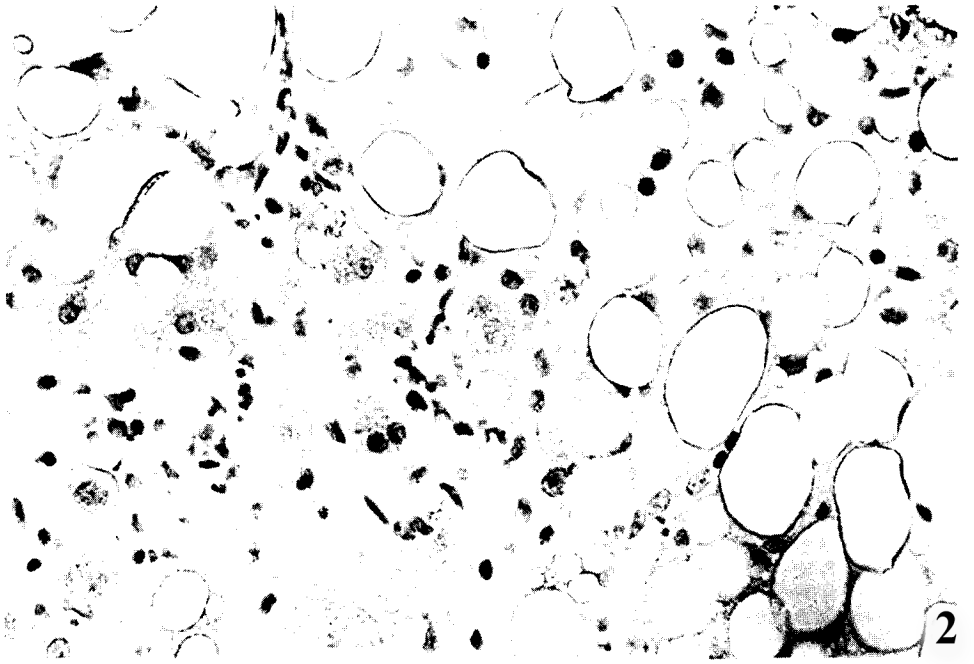
Mannucci P.M., Vigano S. Deficiencies of protein C, an inhibitor of blood coagulation. Lancet. 2: 463, 1982.

Gardiner, J.E., Griffin, J.H. Human protein C and thrombo-embolic diease. Prog. Hematol. 1983. 13: 265-278.

Esmon, C.T., De Bault, L., Carroll, R.C., Comp, P.C., Esmon, N.L. Biochemical and physiological aspects of protein C. Ric. Clin. Lab. 1984 Jul-Sep. 14 (3): 455-468.

M.H. Ma. Ultrastructural pathologic findings of the human hepatocyte. Arch. Path. 94:554-571 (1972).





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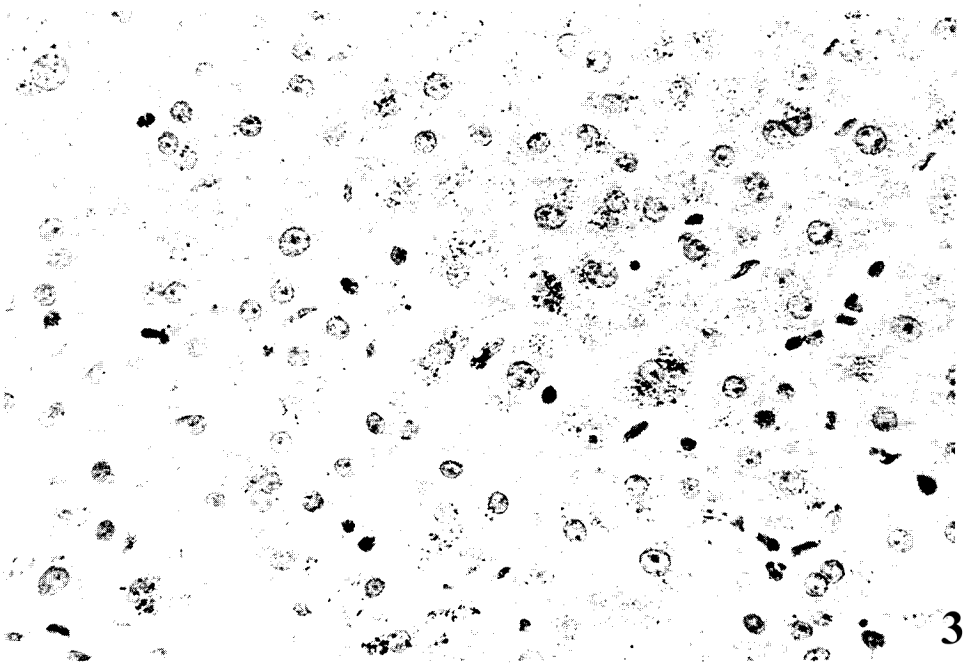

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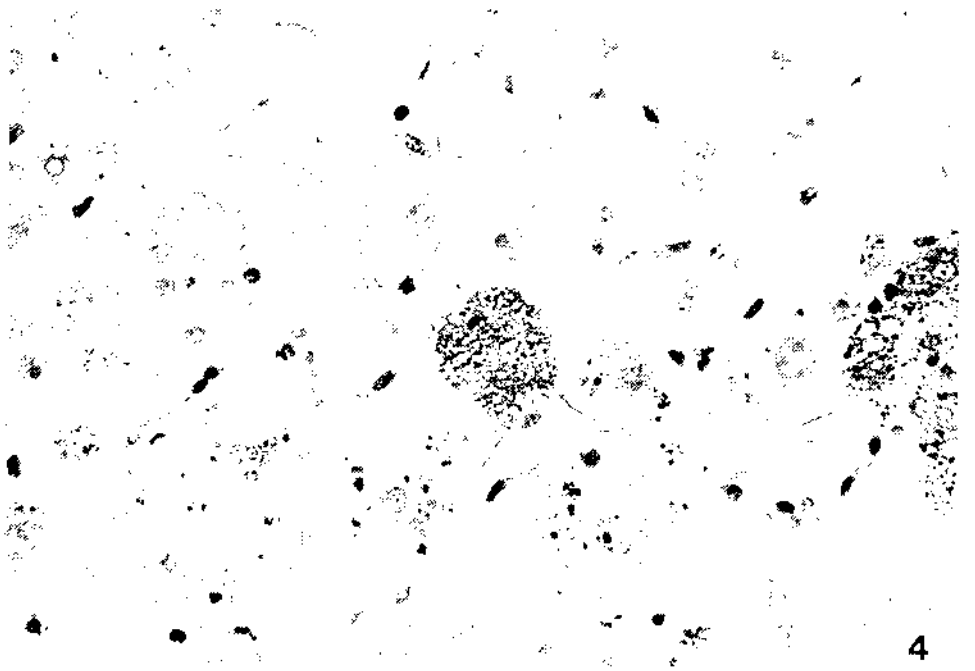
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