Gastroenterologia Japonica
Vol. 22, No. 3

Printed in Japan

—Original Article—

ESTROGEN RECEPTORS IN HEPATOCELLULAR CARCINOMA: IS ENDOCRINE THERAPY FOR HEPATOCELLULAR CARCINOMA LIKELY TO BE EFFECTIVE?

Katsuji KOHIGASHI, M.D., Yoshihiro FUKUDA, M.D. and Hiroo IMURA, M.D.
Second Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

Summary

Specimens of human liver obtained at the time of operation were assayed for cytosolic estrogen receptors by the binding assay and enzyme immunoassay (EIA). Mean estrogen receptor contents determined by the binding assay were 17.8 fmol/mg protein in non-cirrhotic liver, 7.1 in cirrhotic liver, and 0.7 in hepatocellular carcinoma, by EIA the contents were 12.1, 5.9, and 0.8 fmol/mg protein, respectively. There were significant differences among the three groups. In particular, hepatocellular carcinoma specimens contained very little or no detectable amounts of estrogen receptors in either assay. The correlation between the estrogen receptor content determined by the binding assay and that determined by EIA was significant ($r=0.822$, $p<0.001$). It is suggested that the estrogen receptor content decreases with the development of liver cirrhosis and hepatocellular carcinoma and that antiestrogen endocrine therapy for hepatocellular carcinoma may be ineffective.

Key Words: *Binding assay, Enzyme immunoassay, Estrogen receptors, Hepatocellular carcinoma, Liver cirrhosis.*

Introduction

Hepatocellular carcinoma develops from liver cirrhosis in most cases in Japan. Therefore, persistent viral infection, in particular, hepatitis B (HB) virus infection has been considered to play an important role in the development of carcinoma. It is now known, however, that the HB virus does not contain an oncogene in itself, nor is it directly related to endogenous oncogenes. The exact mechanism of hepatocarcinogenesis is still unclear.

On the other hand, liver cirrhosis is associated with active regeneration processes and, under such conditions, either exogenous or endogenous factors may promote hepatocarcinogenesis. Among the endogenous factors, endocrine abnormalities, particularly abnormal estrogen metabolism[1], have been suggested to play some role. This is consistent with case reports on hepatic benign[2–4] and malignant[5] tumors occurring in patients taking oral contraceptives. It has also been shown that exogenous estrogen promotes hepatocarcinogenesis in animals[6–8]. Assum-

Received September 25, 1986. Accepted December 8, 1986.

Address for correspondence: Katsuji Kohigashi, M.D., The Second Department of Internal Medicine, Faculty of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606, Japan.

The authors thank Dinabott Inc. (Tokyo, Japan) for the kind supply of the Abbott ER-EIA monoclonal kit and Dr. H. Ogawa, Daiichi Pure Chemicals Co., for providing us with [^{3}H]DES. This work was supported in part by a Grant-in-Aid for the Special Project Research, Cancer-Bioscience from the Ministry of Education, Science and Culture, Japan.

ing that estrogen has a promoter action, it may exert its effect through a mechanism by which estrogen acts in other target tissues such as uterus and pituitary gland. In other words, the hepatocyte must have estrogen receptors.

Rat[9,10] and human[11,12] liver have estrogen receptors as in reproductive tissues. In particular, Friedman et al.[13] showed that human hepatoma contains estrogen receptors and suggested the potential usefulness of endocrine therapy for hepatoma as for breast cancer.

In this study, we assayed liver specimens obtained from liver cirrhosis, hepatocellular carcinoma and other diseases for estrogen receptors to explore whether abnormal estrogen metabolism takes part in hepatic oncogenesis and whether endocrine therapy would be useful for the treatment of hepatocellular carcinoma.

Materials and Methods

1) Tissues

Liver tissues were obtained at operation in the First and Second Departments of Surgery, Kyoto University, from 32 patients. These specimens were from resected livers or biopsies taken for histological examination with the permission of the patients. Twenty patients had hepatocellular carcinoma associated with liver cirrhosis (19 cases) and chronic hepatitis (one case). There were 12 males with a mean age of 53.7 ± 6.9 (Mean ± SD) years, and 8 females with a mean age of 58.0 ± 11.6 years. The other patients consisted of a male with liver cirrhosis associated with esophageal varices (age 57), a male with cholangiocarcinoma (age 59), a male with gastric cancer (age 43), a female with primary biliary cirrhosis (age 49) and 7 patients (2 males; age 54.0 ± 16.9 and 5 females; age 62.0 ± 14.4) with gallstones. The hepatitis B-surface (HB_s) antigen was positive in 6 out of 20 hepatocellular carcinoma patients and negative in other diseases. α-Fetoprotein was above 500 ng/ml in 6 of the 20 hepatocellular carcinoma patients. None of the patients had a daily alcohol intake of more than 50 g.

From these patients, 45 liver specimens were obtained; 13 of hepatocellular carcinoma, 20 of cirrhotic and 12 of non-cirrhotic liver tissues. Twelve hepatocellular carcinoma tissues were Edmondson grade II and one was grade III. Necrotic tissue was not used. The liver tissue obtained was immediately frozen and stored in liquid nitrogen until use.

2) Chemicals

[Monoethyl-^{3}H] diethylstilbestrol (DES), 104 Ci/mmol, was obtained from Amersham, Buckinghamshire. Tris, ethylenediaminetetraacetate (EDTA) disodium salts, dextran and sodium molybdate were purchased from Nakarai Chemicals Co., Kyoto, and Norit A and bovine serum albumin (BSA) from Sigma Chemical Co., St Louis, Mo. All other reagents used were of analytic grade.

3) Preparation of cytosol

Approximately 0.5–1 g of tissue was used for each assay. All procedures were performed at 0°C. The liver was minced and homogenized with a glass homogenizer in 6 volumes of TE buffer [0.01 M Tris-HCl, 1.5 mM EDTA, 20 mM sodium molybdate, pH 7.4]. The resultant homogenate was centrifuged for 60 min at 105,000×g. The supernatant (cytosol) was removed to avoid lipid contamination. The protein concentration in the cytosol was 10–20 mg/ml, as determined by the method of Lowry et al.[14] using BSA as the standard. Before the binding assay, the cytosol was diluted with a TE buffer to a final protein concentration of 5 mg/ml.

4) Binding assay

Aliquots of hepatic cytosol (200 μl) were incubated with several concentrations of [^{3}H]DES over a range of 0.1–2 nM in the

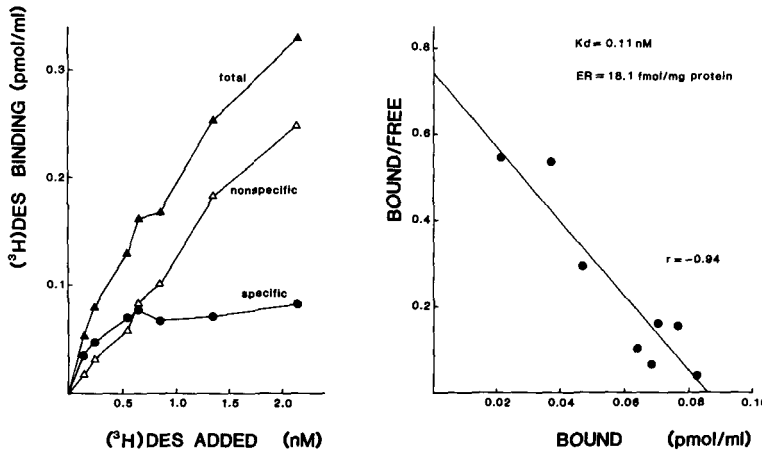

Fig. 1. Specific binding of [³H]DES to liver cytosol from a female patient with gallstones (left) and Scatchard analysis (right). Duplicate 200 μl aliquots of cytosol were incubated with various concentrations of [³H]DES (0.1–2 nM) for 18 hr at 0°C in the presence or absence of 200 fold unlabeled DES. Specific binding was calculated by subtracting the non-specific binding level from the total binding level.

presence (non-specific binding) and absence (total binding) of a 200-fold excess of unlabeled DES for 18 hr. The unbound [³H]DES was removed by using dextran-coated charcoal. The radioactivity in the supernatant was measured by a liquid scintillation counter. Specific binding was obtained by subtracting the non-specific binding level from the total binding level. To determine the estrogen receptor content and dissociation constant (Kd), Scatchard analysis[15] was performed using the specific binding values.

5) Enzyme immunoassay (EIA)

Aliquots of cytosol were assayed for estrogen receptors by an Abbott ER-EIA monoclonal kit (Abbott Laboratories) using monoclonal antibodies to human estrogen receptors.

Both the binding assay and EIA were done in duplicate.

6) Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed using Student's t-test.

Results

Figure 1 shows a representative saturation curve and Scatchard plot using normal liver cytosol obtained from a female patient who had gallstones. Specific binding of [³H]DES was saturable. The Kd calculated from the Scatchard plot was 0.11 nM, and the binding capacity 18.1 fmol/mg protein. The binding was competed with estradiol and DES but not with cortisol, progesterone or testosterone, demonstrating specificity for estrogen (data not shown). Thus the estrogen binding site in the liver cytosol has receptor-like characteristics.

Figure 2 shows the estrogen receptor content in various liver tissues assayed by the binding assay. In non-cirrhotic liver tissue, the estrogen receptor content was 17.8 ± 6.3 fmol/mg protein, and in cirrhotic liver and hepatocellular carcinoma tissue, it was 7.1 ± 6.9 and 0.7 ± 2.3 fmol/mg protein, respectively. There were significant differences in the contents among these the three groups.

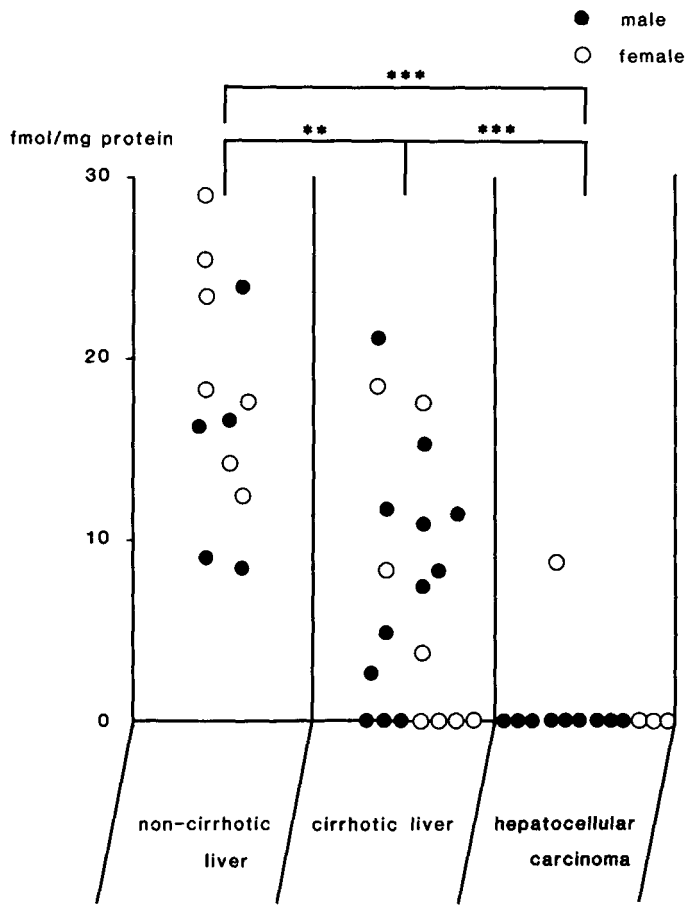

Fig. 2. Estrogen receptor content in the liver cytosol measured by the binding assay. Closed circles show the values for the males and open circles those for the females. **: $p<0.01$, ***: $p<0.001$

The estrogen receptor contents tended to decrease as the disease progressed to liver cirrhosis and hepatocellular carcinoma. The Kd values were less than 1 nM in all specimens. **Figure 3** shows the estrogen receptor content assayed by EIA. In non-cirrhotic liver tissue, it was 12.1 ± 5.7 fmol/mg protein, and in cirrhotic liver and in hepatocellular carcinoma tissue, it was 5.9 ± 3.6 and 0.8 ± 1.3, respectively. As in the results of the binding assay, significant differences were shown among the three groups.

In non-cirrhotic liver tissue, the contents determined by EIA were significantly higher in the females than in the males (15.2 versus 7.9 fmol/mg protein, $p<0.05$). In cirrhotic liver and hepatocellular carcinoma tissues, however, no difference according to sex was evident. Values determined by EIA showed a good correlation with those obtained by the binding assay, as shown in **Figure 4** ($r=0.822$, $p<0.001$).

Figure 5 shows the estrogen receptor content of specimens of hepatocellular carcinoma and adjacent non-tumorous liver tissue in the same patient determined by EIA. Contents of adjacent liver tissue (all cirrhotic liver but one chronic hepatitis) were 5.7 ± 3.0 fmol/mg

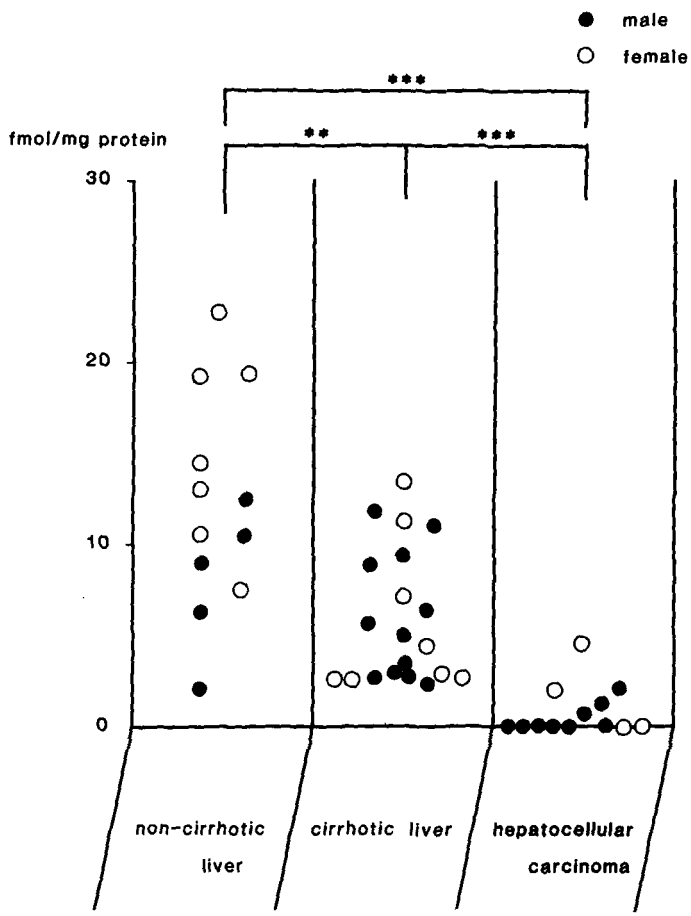

Fig. 3. Estrogen receptor content in the liver cytosol measured by EIA. Closed circles show the values for the males and open circles those for the females. **: $p<0.01$, ***: $p<0.001$

protein and those of hepatocellular carcinoma were 0.8 ± 1.3. In all patients, hepatocellular carcinoma tissue contained fewer estrogen receptors than the adjacent tissue, there being a significant difference between the two groups ($p<0.001$).

There was no relationship between the estrogen receptor content and presence of either HB_s antigen or α-fetoprotein.

Discussion

There have been many reports that oral contraceptive steroids might cause not only hepatic adenoma or focal nodular hyperplasia[2–4] but also hepatocellular carcinoma[5]. Some investigators[6–8] showed that synthetic estrogen could act as a promoter in hepatocarcinogenesis in experimental animals. Moreover, synthetic estrogens have been suggested to be a complete carcinogen[16].

It is generally accepted that estrogens exert their effect through binding with estrogen receptors in target tissues. Chamness et al.[9] and Aten et al.[10] demonstrated estrogen receptors in male and female rat liver. We showed a promotive action of estrogen in hepatocarcinogenesis in the rat and a higher estrogen receptor content in the estrogen-

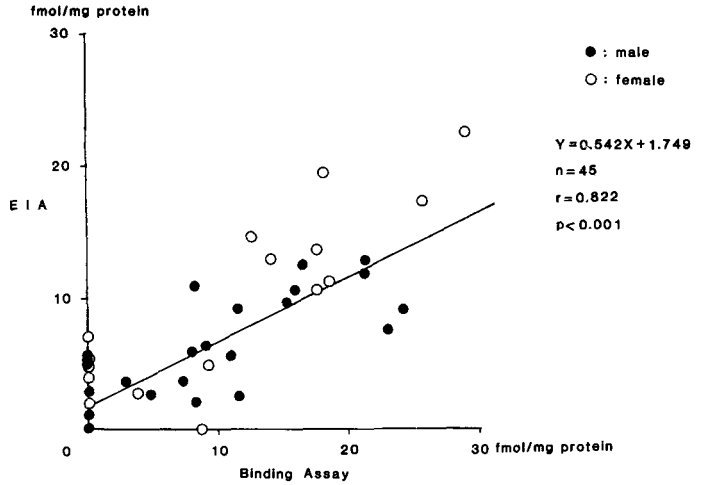

Fig. 4. Correlation between estrogen receptor content determined by binding assay and that determined by EIA.

induced liver tumor than in the control liver tissue[17]. On the other hand, Duffy et al.[11] and Porter et al.[12] demonstrated the presence of estrogen receptors in human liver tissue. Friedman et al.[13] assayed the estrogen receptors in tumorous and adjacent liver tissues. Based on the presence of estrogen receptors in both tissues, they treated patients with hepatoma with progestin and observed a significant regression of the tumor in some patients. The present study demonstrated that cytosol specimens obtained from non-cirrhotic, cirrhotic and carcinomatous liver tissue contain estrogen receptors as measured by the binding assay and EIA. Both procedures gave values with a good correlation. These results suggest that the estrogen binding site in liver tissue has the biologic and immunologic characteristics of estrogen receptors.

The contents of estrogen receptors in the present study were comparable to those reported previously[11–13,18–25]. In these reports specimens of normal liver[12,13,18,22,23], focal nodular hyperplasia[18,22,23], liver cirrhosis[12,13,24,25], adenoma[22], hepatoblastoma[20] or hepatocellular carcinoma[13,19,23–25] were assayed for estrogen receptors by the binding assay. But except for two reports[24,25] the number of liver specimens was very small in these reports. With respect to hepatocellular carcinoma only one[19,23] or five[13] specimens were assayed. On the other hand, we assayed more liver specimens, namely 13 hepatocellular carcinoma, 20 cirrhotic and 12 non-cirrhotic liver specimens using both the binding assay and EIA.

We observed a tendency towards higher contents in females than in males in non-cirrhotic liver patients. This is in agreement with the observation that female rat liver contains almost 3 times more estrogen receptors than male rat liver[26]. The estrogen receptor content in cirrhotic liver varied, ranging from a level undetectable by the binding assay to a level comparable to that in the non-cirrhotic liver. The reason for this variability is unknown, but the grade of cirrhosis, namely the volume of hepatocytes in a given tissue, might have influenced the contents. Histochemical studies of the estrogen receptors in liver tissue would provide an answer to this question.

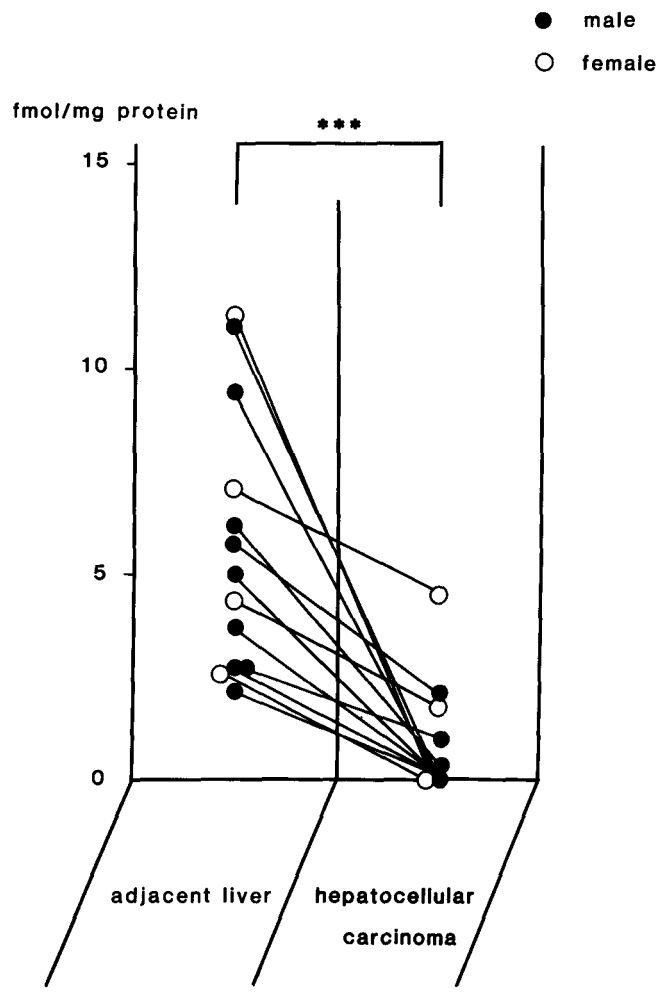

Fig. 5. Estrogen receptors of hepatocellular carcinoma and adjacent liver tissue in the same patients. Estrogen receptor content was determined by EIA. ***: $p<0.001$

Ohnishi et al.[25] recently reported that estrogen receptors could not be detected in seven of the eight hepatocellular carcinomas and five of the seven surrounding cirrhotic tissues and that no hepatocellular carcinoma contained more estrogen receptors than surrounding cirrhotic tissue. These observations are in agreement with our results. But Nagasue et al.[24] reported that estrogen receptors measured by the binding assay could be detected in 12 of the 30 hepatocellular carcinomas and 13 of the 28 surrounding cirrhotic tissues and that in about one third of the hepatocellular carcinoma patients, the estrogen receptor content was higher than that in the cirrhotic liver. These observations are in contrast with our studies, which demonstrated a lower content with the progression of the liver disease. The reason for such a discrepancy is unknown. One possible explanation is that the proteolytic activity in tumor tissue might degrade the estrogen receptors as in the case of human breast cancer[27]. We therefore used a protease inhibitor

such as antipain, leupeptin, chymostatin and phosphoramidon but failed to find any significant effect (unpublished observation). An alternative explanation is the difference of the method used. Nagasue et al.[24] used only the binding assay, using [^{3}H]estradiol as a ligand which is known to bind the sex hormone binding protein and α-fetoprotein. On the other hand, we used both the binding assay using [^{3}H]DES and EIA, and obtained similar results by the two procedures. Francavilla et al.[28] reported that the estrogen receptor content in Morris hepatoma was very low compared with that of normal rat liver. We also failed to detect any significant amount of estrogen receptors in Alexander cells (PLC/PRF/5) which originated from human hepatoma cells (unpublished observation). In such a cell line, the effect of proteolytic enzymes seems minimal.

The results of the present experiment suggest that estrogen has little, if any, role in promoting liver cirrhosis in the developmental process of hepatocellular carcinoma. Although the HB_s antigen was positive in a limited number of cases, liver cirrhosis in our cases seem to develop from viral hepatitis. However, the role of estrogen may be different in hepatocellular carcinoma developing from a different background. MacDonald et al.[18] reported that the estrogen receptor content of focal nodular hyperplasia of the liver developing after the treatment of estrogen was comparable to normal values reported by other investigators. In the cases of Friedman et al.[13] the effect of oral contraceptives could not be completely ruled out. Another type of liver cirrhosis or hepatocellular carcinoma possibly accompanying the high estrogen receptor levels may be the alcoholic type, as chronic alcohol feeding has been reported to raise the hepatic estrogen receptors level in rats[29]. However, in Japan there are a few cases of hepatocellular carcinoma caused by habitual alcohol drinking[30] in contrast to the cases seen in Europe and America.

In either case, we could not demonstrate a high estrogen receptor level in hepatocellular carcinoma cases. The estrogen dependency of hepatocellular carcinoma is suggested to be low and antiestrogen endocrine therapy ineffective.

References

1) Green JRB, et al: Plasma oestrogen in men with chronic liver disease. Gut 1976, 17: 426
2) Baum JK, et al: Possible association between benign hepatomas and oral contraceptives. Lancet 1973, ii: 926
3) Edmondson HA, et al: Regression of liver cell adenomas associated with oral contraceptives. Ann Intern Med 1977, 86: 180
4) Steinbrecher UP, et al: Complete regression of hepatocellular adenoma after withdrawal of oral contraceptives. Dig Dis Sci 1981, 26: 1045
5) Henderson BE, et al: Hepatocellular carcinoma and oral contraceptives. Br J Cancer 1983, 48: 437
6) Taper HS: The effect of estradiol-17-phenylpropionate and estradiol benzoate on N-nitrosomorpholine-induced liver carcinogenesis in ovariectomized female rats. Cancer 1978, 42: 462
7) Yager JD Jr, et al: Oral contraceptive steroids as promoters of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res 1980, 40: 3680
8) Wanless IR, et al: Role of estrogens as promoters of hepatic neoplasia. Lab Invest 1982, 46: 313
9) Chamness GC, et al: Estrogen receptor in rat liver and its dependence on prolactin. Steroids 1975, 26: 363
10) Aten RF, et al: Estrogen receptor in adult male rat liver. Endocrinology 1978, 103: 1629
11) Duffy MJ, et al: Estradiol receptors in human liver. J Steroid Biochem 1978, 9: 233
12) Porter LE, et al: Characterization and quantitation of human hepatic estrogen receptor. Gastroenterology 1983, 84: 704
13) Friedman MA, et al: Hepatomas; hormone receptors and therapy. Am J Med 1982, 73: 362
14) Lowry OH, et al: Protein measurement with the folin phenol reagent. J Biol Chem 1951, 193: 265
15) Scatchard G: The attraction of proteins for small molecules and ions. Ann NY Acad Sci 1949, 51: 660
16) Higashi S, et al: Development of hepatoma in rats following oral administration of synthetic estrogen and progestogen. Gann 1980, 71: 576

17) Kohigashi K, et al: Promotive effect of estrogen on hepatocarcinogenesis and estrogen receptor in male rats. Acta Hepatol Jpn 1986, 27: 1444
18) MacDonald JS, et al: Hepatic estrogen and progesterone receptors in an estrogen-associated hepatic neoplasm. Cancer Chemother Pharmacol 1978, 1: 135
19) Molteni A, et al: Estradiol receptor assays in normal and neoplastic tissues. A possible diagnostic aid for tumor differentiation. Ann Cli Lab Sci 1979, 9: 103
20) Demanes DJ, et al: Hormone receptors in hepatoblastoma: a demonstration of both estrogen and progesterone receptors. Cancer 1982, 50: 1828
21) Bojar H, et al: Does human liver contain estrogen receptors? A comparative study of estrogen binding in human and rodent liver. Klin Wochenschr 1982, 60: 417
22) Porter LE, et al: Estrogen receptor in human liver disease. Clin Res 1983, 31: 287A
23) Bojar H, et al: Steroid receptor status of focal-nodular hyperplasia of the human liver. Klin Wochenschr 1984, 62: 446
24) Nagasue N, et al: Estrogen receptors in hepatocellular carcinoma. Cancer 1986, 57: 87
25) Ohnishi S, et al: Androgen and estrogen receptors in hepatocellular carcinoma and in the surrounding non-cancerous liver tissue. Hepatology 1986, 6: 440
26) Rochman H, et al: Age, sex and gonadal influences on oestrogen receptor content in the rat liver. J Endocr 1985, 106: 95
27) Maeda K, et al: Partial characterization of protease(s) in human breast cancer cytosol that can degrade estrogen and progesterone receptors selectively. Cancer Res 1984, 44: 996
28) Francavilla A, et al: Estrogen binding protein activity in Morris hepatoma 7777 compared with normal rat liver. Gastroenterology 1984, 86: 1410
29) Eagon PK, et al: Alcohol-induced changes in hepatic estrogen-binding proteins: a mechanism explaining feminization in alcoholics. Arch Biochem Biophys 1981, 211: 48
30) Okuda K: Clinical aspects of hepatocellular carcinoma-analysis of 134 cases. In: Okuda K, Peters RL, eds. Hepatocellular carcinoma. John Wiley and Sons, New York, 1976, 387