Secretion of Glucagon in Liver Cell Carcinoma

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Ohneda, A., Kobayashi, T., Nihei, J. and Sakai, T. Secretion of Glucagon in Liver Cell Carcinoma. Tohoku J. exp. Med., 1985, 146 (1), 47-57 — In order to elucidate the response of plasma glucagon in liver cell carcinoma, a clinical study was performed in 12 patients with liver cell carcinoma in addition to 8 patients with liver cirrhosis and 8 normal subjects. Arginine infusion elicited increases in plasma insulin and glucagon in 6 patients with liver cell carcinoma as well as 8 patients with liver cirrhosis compared with the controls. However, the responses of plasma insulin and glucagon in liver cell carcinoma did not exceed those in liver cirrhosis. No glucagon secreting cell was proved in the hepatic cancer tissues from two other patients. Furthermore, no measurable glucagon was demonstrated in the tumor tissues extracted from four other patients with liver cell carcinoma. The extract of the tumors, infused into the pancreatic artery of anesthetized dogs, did not elicit any discernible changes in glucagon and insulin in the pancreatic vein. The present study demonstrates an elevated response of plasma glucagon in liver cell carcinoma. Since the morphological and biochemical studies failed to demonstrate the glucagon secreting cell or glucagon-stimulating material in the tumor tissues, the elevated plasma glucagon response might be interpreted by the increased A-cell function of the pancreas and the decreased degradation of the hormones in the liver. —— liver cell carcinoma; liver cirrhosis; arginine test; tumor extract

It has been recognized that secretion of insulin as well as glucagon is elevated in patients with liver cirrhosis, on the one hand (Megyesi et al. 1967; Collins and Crofford 1969; Marco et al. 1973; Sherwin et al. 1974, 1978; Johnston et al. 1977; Greco et al. 1979; Smith-Laing et al. 1980; Okumura and Hayakawa 1981). However, an increased response of plasma glucagon to arginine has been reported in liver cell carcinoma, whose development is regarded to be closely related to liver cirrhosis, on the other hand (Kihara et al. 1981; Ohneda et al. 1982). Furthermore, it was reported that the glucagon response in liver carcinoma is exaggerated much more than in liver cirrhosis and a relationship to increased glucagon secretion to the development of liver cell carcinoma is observed (Kihara et al. 1981). Therefore, if this is true, there is possibility that glucagon response can predict the occurrence of liver cell carcinoma or figure out its
development. Thus, the present study was undertaken in order to elucidate the response of plasma glucagon in patients with liver cell carcinoma and to evaluate the significance of glucagon measurement in the disease.

**Materials and Methods**

**Clinical study**

In this study, 12 patients with liver cell carcinoma, 8 patients with liver cirrhosis and 6 healthy volunteers were the subjects. Informed consent was observed from all subjects. Among them, 6 patients with liver cell carcinoma, 8 patients with liver cirrhosis and 6 healthy subjects underwent an arginine infusion test. The clinical data for those patients are summarized in Table 1. The diagnosis of liver cell carcinoma or liver cirrhosis was proved by histological examination. The arginine infusion test was carried out after an overnight fast, according to the method described previously (Ohneda et al. 1975). Blood specimens were obtained from the antecubital vein. For hormone measurements, 4 ml of blood were obtained with a heparinized syringe and poured into a glass tube containing 1000 KIU of aprotinin (Trasylol®, Bayer Co.). Plasma was separated by centrifugation immediately after the completion of the experiment and stored at \(-20{\degree}C\) until the assay. Blood glucose was determined by the glucose oxidase method (Teller 1956). Plasma insulin (IRI) was measured by the immunoassay method of Morgan and Lazarow (1962). Plasma glucagon (IRG) was assayed using a specific antiserum (G 21) to C-terminal of glucagon, as described previously (Ohneda et al. 1979).

**Histological study**

Histological examination was carried out for the liver obtained at autopsy in two patients with liver cell carcinoma. In addition to routine staining, the tumor tissue was studied with enzyme immunological procedure capable to stain A, B and D cells of the pancreatic islets, as described previously (Fujiya et al. 1977).

**Tumor extract**

In order to investigate the contents of insulin and glucagon in tumors, the frozen hepatic carcinoma tissues from four other patients were extracted by acid ethanol method (Kenny 1955). Insulin and glucagon contents in the tumors were determined as described above. Furthermore, the tumor tissues were extracted with saline solution and lyophilized for biochemical study.

| Table 1. Clinical data of patients with liver cell carcinoma or liver cirrhosis |
|----------------------------------|-----------------|-----------------|-------------|
| I. Liver cell carcinoma | II. Liver cirrhosis |
| Patients | Age | Sex | Cirrhosis | GTT | Patients | Age | Sex | Varix | GTT |
| 1. MI | 61 | M | + | IGT | 1. NM | 60 | F | + | IGT |
| 2. MA | 63 | M | – | IGT | 2. MS | 61 | F | + | IGT |
| 3. YW | 72 | M | + | DM | 3. KM | 58 | M | + | IGT |
| 4. FO | 49 | M | + | - | 4. YM | 67 | M | + | IGT |
| 5. NH | 63 | M | – | IGT | 5. TM | 49 | M | + | IGT |
| 6. MS | 54 | M | + | - | 6. MC | 74 | F | + | IGT |
| 7. KD | 53 | M | – | DM | 7. TI | 58 | F | + | N |
| 8. TI | 58 | F | + | N |

GTT, glucose tolerance test; DM, Diabetes mellitus; IGT, Impaired glucose tolerance; N, Normal glucose tolerance.
In situ perfusion of canine pancreas

To see the effect of the tumor extract, experiments were performed using a preparation of in situ perfusion of the canine pancreas, as reported previously (Ohneda et al. 1976). The extract from the tumor tissues with acid ethanol or saline solution were administered into the pancreatic artery and blood samples for hormone assay were drawn from the pancreaticoduodenal vein. Blood samples for glucose measurements were obtained from the femoral artery and blood glucose was determined by the glucose oxidase method (Teller 1956). Plasma was separated by centrifugation of blood obtained from the pancreaticoduodenal vein and stored at −20°C until the assay began. Glucagon and insulin in plasma were determined by the method described above.

Statistical analyses

In the present study, values were expressed by mean ± s.e. and statistical analyses were performed by the Student's t-test.

RESULTS

Arginine test

The changes in blood glucose, plasma IRI and plasma IRG of patients with liver cell carcinoma during the arginine test are presented in Fig. 1. In the figure, the values for the healthy controls were presented as shaded areas. In the normal subjects, blood glucose rose from the base line level of 84 ± 3.1 mg/100 ml to a peak of 93 ± 5.0 mg/100 ml at 20 min following the arginine infusion. Plasma IRI was 26 ± 4.7 μU/ml at fasting and increased to 99 ± 11.5 μU/ml 30 min after the infusion.
arginine infusion. Plasma IRG increased from the fasting level of 109±7 pg/ml to a peak of 272±59 pg/ml at 30 min, decreasing to 88±11 pg/ml at 120 min. In the patients with liver cell carcinoma, blood glucose was 88±8 mg/100 ml at fasting and increased to a peak level of 106±8 mg/100 ml at 50 min, returning to the base line level. Plasma IRI was 29±12 μU/ml at fasting and increased to a peak of 81±23 μU/ml at 50 min and then returned to the initial level at 120 min. The plasma levels of IRI in liver cell carcinoma were significantly elevated at 60 and 75 min (p <0.05 and 0.02, respectively). Plasma IRG was slightly elevated at fasting (215±49 pg/ml) and increased markedly after the arginine infusion, reaching a peak of 879±142 pg/ml at 20 min. Thereafter, plasma IRG decreased slowly but remained still elevated significantly throughout 120 min (p <0.001).

The responses of blood glucose, plasma IRI and IRG in 8 patients with liver cirrhosis are presented in Fig. 2. Blood glucose was 84±2 mg/100 ml at fasting and increased to a peak of 98±6 mg/100 ml at 40 min, decreasing thereafter. Plasma IRI was slightly but significantly elevated at fasting (39±8 μU/ml, p <0.05), compared with that of the control group. The arginine infusion exerted a marked increase in plasma IRI, which reached a peak of 148 μU/ml at 20 min and decreased thereafter. The levels of plasma IRI in liver cirrhosis following the arginine infusion was significantly higher than that for the normal subjects.
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Throughout the test (p < 0.005 or less). Plasma IRG in liver cirrhosis was 313 + 37 pg/ml at fasting and significantly higher than that of the controls (p < 0.001). After the arginine infusion plasma IRG increased markedly to a peak of 1478 ± 179 pg/ml at 20 min, decreasing thereafter. The levels of plasma IRG in liver cirrhosis were significantly higher throughout the experiment than those of the control group (p < 0.001).

To compare the responses of plasma glucagon to arginine, the increment area of plasma IRG for 60 min was calculated in these three groups. As shown in Table 2, the increment areas of plasma IRG were elevated in patients with liver cell carcinoma as well as the patients with liver cirrhosis. Furthermore, the increment area for liver cirrhosis was significantly higher than that for liver carcinoma patients (p < 0.05).

### Histological study

Enzyme immunological study did not demonstrate any A, B or D cells in the tumor tissues of two patients with liver cell carcinoma.

### Contents of insulin and glucagon in tumor

Tumors from 4 patients with liver cell carcinoma were extracted by acid ethanol. The hormone contents are presented in Table 3. Glucagon was not demonstrated from any tumors at all. In contrast, insulin was not observed in tumors from 2 patients (YS, YY), while small amount of insulin was determined in two other patients (MS, SN).

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**TABLE 2. Incremental area of plasma glucagon in arginine infusion test**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>Glucagon area*</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Normal</td>
<td>6</td>
<td>15.40 ± 0.96</td>
<td>I : II p &lt; 0.01</td>
</tr>
<tr>
<td>II. Liver cell carcinoma</td>
<td>6</td>
<td>44.98 ± 7.88</td>
<td>I : III p &lt; 0.001</td>
</tr>
<tr>
<td>III. Liver cirrhosis</td>
<td>8</td>
<td>68.77 ± 7.17</td>
<td>II : III p &lt; 0.05</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M., \( \sum_{t=0}^{60} \Delta \text{IRG}, \text{ng} \cdot \text{min} \cdot \text{ml}^{-1} \)

**TABLE 3. Contents of insulin and glucagon in tumor tissue from patients with liver cell carcinoma**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Insulin (( \mu \text{U/g} ))</th>
<th>Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>YS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>YY</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>SN</td>
<td>225</td>
<td>0</td>
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</table>
Fig. 3. Changes in blood glucose, plasma IRI and IRG in the pancreaticoduodenal vein (PV) of an anesthetized dog following the infusion of tumor extract by acid ethanol.

Fig. 4. Changes in blood glucose, plasma IRI and IRG in the pancreaticoduodenal vein (PV) of an anesthetized dog following the infusion of tumor extract by saline solution. In addition to tumor extract (YS and SN), a saline extract of liver (Hep) was infused.
Effect of tumor extract on insulin and glucagon secretion

In order to see if the tumor tissue contains any substances to stimulate the release of insulin or glucagon from the pancreas, the response of the endocrine pancreas was investigated, using in vivo perfusion system of the canine pancreas. In Fig. 3, a typical result is presented. Blood glucose did not change throughout the experiment. No discernible changes were observed in plasma IRI and plasma IRG in the pancreatic vein. Since the substances with a large molecular size cannot be extracted by acid ethanol, the tumors were extracted with saline solution and lyophilized. When the saline extracts of the tumor tissues were infused into the pancreatic artery, blood glucose did not change at all (Fig. 4). Furthermore, the saline extracts did not elicit any changes in plasma IRI or IRG in the pancreatic vein. However, the saline extract from the liver induced an abrupt rise of plasma IRI in the pancreatic vein.

DISCUSSION

A lot of results have been published concerning the endocrine function of the pancreas in liver cirrhosis (Megyesi et al. 1967; Collins and Crofford 1969; Marco et al. 1973; Sherwin et al. 1974, 1978; Johnston et al. 1977; Greco et al. 1979; Smith-Laing et al. 1980; Okumura and Hayakawa 1981). According to those results, liver cirrhosis is accompanied by exaggerated release of insulin and glucagon. In this connection there are many reports, which state the relationship of liver cirrhosis to liver cell carcinoma (Gall 1960; Anthony 1973; Johnson et al. 1978; Okudaira 1983). Especially in Japan, most liver cell carcinoma has been proved to develop on a basis of liver cirrhosis (Okudaira 1983; Matsushita 1981). In the present study, 4 of 6 patients with liver cell carcinoma revealed liver cirrhosis. Therefore, elevation of the endocrine function of the pancreas can be expected in patients with liver cell carcinoma. Indeed, a marked increase in glucagon release was demonstrated (Kihara et al. 1981; Ohneda 1982), and consequently it is presumed that measurement of plasma glucagon provides possibility as a tumor marker in liver cell carcinoma.

In the present study, increased responses of plasma insulin and glucagon were observed in patients with liver cell carcinoma, in comparison with the control group. However, the exaggeration of both plasma insulin and glucagon in the patients with liver cancer did not exceed those in the patients with liver cirrhosis. Therefore, the present study does not support the hypothesis that measurement of plasma glucagon provides a tumor marker for liver cell carcinoma. From the present study it is demonstrated that a much more increased response of plasma glucagon than plasma insulin might be characteristic of liver cell carcinoma.

Several factors are considered concerning the increased response of plasma glucagon observed in hepatoma compared with the normal subjects. At first, the increased response observed in liver cell carcinoma can be accounted for by a
similar mechanism to liver cirrhosis. According to the observations reported, the increased response of plasma glucagon in liver cirrhosis derives from the decreased degradation of glucagon in the liver (Marco et al. 1973; Smith-Laing et al. 1980) and the increased release of glucagon from the pancreas (Greco et al. 1974; Marco et al. 1973; Sherwin et al. 1974, 1978). Since most of liver cell carcinoma develops on the basis of liver cirrhosis as mentioned above (Anthony 1973; Johnson et al. 1978; Okudaira 1983), it is presumed that the increased response of plasma glucagon in liver cell carcinoma is resulted from the exaggerated A cell function of the pancreas, in addition to the decreased degradation of glucagon in the liver.

Immunohistochemical study did not demonstrate any insulin- or glucagon-secreting cells in the tumor tissue. Furthermore, no glucagon was detected in the extract from the tumor tissue, although a minute amount of insulin was measured in the extract. Therefore, a possibility that insulin as well as glucagon is secreted from the tumor tissue can be excluded in liver cell carcinoma.

Recently, the presence of glucagon-degrading substances was demonstrated in plasma of animals with liver injury (Tsubouchi et al. 1983) or in the salivary gland (Tahara et al. 1983) and this has been considered to induce artifact in glucagon measurement. In order to investigate the substances which bring about falsely elevated plasma glucagon, gel filtration of plasma from the patients with liver cell carcinoma or liver cirrhosis was carried out (Ohneda et al. 1982). However, chromatography did not reveal any increase in fractions corresponding to such degradating substances in plasma. Therefore, the possibility that elevated plasma glucagon derives from glucagon-degradating substances seems unlikely in patients with liver cell carcinoma or liver cirrhosis.

Another possibility considered is that some substances secreted from the tumor tissue stimulate glucagon secretion from the pancreas. In order to prove the validity, the tumor tissues were extracted by acid ethanol as well as saline solution, and the response of glucagon secretion from the pancreas to the extract was investigated. The previous studies from our laboratory indicate that the canine pancreas with in situ perfusion functions in response to various secretagogues for insulin and glucagon such as amino acids (Ohneda et al. 1980), vasoactive intestinal peptide (Ohneda et al. 1977) and caerulein (Ohneda et al. 1978). As shown in Figs. 3 and 4, however, neither extract by acid ethanol nor saline solution elicited the increase in plasma glucagon in the pancreatic vein of anesthetized dogs. Therefore, the assumption that pancreatotropic substance secreted from the tumor tissue induces an elevated response of glucagon secretion might be ruled out.

In contrast to glucagon release, a saline extract of the liver tissue exerted an increased release of insulin, as shown in Fig. 4. The same liver tissue extracted with acid ethanol did not reveal any changes in plasma insulin in the pancreatic vein (data not shown). Therefore, the hepatic extract might contain protein
fraction(s) which are solved in saline solution and stimulate insulin release. The material should be identified in future.

Recently glucagon in the gut has been designated as extrapancreatic glucagon (Vranic et al. 1974; Matsuyama and Foa 1974), which cannot be distinguished by immunoassay from pancreatic glucagon. Therefore, an increased response of plasma glucagon observed in liver cell carcinoma might derive from extrapancreatic glucagon. However, a preliminary study from our laboratory failed to demonstrate any exaggerated response of extrapancreatic glucagon to arginine in dogs with chronic portocaval shunt (Ohneda 1983). Therefore, participation of extrapancreatic glucagon in elevated plasma response seems unlikely in liver cell carcinoma.

In brief, in the present study elevated responses of plasma glucagon and insulin to arginine were observed in patients with liver cell carcinoma, similarly to those with liver cirrhosis. These changes in response of plasma hormones in liver cell carcinoma might be interpreted by the increased response of pancreatic endocrine system and the decreased degradation of these hormones in the liver, as reported in liver cirrhosis.

Acknowledgments

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References


