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Glucagon-like Immunoreactivity in Chronic Renal Failure

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Summary. **The modulation of glucagon-related peptides, plasma glucagon-like immunoreactivity (GLI) and plasma immunoreactive glucagon (IRG) were studied in 44 patients with chronic renal failure (CRF). The plasma levels of GLI and IRG during fasting were higher in the CRF patients than in the healthy control subjects and correlated well with renal dysfunction. Both plasma GLI and IRG levels increased after arginine infusion test. Following oral glucose ingestion, the response of plasma GLI levels was much greater in CRF than in the controls, but the plasma IRG levels in CRF were not decreased despite significant reduction in the controls. On gelfiltration of the plasma in CRF during fasting, both GLI and IRG were eluted at the position of 8-9 Kd, and the ratio of GLI to IRG in this fraction was 11.2. The authentic glucagon (3.5 Kd) appeared only in samples following arginine infusion. The 8-9 Kd GLI and IRG increased after oral ingestion of glucose, but not after arginine infusion. By electrofocusing column chromatography of the pooled 8-9 Kd fraction, GLI showed 3 peaks at pI 4.42, 5.12 and 5.94, and IRG showed a peak at pI 5, 12. These pIs were compatible to the pIs of peak I GLI obtained from canine intestinal extracts. Therefore, it is suggested that this large molecule GLI, especially prevalent in CRF patients, originates from the intestinal tract.**

INTRODUCTION

Various hormones including pancreatic hormones in plasma have been known to increase during chronic renal failure (CRF).[1] An increase in plasma levels of immunoreactive glucagon (IRG) is well known in CRF as well, where an increase in 9 Kd IRG predominates over authentic glucagon.

Recent advances in molecular biology have elucidated whole sequences of the preproglucagon molecule consisting of proglucagon-related peptide, glicentin, glucagon, oxyntomodulin, and glucagon-like polypeptide (GLP-1 and GLP-2). The development of radioimmunoassay (RIA) for glucagon has disclosed 2 kinds of glucagon-related immunoreactivities: immunoreactive glucagon (IRG) and glucagon-like immunoreactivity (GLI). IRG is referred to as the peptide measured by RIA with the C-terminal directed antibody, which recognizes the peptide having the unmasked C-terminal sequence of glucagon including glucagon itself. GLI is the peptide crossreacted equally with the N-terminal directed and C-terminal directed antibodies, irrespective of masking of the C-terminal of the glucagon molecule, representing mostly glicentin, oxyntomodulin and glucagon. In the narrowest sense of GLI, the value subtracting IRG from GLI is used.

Abundant amounts of GLI were found in the intestine and even in plasma, whose concentration is far higher than IRG. Although high plasma levels of many hormones including IRG in CRF have been generally ascribed to decreased renal clearance, there is no report on GLI in CRF.

In this paper, we aim to study the modulation of plasma levels of IRG and GLI, and their molecular forms following oral ingestion of glucose and intravenous infusion of arginine in CRF patients.

MATERIAL AND METHODS

Subjects

27 dialyzed and 17 undialyzed chronic renal failure (CRF) patients and 10 healthy controls were tested for the fasting plasma IRG and GLI levels. Serum creatinine and creatinine clearane were also examined. None of subjects had diabetes or other hormonal disease.

Intravenous arginine test and oral glucose loading test

Both tests were performed on the 6 undialyzed CRF patients and 8 healthy control subjects. The arginine test was done during fasting by intravenous infusion of 300 ml of 10% arginine hydrochloride solution for 30 min. The oral glucose test was carried out during fasting by the ingestion of 75 g glucose in 150 ml solution. Venous blood was taken at 0, 30, 60, 90, and 120 min after initiation of the tests. The arginine test was performed under careful observation and concluded without any trouble including hyperkalemia in the CRF patients.

Sampling

Blood samples were immediately poured into chilled tubes containing 500 KIU aprotinine and 1mg EDTA per milliliter of blood and centrifuged at 4℃. The plasma was stored at −20℃.

Assay procedures

IRG was measured by RIA of the double antibody method using the glucagon C-terminal specific antibody, OAL123 (GLUCAGON RIA kit, Daiiti Radioisotope Laboratories, Tokyo, Japan). GLI was measured with the glucagon N-terminal specific antibody, OAL 196 (Otsuka Assay Laboratories, Tokuyama, Japan) by RIA of dextran coated charcoal method.

Gelfiltration

1.5 or 2 ml plasma was applied on 1×100 cm Bio-Gel P-30 (Bio-Rad, California, USA) column chromatography. The samples were eluted under gravity with 0.2 M glycine buffer, pH 8.8 containing 0.1% bovine serum albumin at a rate of 3 ml/hr at 4℃. Each 2 ml fraction was collected and lypholized. The residues were reconstituted in 0.5 ml distilled water and assayed for IRG and GLI.

Isoelectric focusing column chromatography

The pooled peak fraction of 8-9 Kd GLI from gelfiltration was subjected to isoelectric focusing column chromatography (LKB, Stockholm, Sweden) by means of a sucrose density gradient with ampholine (pH range; 3.5-10.0). Isoelectric focusing chromatography was carried out at 400 volts for 48 h at 4℃. Each 2 ml fraction was collected and assayed for IRG and GLI. PH was also measured by pH meter in each fraction at 4℃.

Statistical analysis

All data were represented as the mean±SEM. Statistical significance was evaluated using the Student's t-test. P values less than 0.05 were considered significant.

RESULTS

Fasting plasma GLI and IRG levels

The GLI levels in plasma during fasting were 2255±233 pg/ml in dialyzed CRF patients ($p<0.005$ vs. control), 1094±107 pg/ml CRF patients ($p<0.005$ vs. control), and 433±25 pg/ml in healthy control subjects, respectively. The IRG levels of plasma during fasting were 240±11 pg/ml in dialyzed CRF patients ($p<0.005$ vs. control), 154±12 pg/ml in undialyzed CRF patients ($p<0.005$ vs. control), and 53±6 pg/ml

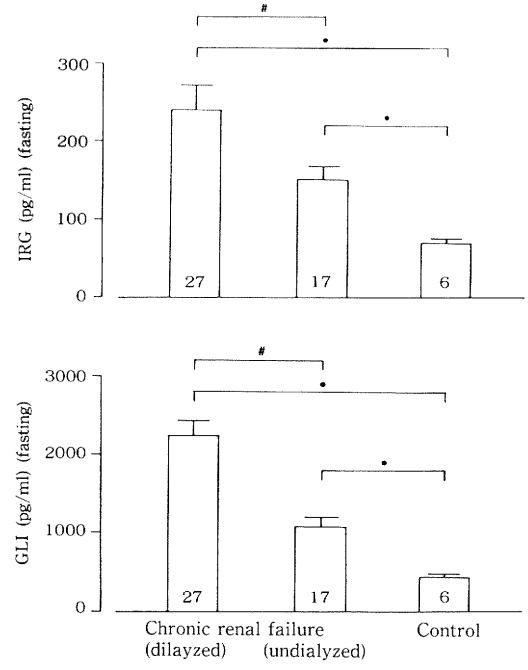

Fig. 1. Plasma IRG (upper panel) and GLI (lower panel) levels during fasting in 27 dialyzed chronic renal failure (CRF) patients (left columns), 17 undialyzed CRF patients (middle columns), and 6 healthy control subjects (right columns). *$p<0.005$ vs. control, #$p<0.005$ between dialyzed and undialyzed CRF patients.

in healthy control subjects, respectively. Both plasma IRG and GLI levels were significantly elevated in the renal failure group against the healthy controls (Fig. 1).

Correlation of GLI and IRG levels in plasma during fasting with renal function

Renal function was evaluated by serum creatinine levels or creatinine clearance in the CRF patients and the healthy control subjects. The plasma IRG and GLI levels were correlated positively with serum creatinine levels (IRG: $r=0.61$, $p<0.005$; GLI: $r=0.58$, $p<0.005$) and correlated inversely with creatinine clearance (IRG: $r=-0.39$, $p<0.05$; GLI: $r=-0.61$, $p<0.005$), respectively (Fig. 2).

Responses of plasma GLI and IRG to intravenous arginine infusion and oral glucose tolerance test

In the undialyzed CRF patients, the plasma GLI levels during fasting (919±217 pg/ml) increased by 75% to the peak of 1616±522 pg/ml and the fasting plasma IRG levels (145±9 pg/ml) increased by 146% to the peak of 358±42 pg/ml 30 min after arginine infusion. Similar increases in plasma GLI and IRG levels were found in the healthy control subjects with GLI from 407±26 pg/ml to 662±44 pg/ml at the peak (62% rise) and IRG from 62+10 pg/ml to 221±37 pg/ml at the peak (256% rise). After oral glucose ingestion, the plasma GLI levels (1111±210 pg/ml) in CRF patients markedly increased by 82% to a peak of 2028±384 pg/ml after 60 min, but the plasma GLI levels in healthy subjects (443±10 pg/ml) increased slightly by 18% to a peak of 525±47 pg/ml after 60 min. In patients with CRF, the plasma IRG levels during fasting (163±9 pg/ml) were unchanged after glucose challenge, but those of healthy subjects (53±6 pg/ml) were slightly suppressed by 23% to a nadir of 41±1 pg/ml after 60 min (Fig. 3).

Elution profiles of the plasma of dialyzed CRF patients

Two major peaks of GLI and IRG were found in the plasma of dialyzed CRF patients. The first peak containing GLI and IRG was found in the void volume

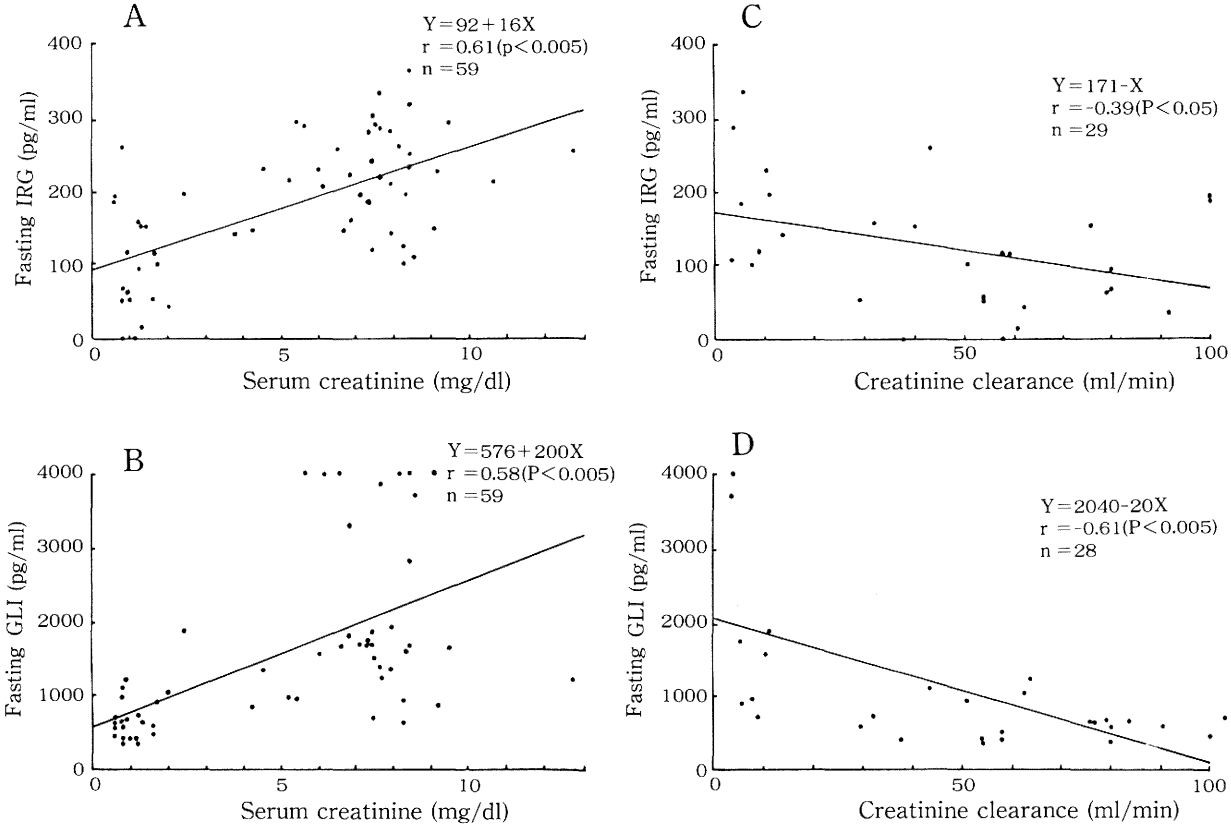

Fig. 2. Correlation of plasma IRG (A) or GLI (B) levels during fasting to plasma creatinine levels, and correlation of plasma IRG (C) or GLI (D) levels during fasting to creatinine clearance.

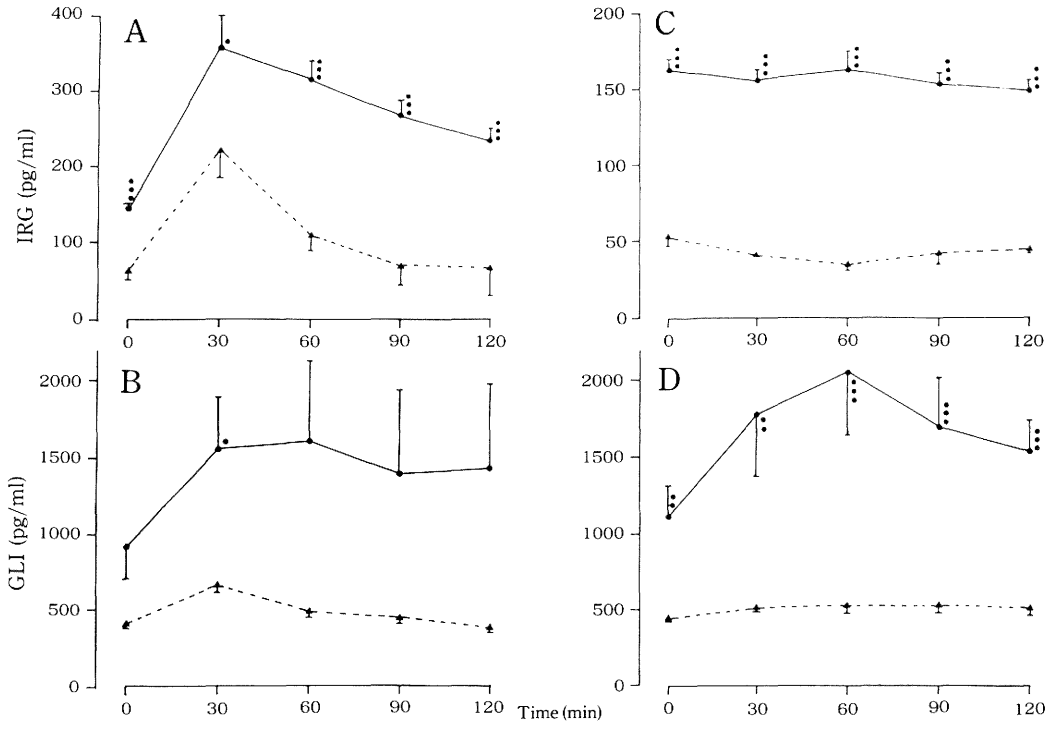

Fig. 3. Responses of plasma IRG (A) and GLI (B) levels after 30 g intravenous arginine infusion, and responses of plasma IRG (C) and GLI (D) levels after 75 g oral glucose ingestion in 6 undialyzed CRF patients (solid line) and 8 controls (broken line). *$p<0.05$, **$p<0.01$, ***$p<0.005$ vs. control in each sampling time

(mol wt >150,000), which was also found in the healthy control subject. The second peak containing GLI and IRG was eluted in the position between cytochrome-C (mol wt 12,000) and ^{125}I-insulin (mol wt 6,000) marker. The molecular weight of the second peak GLI and IRG was estimated to be 8-9 kd. The value of GLI was ten times higher than IRG in the second peak, and the ratio of sum GLI to sum IRG in the second peak was 11.2. This second peak appeared only in the plasma of CRF patients. Authentic glucagon (mol wt 3,500) was neither found in the plasma during fasting of CRF patients nor in the normal subjects (Fig. 4).

Elution profiles of the plasma obtained after arginine infusion and oral glucose test in an undialyzed CRF patient

Since the administration of amino acid to dialyzed patients is dangerous, arginine test was performed only on undialyzed CRF patients.

The elution profile of plasma of undialyzed CRF patients showed the same two peaks of GLI and IRG as was shown in the dialyzed CRF patients. After arginine infusion, three major peaks of GLI and IRG were eluted. The first and second peaks of GLI and IRG were found at the same positions as those obtained from the plasma during fasting. The third peak of GLI and IRG was eluted at the position of ^{125}I-glucagon marker. The both values of GLI and IRG were equal in this fraction; this peak was therefore regarded as authentic glucagon. After glucose ingestion the second peak of GLI and IRG were markedly increased in the CRF patient, whereas this second peak was not found in the healthy control (Figs. 5 and 6).

Isoelectric focusing chromatography analysis

Isoelectric focusing chromatography of the pooled second peak fraction from the plasma of dialyzed CRF patients showed three peaks for GLI and one peak for IRG. The isoelectric points of GLI were pI 4.42, 5.12, and 5.94 and that of IRG was pI 5.12 (Fig. 7).

DISCUSSION

The present study showed that plasma levels of IRG

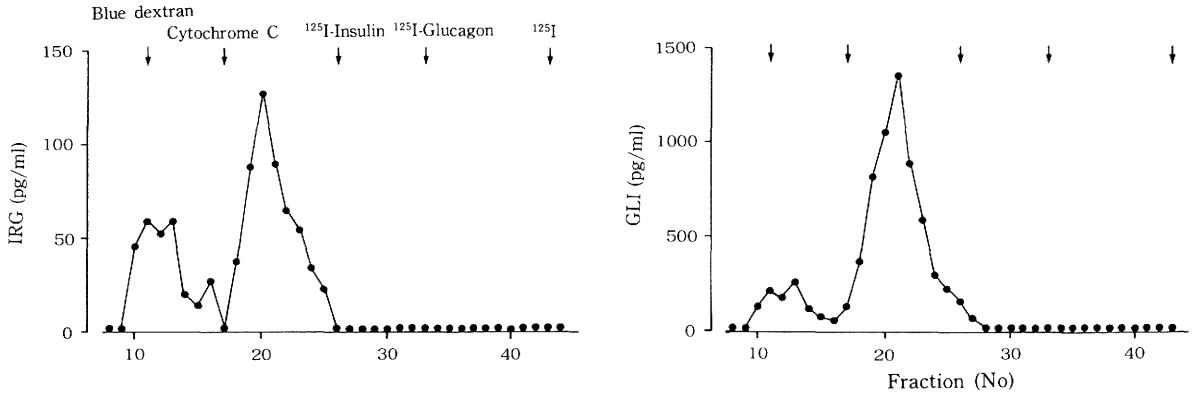

Fig. 4. Elution profiles of plasma IRG (A) and GLI (B) on Bio-Gel P-30 column chromatography in a dialyzed CRF patient. Sample was taken during fasting. The column was calibrated with blue dextran (mol wt >150,000), cytochrome-C (mol wt 12,000), ^{125}I-insulin (mol wt 6,000), and ^{125}I-glucagon (mol wt 3,500).

and GLI during fasting were markedly higher in CRF than in the control. The increases in these peptides correlated well with an increase in plasma creatine levels and reduction in creatinine clearance, suggesting close relations to renal dysfunction. High values of plasma GLI in renal failure have also been reported by several other investigators.[2-4]

On gelfiltration, both IRG and GLI in the plasma during fasting of normal subjects were found only at void volume, but in CRF these were found at the 9 kd position besides the void volume fraction. It should be noted that no glucagon moiety was found in the plasma during fasting of either normal subjects or CRF patients. Valverde et al.[8] and Kuku et al.[4] found IRG 3.5 Kd as well as IRG 9 Kd in CRF. These data are mostly compatible with ours, excepting their finding of small amount of 3.5 Kd IRG. A void volume fraction of IRG was detected only by OAL 196 antibody but not by glucagon N-terminal specific antibody K4023 (Novo, Denmark) (data not shown). Therefore, this can be considered a nonspecific finding. Weir et al. found IRG in the γ-globulin fraction and referred to it as a nonspecific interfering factor.[5] Schenk and Grubb found that the void volume fraction IRG was a nonspecific binding of labeled antigen to Fc fragment of immunoglobulin G.[6] Tsubouchi et al. demonstrated that apparent IRG at the void volume fraction in the liver disease is a degradation activity for glucagon.[7] However, we could not detect any degradation activity in the void volume fraction (data not shown).

Our finding that the 9 kd IRG in the circulating plasma in the CRF patients is a major component is compatible with other reports.[2,3,4,8] In contrast with normal subjects, a major fraction of GLI in the CRF patients was found in the same region as 8-9 Kd IRG, but this GLI fraction was 15 times higher compared to the IRG fraction. Since this fraction was found only in CRF patients and was not thought to be metabolized in the liver,[9] the kidney may play an important role in metabolizing 8-9 Kd GLI and IRG.

The 3500 molecular weight component of GLI and IRG corresponding to authentic glucagon was found only after arginine infusion in both CRF patients and normal subjects. In contrast, the 8-9 Kd of GLI and IRG were unchanged after arginine stimulation in CRF patients. Kuku et al.[4] and Jaspan et al.[10] reported that 8-9 Kd IRG after arginine infusion was similarly unchanged. These results indicate that authentic glucagon is slight, if at all, in the plasma during fasting, and that only arginine stimulation may induce a response of authentic glucagon.

On the other hand, after the ingestion of glucose, the increases in plasma GLI levels were more marked in CRF than in the control, while plasma IRG levels in CRF were unchanged, in contrast with the decline of those in the control. We found 8-9 Kd of GLI and IRG in the plasma of total gastrectomized patients only after oral glucose ingestion (data not shown). Valverde et al. also reported an increase in 9 kd IRG in a pancreatectomized dog.[11] At any rate, the discrete responses of 8-9 kd GLI in CRF between arginine infusion and oral glucose ingestion test suggest that the source of this GLI fraction is the intestine.

Isoelectric focusing chromatography of the 8-9 Kd fraction in CRF patients showed three GLI and one IRG components. Conlon et al.[12] reported that the isoelectric points of peak I GLI of the canine intesti-

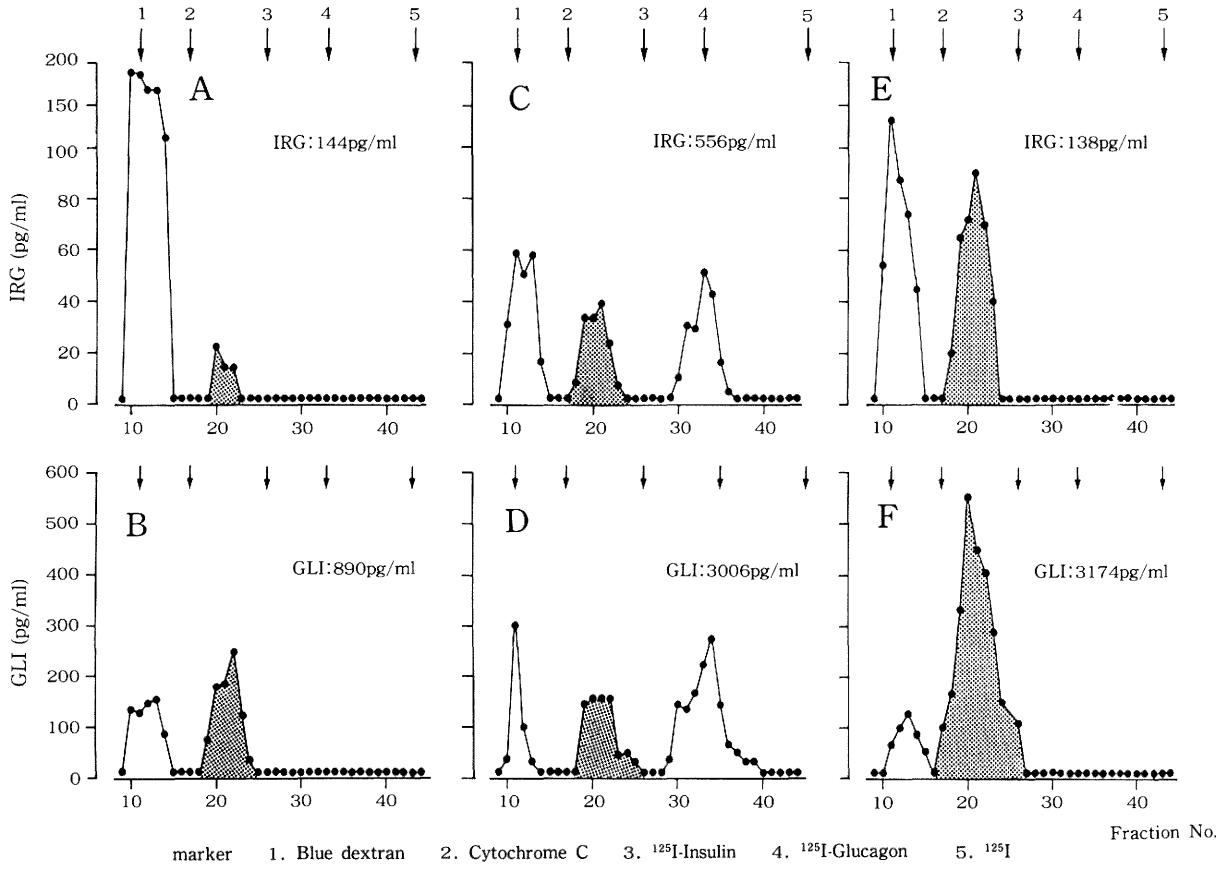

Fig. 5. Elution profiles of plasma IRG (A, C, and E) and GLI (B, D, and F) on Bio-Gel P-30 column chromatography in an undialyzed CRF patient. Samples were taken during fasting (A and B), after intravenous arginine infusion (C and D), and after oral glucose ingestion (E and F). Shadow areas show the 8-9 Kd fraction of IRG and GLI. Column was calibrated as in Fig. 4.

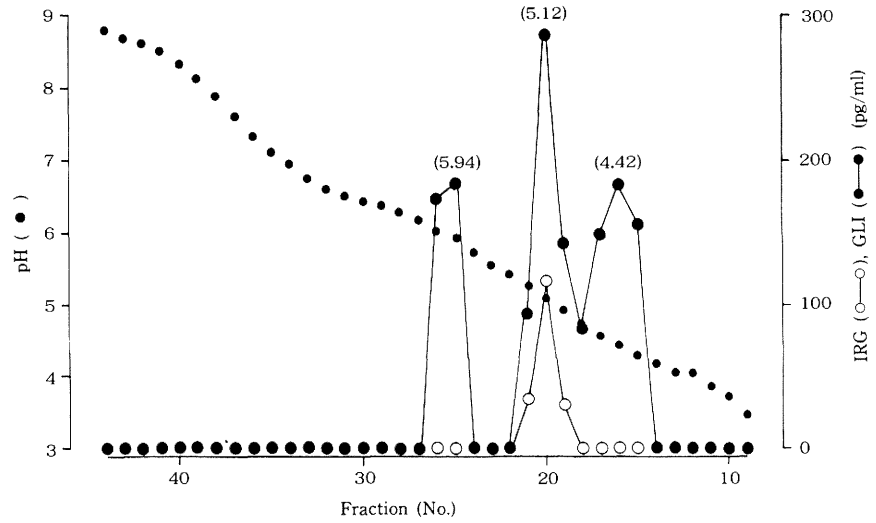

Fig. 7. Electrofocusing column chromatography of pooled 8-9 Kd GLI fraction on Bio-Gel P-30 column in a CRF patient. GLI (larger closed circle), IRG (opened circle), and pH (smaller closed circle) were assayed in each fraction.

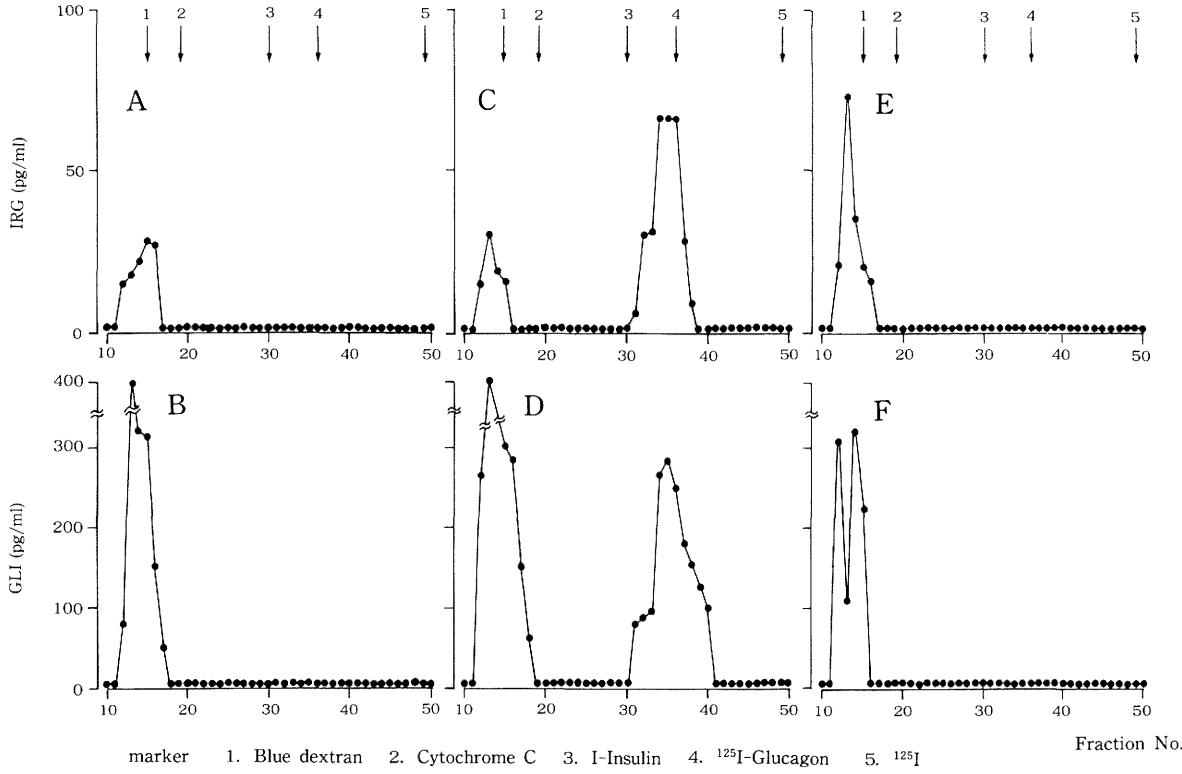

Fig. 6. Elution profiles of plasma IRG (A, C, and E) and GLI (B, D, and F) on Bio-Gel P-30 column chromatography in a healthy control subject. Samples were taken during fasting (A and B), after intravenous arginine infusion (C and D), and after oral glucose ingestion (E and F). Column was calibrated as in Fig. 4.

nal extracts were 4.7, 5.1, 6.1 and 6.9. The former three isoelectric points were similar to our results. Together with these results, it is suggested that the 8-9 Kd GLI in the CRF patients is derived from the intestine, and that the increase in these materials by oral glucose loading resulted from stimulation to the intestine.

Chronic renal failure is a long term process in metabolic changes. Despite increases in plasma GLI and IRG, none of the CRF patients in this study developed diabetes. This might indicate that GLI and IRG increases in CRF have little effect on the glucose metabolism, and that these resulted from metabolic changes and lowered clearance in chronic renal failure.

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