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Analysis of Human Insulin Analogues in Vitro, Using Gel Chromatographic Method

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ABSTRACT. The incubation medium and incubated human pancreas were gel chromatographed on the Bio-Gel P-30 column after extraction with acid ethanol. The extracted immunoreactive insulin (IRI) was successfully separated into two peaks at the position of 6000 molecular weight region. These two peaks corresponded to those which were detected in human serum. These findings suggest that the two groups of insulin are directly secreted from human pancreatic tissue. But the incorporated [^{3}H] leucine peak into acid-ethanol extractable protein did not elute out at the same position as each insulin peak. Therefore, the measurement of [^{3}H] leucine incorporation into acid-ethanol extractable protein is not a good indicator to evaluate insulin biosynthesis.

Key words : human insulin analogues — gel chromatography

The presence of two kinds of immunoreactive insulin (IRI) was reported in human sera after extraction and gel filtration in ealier report.[1] The early eluting insulin is not released with the stimulation of glucose, but the late eluting insulin is secreted with the same stimulation.[2] But it is not clarified whether each insulin is released from human pancreatic B-cell directly or a form of insulin released is converted to two types of insulin in peripheral circulation.

The measurement of labeled amino acids incorporation into proinsulin and insulin is now one of the well established methods to estimate the insulin biosynthesis.[3—6] Therefore, the incubation study was undertaken to elucidate the presence of insulin analogues in incubation medium and incubated pancreatic tissue, and to reassess the concordance of the peak of insulin with that of incorporated [^{3}H] leucine radioactivity, using gel chromatography after acid-ethanol extraction.

MATERIALS AND METHODS

Subjects

Human pancreas was taken from a patient with esophageal cancer and two

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patients with gastric cancer. All three patients had no history of diabetes mellitus.

Incubation

Piece of the panctreatic tissue (wet weight : approximately 50 mg) were incubated for 30 minutes at 37°C (95% O_2/5% CO_2) in Krebs Henseleit buffer solution containing 3.0 mg/ml of glucose, and additional 30 minutes in the medium containing 0.6 mg/ml of glucose. After this, the tissue was incubated at 37°C (95%O_2/5%CO_2) in the medium containing 3.0 mg/ml of glucose and [^{3}H] leucine (L-[4,5-^{3}H(N)]-, 100 μCi, 5.0 Ci/mmol, New England Nuclear) for another 2 hours and 8 hours, respectively. The other pieces of pancreas from each patient were incubated for 2 hours at 37°C (95%O_2/5%CO_2) in the medium containing 3.0 mg/ml glucose and [^{3}H] tryptophan (L-[SIDE CHAIN-2,3-^{3}H(N)]-, 100 μCi, 15.0 Ci/mmol, New England Nuclear), after the same preincubation. All incubation medium contained 0.5% bovine serum albumin (Sigma) to prevent the adsorption of insulin molecules.

Extraction and gel chromatography

After the incubation, the medium was extracted by the reported method.[2] The pancreatic tissue was homogenized by Polytron PT 10-35 KINEMATICA (Switzerland) for one minute at setting 7 in a silliconized glass tube with 8 ml of acid-ethanol (350 ml, 95% ethanol; 7 ml, conc. HCl; 153 ml, distilled water). After these procedures, extraction and gel filtration were performed according to the reported method.[2] The column was calibrated with ^{125}I-labeled C-peptide (Daiichi Radioisotope Lab.) and ^{125}I-labeled glucagon (Dainabot Radioisotope Lab.), besides porcine ^{125}I-labeled proinsulin and porcine ^{125}I-labeled insulin.

Peak I represents early eluting peak of insulin and Peak II represents later eluting peak of insulin.

Assay for immunoreactive insulin and c-peptide immunoreactivity

Assay for immunoreactive insulin and C-peptide immunoreactivity was performed according to the reported method.[7,8] The specificity of the insulin antiserum employed in this study had elucidated by Heding.[9]

Counting for [^{3}H] radioactivity

0.1 ml of the fraction was counted for [^{3}H] radioactivity in the liquid scintillation counter (Packard, Tri-carb. Model 3385), using Bray's scintillator liquid.[10]

RESULTS

All pancreatic samples were incubated for 2 and 8 hours, respectively. In the all incubation studies of the pancreas incubated for 2 hours, two groups of insulin were detected both in the incubated medium (Fig. 1, A') and in

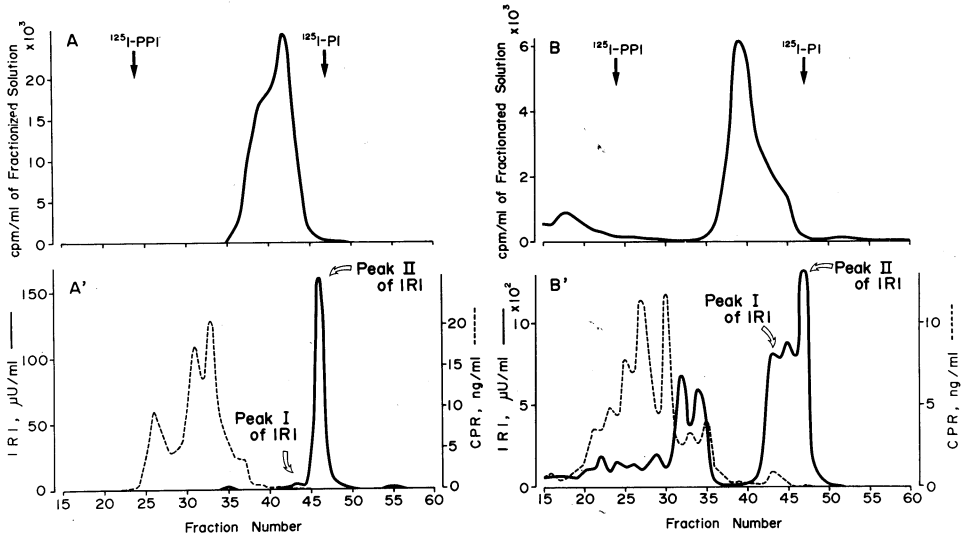

Fig. 1. Elution profiles of incorporated [^{3}H] leucine radioactivity, insulin and C-peptide extracted from incubation medium and incubated human pancreas (I.M., 69 y.o., male) on the Bio-Gel P-30 column. Krebs Henseleit bicarbonate buffer (pH 7.4, 95% O_2/5% CO_2) with 3.0 mg/ml glucose and [^{3}H] leucine for 2 hours at 37°C. A : Elution profile of incorporated [^{3}H] leucine radioactivity extracted from incubation medium. A' : Elution profile of insulin and C-peptide extracted from incubation medium. B : Elution profile of incorporated [^{3}H] leucine radioactivity extracted from incubated human pancreas. B' : Elution profile of insulin and C-peptide extracted from incubated human pancreas. PPI represents porcine proinsulin. PI represents porcine insulin. Detection level : 0.8 μU/ml for insulin, 0.04 ng/ml for C-peptide.

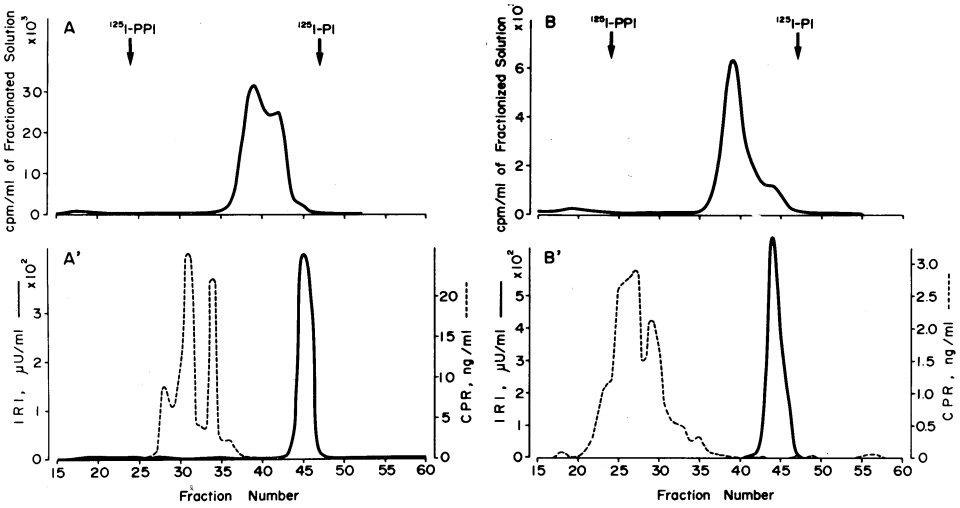

Fig. 2. Elution profiles of incorporated [^{3}H] leucine radioactivity, insulin and C-peptide extracted from incubation medium and incubated human pancreas (I.M., 69 y.o., male)

on the Bio-Gel P-30 column. Krebs Henseleit bicarbonate buffer (pH 7.4, 95% O_2/5% CO_2) with 3.0 mg/ml glucose and [^{3}H] leucine for 8 hours at 37°C. A : Elution profile of incorporated [^{3}H] leucine radioactivity extracted from incubation medium. A' : Elution profile of insulin and C-peptide extracted from incubation medium. B : Elution profile of incorporated [^{3}H] leucine radioactivity extracted from incubated human pancreas. B' : Elution profile of insulin and C-peptide extracted from incubated human pancreas. PPI represents porcine proinsulin. PI represents porcine insulin. Detection level : 0.8 μU/ml for insulin, 0.04 ng/ml for C-peptide.

the incubated pancreas (Fig. 1, B'). In Fig. 1, A', the early peak (Peak I) was reassured with the increased sample size. The peak of [^{3}H] leucine radioactivity was not corresponded to the peak of insulin (Fig. 1, A, A'; B, B'). On the contrary, 8 hours incubation study showed only one peak of insulin both in the incubation medium (Fig. 2, A') and in the incubated pancreatic tissue (Fig. 2, B'). The peak of insulin was also not corresponded to that of [^{3}H] leucine radioactivity (Fig. 2, A, A'; B, B').

In the case of female patient with gastric cancer, the correspondence between the insulin and [^{3}H] leucine peak was not recognized. In the same case, the incubation study with [^{3}H] tryptophan was also performed with the same conditions. The minor peak of [^{3}H] tryptophan corresponded to the peak of insulin in the incubation medium, contradictorily (Data not shown), though proinsulin, insulin and C-peptide have no tryptophan molecule.

The peak of [^{3}H] leucine radioactivity did not corresponded to those of ^{125}I-labeled proinsulin, ^{125}I-labeled insulin, ^{125}I-labeled C-peptide and ^{125}I-labeled glucagon in all experiments. The qualification of the peaks of C-peptide immunoreactivity and proinsulin had been reported with the same system.[11]

DISCUSSION

It has been well established that proinsulin is enzymatically converted to insulin with liberation of the connecting peptide, and the C-peptide remains packaged with insulin in secretory granules and both are subsequently released together into the circulation.[12,13] On the contrary, the circulating form of insulin and C-peptide molecules is obscure.

The presence of two kinds of insulin was reported in human sera after extraction and gel filtration.[1,2] But it is not clarified whether each insulin is directly released from human pancreatic B-cell or one form of insulin molecule released is converted to two types of insulin in peripheral circulation. In the present study, two peaks of insulin were detected in some of the samples of both medium and pancreatic tissue. These insulin peaks were corresponded to those extracted from human serum. Therefore, two kinds of insulin should be released directly from the human pancreatic tissue.

It is well known that the labeled amino acids are incorporated into proinsulin and insulin in incubated pancreatic slices[3,4] and pancreatic islets.[5,6] These facts have been utilized for elucidation of insulin biosynthesis by many investigators.[14–18] But in the present study, the peak of [^{3}H] leucine

radioactivity is not corresponded to that of insulin. [^{3}H] leucine might be incorporated into acid-ethanol extractable protein, besides proinsulin, insulin and glucagon. Moreover, the minor peak of [^{3}H] tryptophan is corresponded to the peak of insulin in the incubation medium contradictorily (Data not shown). These facts are not compatible with the reported result.[6] The difference between the peak of incorporated [^{3}H] leucine and that of insulin should be verified in future. Therefore, the estimation of incorporated radioactivity of [^{3}H] leucine might be inadequate to evaluate the insulin biosynthesis, using this way of study.

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