Measurement, Characterization, and Source of Somatostatin-like Immunoreactivity in Human Amniotic Fluid

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ABSTRACT Somatostatin-like immunoreactivity (SLI) is widely distributed in tissues and biological fluids. To determine whether SLI is also present in amniotic fluid, samples obtained by amniocentesis from 30 normal and 27 abnormal pregnancies were studied by radioimmunoassay. Direct incubation of [^{125}I-Tyr^{1}]tetradecapeptide somatostatin (SRIF) with amniotic fluid resulted in 89% tracer degradation. Damage was reduced to <5% when samples were acidified and boiled before the assay. With this technique, SLI was detectable in all normal amniotic fluid samples; the mean level at 15–20 wk of gestation (320±55 pg/ml, n = 15) being 4.5 times higher than the mean at 32–43 wk (70±12 pg/ml, n = 15) ($P < 0.001$). In cases of preeclampsia (n = 6), gestational diabetes (n = 5), anencephaly (n = 1), and meningomyelocele (n = 1), SLI values were in the normal range, but in one juvenile diabetic and one patient with chronic renal failure, SLI was undetectable (<10 pg/ml). In a pair of monochorionic diamniotic twins, SLI levels were very different (33 and 197 pg/ml), which suggests that fetal factors are more important than materno-placental ones in determining amniotic fluid SLI. Serial dilutions of amniotic fluid showed parallelism with standard SRIF. When concentrates of pooled amniotic fluid were chromatographed on Sephadex G-25 columns, all SLI eluted in the void volume ahead of SRIF even after treatment with 8 M urea and dithiothreitol. This "big" SLI incubated in amniotic fluid showed 100% stability over 24 h at 37°C, whereas SRIF was rapidly inactivated ($t_{1/2} \cong 7$ min). Extracts of placenta and fetal membranes contained no SLI, but small amounts (6–20% of total amniotic fluid SLI) were found in cells from fresh fluid. Radioimmunoassay of SLI in extracts of seven paired cord arterial and venous plasma samples showed no arteriovenous gradient consistent with fetal origin of cord blood SLI. It is concluded that (*a*) amniotic fluid contains SLI which is of fetal origin and (*b*) normal levels vary with gestational age. The SLI has a higher molecular weight (≥5,000) and is more stable in amniotic fluid than SRIF.

INTRODUCTION

The cyclic tetradecapeptide somatostatin (SRIF),[1] originally isolated from the hypothalamus as a growth hormone-inhibiting factor (1), has been found to exhibit a wide spectrum of biological actions and to be distributed outside the hypothalamus in sites such as extrahypothalamic brain and spinal cord, peripheral nervous system, pancreas, and gastrointestinal tract (2–5). In addition, SRIF and somatostatin-like immunoreactivity (SLI) have also been reported in biological fluids such as blood (6–10), cerebrospinal fluid (11), and urine (12), but their presence in amniotic fluid has not been described previously.

A number of studies have indicated that the placenta and amniotic fluid may be a source of peptide hormones such as thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and ACTH (13–15), a finding of both practical and theoretical importance because the placental peptide-producing cells may share a common embryological origin with the brain-, gut-, and cutaneous peptide-producing cells (16).

In this study we report (*a*) the presence of SLI in human amniotic fluid, (*b*) the characterization of this

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[1] *Abbreviations used in this paper:* OGTT, oral glucose tolerance test; SLI, somatostatin-like immunoreactivity; SRIF, tetradecapeptide somatostatin.

material by gel filtration, (*c*) the possible origins of the SLI, and (*d*) levels of amniotic fluid SLI in normal and complicated pregnancies.

METHODS

Samples

Amniotic fluid from normal pregnancies. Amniotic fluid samples were obtained by amniocentesis performed for other well-established reasons. 15 samples were obtained between 15 and 20 wk of gestation in apparently normal pregnancies undergoing saline termination. 15 additional samples were collected from normal pregnancies between 32 and 43 wk of gestation where amniocentesis was performed for determination of lecithin:sphingomyelin ratio, fetal age, etc.

Amniotic fluid from abnormal pregnancies. 21 samples were obtained between 35 and 40 wk from abnormal pregnancies as follows: six cases complicated by preeclampsia; and eight cases of maternal diabetes which included five patients with an abnormal oral glucose tolerance test (OGTT) only, one with gestational diabetes requiring insulin, one with long-standing insulin-dependent diabetes, and one with chronic renal failure and an abnormal OGTT. All these pregnancies had a normal outcome, although the last two were complicated by fetal distress. One sample was obtained from a pregnancy complicated by a severe meningomyelocele which resulted in death shortly after birth, and another sample was collected from a patient who delivered an anencephalic fetus. Finally, there were five samples from three instances of twin pregnancies (monochorionic diamniotic, dichorionic diamniotic, and monochorionic monoamniotic twins). The three sets of twins were normal at birth.

Placental and fetal tissue. Five placentas, together with placental and fetal membranes, were obtained immediately post-partum (36–40 wk gestation). In addition, two placentas and membranes were collected after hysterotomy for termination of pregnancy at 14 and 18 wk of gestation, respectively. The stomach of the 18-wk-old fetus was obtained for characterization of fetal gut SLI.

Cord blood. Seven pairs of arterial and venous umbilical cord blood were obtained immediately post-partum at 36–40 wk of gestation.

The protocols used in these studies were reviewed and approved by the Medical Evaluation Committees at the Royal Victoria and Montreal Children's Hospitals, McGill University, Montreal. Informed consent was obtained from all patients studied.

Radioimmunoassay of SLI

SLI was measured by radioimmunoassay with a rabbit anti-SRIF antibody, [^{125}I-Tyr^{1}]SRIF label, and synthetic SRIF standards as previously described (5).

Amniotic fluid. In initial studies, 0.1 ml of unmodified amniotic fluid was incubated for assay. Under these conditions, however, [^{125}I-Tyr^{1}]SRIF underwent extensive incubation damage as assessed by chromatoelectrophoresis (17) and excess antibody binding. With both these criteria, only 11% of the tracer remained intact after a 24-h period of incubation with amniotic fluid compared with 95% of intact tracer when incubated with buffer alone. When the amniotic fluid samples were acidified to pH 1.5 with 1 N HCl, boiled for 5 min, neutralized, and then incubated with [^{125}I-Tyr^{1}]SRIF, damage was reduced to <5% and was comparable to that with buffer alone. As a result of these findings, all samples of amniotic fluid were collected in tubes at 0°C, immediately acidified, boiled, and neutralized before the assay. Mean assay sensitivity was 1 pg. Within-assay precision, expressed as a coefficient of variation, was ±5% over the range 5–100 pg SRIF. Between-assay precision over the same range was <±11%. Recovery of 100 pg synthetic SRIF added to amniotic fluid ranged from 96 to 101%.

Amniotic fluid cells. Amniotic fluid cells were harvested from freshly obtained amniotic fluid by centrifugation (2,000 rpm for 20 min) at 4°C. After removal of the supernate, the pellet was washed in saline to remove contaminating amniotic fluid, recentrifuged, and the final pellet which contained the precipitated cells was extracted by sonication in 1 M acetic acid, boiled, neutralized, and assayed for SLI.

Plasma SLI. Radioimmunoassay of SLI in plasma was performed by a single-step extraction procedure.[2] 0.5 ml of plasma was mixed with 1 ml acid-ethanol (95 parts absolute ethanol, 5 parts 1 N HCl) on a vortex stirrer, the precipitated proteins were separated by centrifugation, and the supernate was assayed directly for SLI (18). By this method, incubation damage to [^{125}I-Tyr^{1}]SRIF as assessed by chromatoelectrophoresis and excess antibody binding was <10%. Recovery of synthetic SRIF (100–500 pg/ml) added to plasma was 94±1.5% (n = 10). Assay sensitivity was 30 pg SLI/ml plasma. Within-assay precision, expressed as a coefficient of variation, was ±8% over the range 50–1,000 pg/ml.

Tissue SLI. For determination of SLI in placenta and placental and fetal membranes, tissue samples (≅5–10 g wet wt) were rinsed in saline to remove contaminating blood and amniotic fluid, finely minced with scizzors in 1 M acetic acid at 0°C, and extracted by sonication and boiling as previously described (5). The same procedure was used for extraction of fetal stomach. The extracts were neutralized and assayed.

Gel filtration

For gel filtration studies, 30- to 40-ml pools of amniotic fluid were concentrated by lyophilization and chromatographed on Sephadex G-25 (fine) (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) columns (1.3 × 40 cm) eluted with a 6-M urea–0.05-M PO_4 buffer, pH 7.5 (5). Identical conditions were used for gel filtration of fetal stomach extracts. SLI in the eluting fractions was measured by radioimmunoassay.

Calculation of assay data and statistical analysis

Results of assays including assay sensitivity and precision were computed with the Faden and Rodbard program (19). To check for parallelism between serial dilution slopes of unknown substances and SRIF, the residual variances of the two curves were tested for homogeneity by an f test, and the lines were tested for parallelism by a t test both with the aid of the program (19). Significant nonparallelism was indicated by a P value > 0.05 in the t test. Results are expressed as mean±SE. For comparison of arterial and venous umbilical cord blood levels of SLI, the paired Student's t test was employed. The nonpaired t test was used for all other analyses.

RESULTS

SLI in normal amniotic fluid. Detectable levels of SLI were found in all the samples of amniotic fluid from the 30 normal pregnancies, with values ranging

[2] Patel, Y. C., T. W. Wheatley, D. Fitz-Patrick, and J. Brock. Manuscript submitted for publication.

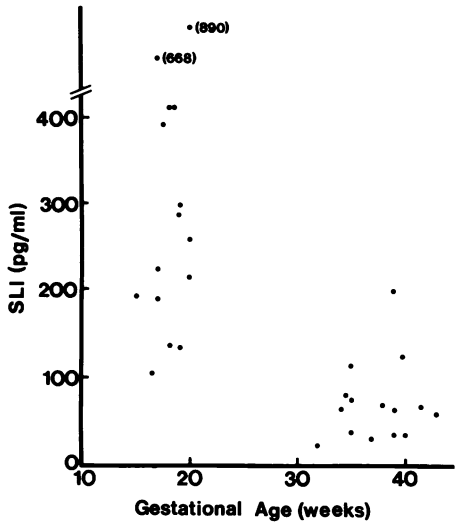

FIGURE 1 SLI concentration in amniotic fluid of normal pregnancies as a function of gestational age.

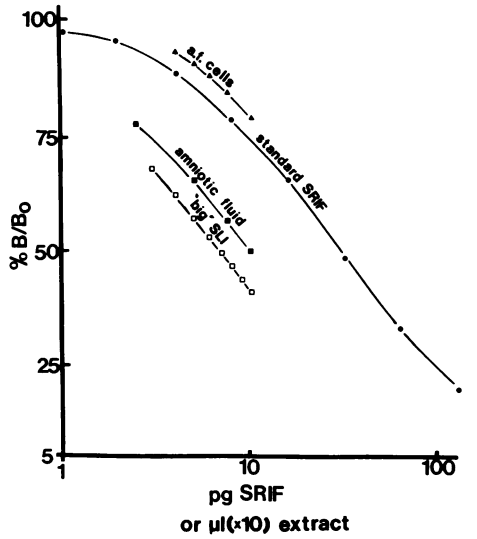

FIGURE 2 Comparison of SRIF standard curve with inhibition curves obtained by assay of serial dilutions of amniotic fluid, extracts of amniotic fluid cells (a.f. cells), and column fractions (20 and 21 in Fig. 3, upper panel) containing big SLI. The radioactivity bound to antiserum in the absence of SRIF (B_0, 38%) was assigned a value of 100, and all other results were expressed as a percentage (B/B_0).

from 22 to 890 pg/ml (Fig. 1). The mean SLI level for the 15 samples obtained between 15 and 20 wk of gestation was 320±55 pg/ml being more than four times greater than the mean value of the 15 samples obtained between 32 and 43 wk (70±12 pg/ml, $P < 0.0002$).

SLI in amniotic fluid of abnormal pregnancies. The mean amniotic fluid SLI concentration in pregnancies complicated by preeclampsia was 50±7 pg/ml, and in those with an abnormal OGTT the mean level was 81±19 pg/ml. Neither value was significantly different from the mean SLI level (70±12 pg/ml) in normal pregnancies of the same gestational age. Amniotic fluid SLI concentration in the patient with gestational diabetes requiring insulin was 103 pg/ml, whereas the levels were undetectable (<10 pg/ml) in the juvenile-onset diabetic patient whose insulin requirements had declined markedly between 34 and 37 wk of gestation and in the patient with chronic renal failure, abnormal OGTT, and evidence of fetal distress. The two cases of fetal disorders (meningomyelocele and anencephaly) had amniotic fluid SLI levels of 77 and 85 pg/ml, respectively. In the three twin pregnancies studied, the five samples contained the following SLI values: 33 and 197 pg/ml (monochorionic and diamniotic); 90 and 123 pg/ml (dichorionic and diamniotic); and 156 pg/ml (monochorionic and monoamniotic).

Characterization of amniotic fluid SLI. To further characterize the SLI material in amniotic fluid, serial dilutions of fluid were assayed, and the resultant inhibition curves (Fig. 2) showed parallelism with curves obtained using standard SRIF. Gel filtration of amniotic fluid concentrates showed the presence of only one peak of immunoreactivity eluting in the void volume (Fig. 3, upper panel). There was no immunoreactive material corresponding to synthetic SRIF. Treatment of the high molecular weight SLI material with 8 M urea at 100°C for 5 min followed by incubation with dithiothreitol for 3 h at 50°C in an atmosphere of N_2 (20) maintained the elution position in the void-volume region with no conversion to a lower molecular weight immunoreac-

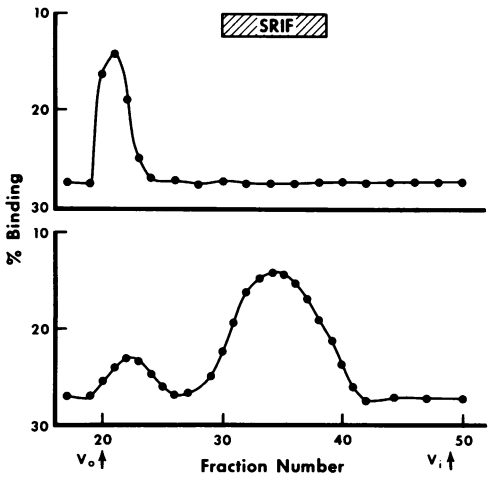

FIGURE 3 Elution pattern of SLI after Sephadex G-25 gel chromatography of concentrated amniotic fluid (upper panel) and human fetal stomach extract (lower panel). A 1.3 × 40-cm column was eluted with a 6-M urea–0.05-M PO_4 buffer, pH 7.5. SLI in the fractions was determined by radioimmunoassay. Synthetic SRIF was eluted in fractions 31–37 as indicated. %Binding, percentage of total counts bound. V_o, void volume; V_i, salt volume.

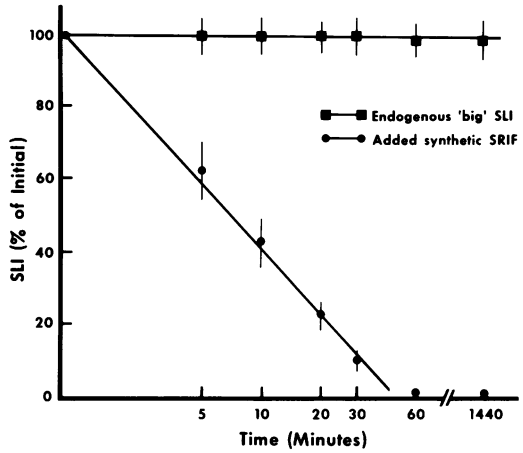

FIGURE 4 Comparison of the stability of amniotic fluid big SLI and synthetic SRIF in amniotic fluid at 37°C. Data are the average of four experiments (mean±SEM).

tive form. Assay of serial dilutions of the void-volume material showed parallelism with standard SRIF (Fig. 2).

Characterization of human fetal gastric SLI. Sephadex G-25 gel filtration of fetal stomach extracts revealed two peaks of immunoreactivity (Fig. 3, lower panel), one in the void volume (≅20% of total immunoreactivity) coeluting with "big" amniotic fluid SLI, the other peak (≅80% of total immunoreactivity) corresponding to SRIF.

Stability of big somatostatin and SRIF in amniotic fluid. To determine the stability of big SLI in amniotic fluid, freshly obtained (untreated) amniotic fluid was divided into 10 0.5-ml aliquots, one of which was prepared for assay immediately and the remainder were incubated at 37°C for different periods of time up to 24 h and assayed after acidification, heating, and neutralization. The concentration of SLI in the amniotic fluid did not alter significantly over the period of incubation (Fig. 4). The fact that the immunoreactivity was composed entirely of big SLI, suggests that the large molecular weight form of SLI is stable in amniotic fluid for at least 24 h at 37°C.

In a second experiment performed in parallel with the one described above, aliquots of the same amniotic fluid, but with 1 ng/ml of synthetic SRIF added, were incubated at 37°C for up to 24 h. Samples were removed at the same time points as in the first experiment and assayed in the same manner. By subtracting the values of SLI obtained in the first experiment from that of the corresponding tube in the second experiment it was possible to determine the levels of SRIF in the latter (Fig. 4). This experiment showed that, unlike big SLI, SRIF is rapidly degraded in amniotic fluid at 37°C with a $t_{1/2}$ of ≅7 min.

Assay of placenta and placental membranes for SLI. SLI was undetectable (<0.0001 ng/mg wet wt) in extracts of placenta and placental and fetal membranes.

TABLE I
Concentration of SLI in Seven Pairs of Umbilical Cord Arterial and Venous Plasma Samples

	SLI concentration	
	Arterial plasma	Venous plasma
	pg/ml	*pg/ml*
	75	84
	75	99
	204	171
	267	216
	474	237
	123	30
	159	177
Mean±SE	197±53	145±29

SLI in amniotic fluid cells. Extracts of amniotic fluid cells were found to contain small amounts of SLI and gave parallel inhibition curves to synthetic SRIF (Fig. 2). To determine the magnitude of the contribution of these intact cells to the total amount of SLI in amniotic fluid, six fluids were assayed before and after removal of the cells by centrifugation. Regardless of gestational age, removal of the cells resulted in a 6–20% decrease in SLI values.

Arterial and venous umbilical cord blood SLI. Table I shows a comparison of the SLI concentrations in seven pairs of arterial and venous blood samples obtained from the umbilical cord. There was no significant arteriovenous gradient of SLI ($P = 0.29$), although in two of the pairs, the arterial blood SLI levels exceeded the venous levels by 200 and 410%, respectively.

DISCUSSION

In this study, human amniotic fluid has been shown to contain SLI which on gel chromatography was not SRIF but material(s) with a molecular weight ≥5,000 (big SLI). High molecular weight forms of immunoreactive somatostatin have been described before in normal tissues, in blood, and in a case of pancreatic somatostatinoma (2, 5, 10, 21, 22) and interpreted in different studies as representing a prohormone (5), aggregated SRIF (23), or protein-bound SRIF (24). Biosynthesis studies with pancreatic islets and hypothalamic slices have provided evidence for a big somatostatin precursor molecule (25, 26). Our finding of big SLI in amniotic fluid which did not dissociate to a lower molecular weight immunoreactive form with boiling 8 M urea and dithiothreitol excludes noncovalently bound SRIF as a constituent of the big material and suggests that it likely represents a prohormone. Gel filtration of human fetal stomach extracts also revealed the presence of a high molecular weight peak of SLI similar to that found in amniotic fluid. Whether the big SLI of amniotic fluid is identical to big SLI in tissues

and in plasma remains to be determined. Because big SLI in tissues (e.g., Fig. 3) and in blood has been reported to coexist with SRIF (2, 5, 10, 21, 22) our finding of only big SLI in amniotic fluid is of interest. Although it is possible that only big SLI is released into the amniotic fluid, the more likely explanation is the selective degradation of SRIF ($t_{1/2}$ of $\cong$7 min) and the relative stability of big SLI in amniotic fluid (Fig. 4).

There is growing evidence that the placenta may produce peptide hormones, e.g., thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, ACTH, and prolactin (13–15, 27). In this study, however, SLI could not be demonstrated in extracts of placenta and placental and fetal membranes, which argues against these tissues as a source of amniotic fluid SLI. In addition, the absence of an arteriovenous gradient of SLI in cord blood is evidence that the placenta neither secretes SLI into the cord blood nor degrades it, which suggests that amniotic fluid SLI is not of placental (or maternal) origin and probably comes from the fetus. That fetal factors are more important than materno-placental ones in determining amniotic fluid SLI is also indicated by the finding of different levels of SLI in the amniotic fluids of a pair of twins with a monochorionic diamniotic placenta.

The cells normally found in amniotic fluid are derived from the fetal skin as well as the respiratory, genitourinary, and gastrointestinal tracts (28) and were found in this study to make a contribution of up to 20% of total amniotic fluid SLI. Because somatostatin cells are present in the mucosa throughout the gastrointestinal tract (3) it seems likely that the desquamated cells of the fetal gut are mainly responsible for the SLI detected in the cellular fraction of amniotic fluid, although contributions from cells of other origin which have not been closely examined for SLI cannot be excluded.

Apart from fetal cells, the fetal circulation is another potentially important source of amniotic fluid SLI (28). SLI is certainly present in fetal blood as indicated by our measurements in umbilical cord arterial blood (Table I), and, furthermore, the mean blood value (197$\pm$53 pg/ml) was more than twice the mean amniotic fluid SLI concentration in the third trimester (70$\pm$12 pg/ml) (Fig 1). In the first half of pregnancy, the fetal skin is freely permeable, and amniotic fluid can be regarded as an extension of the fetal extracellular fluid (28). Diffusion of SLI from fetal blood via fetal skin, umbilical cord, and fetal placental vessels may thus account for the high levels of amniotic fluid SLI found in early pregnancy. After mid-gestation the permeability of the fetal skin is decreased, presumably resulting in decreased delivery of SLI to the amniotic fluid which also steadily increases in volume as a result of the contribution of fetal urine (29). These events may provide an explanation of the sharp drop in amniotic fluid SLI concentration between early and late pregnancy (Fig. 1). In a preliminary study of neonates, we have been unable to demonstrate big somatostatin in urine, findings comparable to those reported for adult urine (12), although it is recognized that in early pregnancy the fetal kidney may not exclude big SLI from urine.

Because somatostatin is present in high concentrations in the brain, spinal cord, pancreas, and gut (2, 5), and because amniotic fluid SLI measurements may reflect fetal somatostatin function, the values of amniotic fluid SLI are of particular interest in disorders of these systems. In the cases of gestational diabetes, the SLI levels were comparable to those of normal pregnancies. However, in one severe juvenile-onset diabetic patient with rapidly falling insulin requirements, SLI was undetectable as was also the case in the patient with chronic renal failure and mild diabetes. In both cases there was evidence of fetal distress at birth. In the two cases of anencephaly and meningomyelocele, respectively, SLI levels were in the normal range. Further studies in similar cases of fetal and maternal abnormalities should help to delineate the value of amniotic fluid SLI measurements in the assessment of fetal somatostatin function and in the diagnosis of some fetal disorders.

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