Extraction of Anemia-inducing Substance from the Human Placenta and Its Chemical Nature

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A number of fractions were obtained from aqueous extracts of the human placenta. These fractions were tested for anemia-inducing activity, and their chemical and physical properties were examined.

The results are summarized as follows:

- 1) An anemia-inducing substance (P-62) was extracted from the human placenta with 60% (v/v) ethanol and was precipitated at full saturation with ammonium sulfate.
- 2) Intravenous injection of P-62 produced anemia in rabbits even in a small dose of 0.02 mg per kg of body weight.
- 3) The substance was almost homogeneous in the electrophoretic pattern.
- 4) Chemical analysis showed that P-62 was a mucoprotein.

It was found by Kozawa *et al.*¹ that intravenous injection of gastric juice from patients with gastric cancer produced anemia in rabbits. The phenomenon was termed KIK reaction by them. The nature of the factor responsible for this reaction was partly elucidated by Masamune and Kawasaki,² Harima³ and Ogata.⁴ It has been shown that the anemia-inducing substance was contained not only in gastric juice of patients with gastric cancer but also in the cancerous gastric tissue.⁵⁻¹⁰ As previously reported by Oh-Uti and his co-workers,¹¹⁻¹⁵ the human placenta was found to have immunologic characteristics similar to those of gastric cancer tissue. With a view of seeing whether the human placenta contains such an anemia-inducing factor, we have carried out the present work.

METHODS AND RESULTS

Preparation of placental fractions

Human placentas were kindly supplied from Prof. Shinagawa's obstetrical clinic, and after the blood was washed out under running water, they were mechanically freed from the amnion and decidua, cut into pieces, and homogenized by a Waring blender.

Extraction. From 3 human placentas 845 g of the tissue were obtained and extracted with 1.5 volumes of distilled water containing 2-3 drops of 5% thymol alcohol, by shaking for 2 days in a cold room at 2°C. The residue was treated further twice in the same way with decreasing volumes of water. The pooled aqueous extract of 3,000 ml was evaporated under reduced pressure to about 1/3 of the original volume and dialyzed against running water through a cellophane membrane for 48 hours.

Fractionation. The dialyzed solution (inner fluid) was evaporated to about 1/10 in volume (114 ml), and then barium acetate was added to a final concentration of 3.5%. The precipitate (crude P-8) was separated by centrifugation, dissolved in 100 ml of water, and crystalline Na_2SO_4 , just sufficient to carry down all the barium ion, was added. The whitish precipitate of BaSO_4 was discarded after centrifugation. The supernatant was dialyzed for 3 days, evaporated to a syrup and 10 volumes of absolute ethanol were added. The resulting solution was placed in a refrigerator for several hours. The precipitate (P-8) was washed in ethanol and dried in a desiccator.

To the supernatant obtained after $\text{Ba}(\text{AcO})_2$ addition, ice-cooled 99% ethanol was added drop by drop until the solution contained 20% ethanol by volume. The solution was again placed in a refrigerator for several hours.

The resulting precipitate (Cr. P-20) was washed with 20% ethanol several times, and then further purified in the same manner as described for Cr. P-8. The precipitate obtained (P-20) was stored in a desiccator. When the supernatant and the 20% ethanol-washing of Cr. P-20 were combined, they yielded a volume of 219 ml altogether. The mixture was then chilled. After that 99% ethanol was added until the solution was of 40% (v/v) alcohol concentration. After cooling, the precipitate produced (Cr. P-40) was removed, and ethanol was further added to the supernatant to make the concentration of ethanol 60% (v/v), and the resulting solution was again cooled and the precipitate (Cr. P-60) was removed. These two precipitates (Cr. P-40 and Cr. P-60) were dissolved in water, Na_2SO_4 was added, and purified by the same procedure as described for Cr. P-8. The supernatant containing 60% ethanol was evaporated to about 100 ml, and crystalline Na_2SO_4 , just sufficient to carry down all the barium ion, was added. Again the purification process of Cr. P-8 was repeated and the resulting precipitate (P-61) was dried and stored in a desiccator. P-61 (410 mg) was dissolved in 100 ml of distilled water and then saturated fully with ammonium sulfate. The mixture was allowed to stand at room temperature for 3 hours and then centrifuged. The precipitate (Cr. P-62) was washed twice with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, and added with a small amount of distilled water and dialyzed against distilled water, while the supernatant and washings of Cr. P-62, were combined together and submitted also to dialysis. After completion of dialysis, the two dialyzed fractions were each condensed under reduced pressure to syrup to which alcohol was added and the mixture was dried to powder. The powder from the precipitate by $(\text{NH}_4)_2$ -

Fig. 1. Scheme of the fractionation procedures of anemia-inducing substance from human placenta.

SO₄-saturation, Cr. P-62, amounted to 185 mg and the powder from the supernatant by (NH₄)₂SO₄-saturation, P-63, to 89 mg. The procedure of preparation is shown in Fig. 1.

Purification. The powder of Cr. P-62 was further purified by Gel-filtration on a column of Sephadex G-150 according to Millin's method.¹⁶ Sephadex G-150 was exhaustively washed with water and filled in a column of 45×2 cm without salt or organic solvent. Cr. P-62 was dissolved in water to 4%, placed on a column and developed with water. Fractions of 5 ml were received with a fraction collector. From each tube of the collector, 0.3 ml was taken and examined with Folin-Ciocalteu's, Anthrone and Nessler's reagents.

As illustrated in Fig. 2, a single peak of sugar containing protein was found between the 10th and 40th tubes of the elution. These fractions were combined,

Fig. 2. Gel filtration diagram of Cr. P-62 on a column (45×2 cm) of Sephadex G-150. Five ml of the fraction were collected at a flow rate of about 20 ml/hr. Aliquots of 0.1 ml each were analyzed for the Folin-Ciocalteu reaction (—o—o—), Nessler reaction (-----) and the Anthrone reagent (—x—x—).

concentrated, and then lyophilized. Grayish whitish powder obtained in this way was termed P-62 and regarded as purified anemia-inducing substance.

Anemia-inducing activity

The anemia-inducing activity of the placental preparation was assayed¹⁷ in principle according to Iwatsuru and Kato's¹⁸ method. Various amounts of placental fractions were dissolved in physiological saline and injected into the auricular vein of rabbits weighing 2–3 kg. The erythrocytes of venous blood were counted before and 1, 2, 3, 4 and 5 hours after the injection to determine the minimum dose per kg of body weight, necessary to cause a 10% decrease of erythrocytes.

P-62 fraction induced marked anemia in rabbits even in a small dose of 0.02 mg per kg of body weight. These results are shown in Tables 1 and 2.

Chemical properties

Electrophoretic examination. The anemia-inducing substance from human placentas, namely P-62, was examined using a Tiselius apparatus (HTD-1 type, Hitachi Ltd.). As shown in Fig. 3, P-62 was proved to be relatively homogeneous in the electrophoretic pattern.

*Paper partition chromatography of sugar and aminosugar.*¹⁹ (a) Five mg of the anemia-inducing substance, P-62, were sealed in a tube with 0.5 ml of 1N H₂SO₄ and heated at 100°C for 5 hours. The hydrolysate was adjusted to pH 5.0–6.0 with saturated Ba(OH)₂ and centrifuged. The precipitate was washed with three 2 ml portions of water warmed to 70°C. The supernatant and washings were united and filtered under suction, and the filtrate was evaporated *in vacuo* over CaCl₂ to dryness. The residue was dissolved in 0.05 ml of water, and was applied onto 12×60 cm

TABLE 1. *Anemia-inducing effect of each placental fraction on rabbits*

Fraction	Number of examinations	Substance injected (mg per kg body weight)	Results	
			Anemia (+)	Anemia (-)
P-3	3	1.0	0	3
	2	3.0	0	2
P-20	3	1.0	0	3
	2	3.0	0	2
P-40	4	1.0	0	4
	3	3.0	0	3
P-60	3	1.0	0	3
	3	1.0	0	3
P-61	1	0.4	1	0
	1	0.5	1	0
	1	1.0	1	0
	1	1.25	1	0
P-62	45	Given in Table 2	37	8
P-63	3		2	1
	3		1	2

TABLE 2. *Anemia-inducing effect of P-62 on rabbits*

P-62 (mg per kg body weight)	Number of examinations	Results	
		Anemia (+)	Anemia (-)
0.01	6	3	3
0.02	15	15	0
0.025	5	3	2
0.03	2	2	0
0.04	1	0	1
0.05	6	5	1
0.25	3	3	0
0.5	7	6	1
Total	45	37	8

pieces of Toyo Roshi filter paper No. 2. Descending paper partition chromatography was made with butanol-pyridine-water mixture (5:3:2 by volume) according to the principle of Masamune and Yoshizawa. The sample was spotted 10 cm apart from the upper margin and developed for 24 hours. Aniline hydrogen phthalate was used as the indicator.

As shown in Fig. 4 A, P-62 gave spots at the positions corresponding to aminosugars (galactosamine and glucosamine), galactose, glucose, mannose and xylose and/or L-fucose in this order from above. The spots of aminosugars were connected to each other and the lower spot had some red tint suggesting overlapping of two sugars, L-fucose and xylose.

Fig. 3. Electrophoretic pattern of 1% solution of the placental anemia-inducing substance, P-62, in 0.1 M phosphate buffer (pH 7.8, $\mu=0.2$) after 40 and 60 minutes. Temperature 15°C , current 6 mA, ascending limb.

Fig. 4. Chromatograms of P-62.

Hydrolysis: (A) (B) 1N H_2SO_4 , 5 hours, (C) 2N HCl, 14 hours

Solvent: (A) (C) Butanol-pyridine-water (5:3:2)

(B) n-Butylacetate-acetic acid-ethanol-water (3:2:1:1)

Chromatography in all cases was developed by descending irrigation for 24 hours at $20 \pm 1^{\circ}\text{C}$. Indicator, aniline hydrogen phthalate. Reference runs were done with a 0.002 ml portion of 1% solutions with respect to individual sugars and aminosugars. a: P-62. b: reference run. 1: galactosamine hydrochloride. 2: glucosamine hydrochloride. 3: galactose. 4: glucose. 5: mannose. 6: xylose. 7: L-fucose.

(b) Next, the hydrolysate was developed with a mixture of n-butylacetate, acetic acid, ethanol and water in a volume proportion of 3:2:1:1. The chromatograms obtained (Fig. 4 B) had weakly demonstrated spots of xylose and L-fucose.

(c) Then, 5 mg of P-62 was hydrolyzed with 0.5 ml of 2N HCl at 100°C for 14 hours, and the hydrolysate was freed from most of hydrochloric acid by keeping it in a vacuum desiccator over anhydrous calcium chloride and solid caustic soda at 40°C , with an occasional addition of a few drops of water. When pH of the solution reached a value of about 4, the solution was centrifuged and the pellet

was washed with water. The supernatant combined with the washing was evaporated to dryness. The paper chromatography was developed with the same method as described in (a).

As shown in Fig. 4C, galactosamine and glucosamine were detected.

Quantitative analysis

The results of analysis were listed in Table 3 and compared with analytical data of anemia-inducing substances from gastric juice of patients with gastric cancer (KIK factor II) and from cancerous gastric tissue (MPRI).

TABLE 3. Analysis of P-62 and of other anemia-inducing substances

Analysis	P-62	KIK factor II	MPRI
N*	12.3	11.2	12.1
Total hexosamine**	3.7	5.7	4.9
Galactosamine/ glucosamine +	1/3		
Mannose ±	4.4	3.5	
Glucose ±	2.3	1.9	6.4
Galactose ±	1.5	2.9	
L-Fucose §	0.8	1.1	0.9
Xylose †	1.0	0.9	
Sialic acid ‡	2.8	2.6	2.0
Ash ⊥	2.0	1.7	1.0

* Micro Kjeldahl, ** Blix²⁰, + Roseman & Paffner,²¹ ± Masamune & Sakamoto,²² § Dische & Shettles²³, † Masamune & Sakamoto,²⁴ ‡ Werner & Odin,²⁵ ⊥ Pregl without use of H₂SO₄

DISCUSSION

Masamune and Kawasaki,² Harima³ and Ogata⁴ fractionated gastric juice from patients with gastric cancer in order to detect fractions with anemia-inducing activity (KIK reaction), and obtained two active substances which were each electrophoretically almost homogeneous. On the basis of their analytical examination, they concluded that one of the substances (KIK factor I) was a mucopolysaccharide and the other (KIK factor II) a mucoprotein. In recent studies⁵⁻¹⁰ it was found that another anemia-inducing substance was contained in the cancerous gastric tissue (MPR-I), and it belonged to mucoprotein.

As previously reported by Oh-Uti *et al.*,¹¹ Ogawa,¹² Kobayashi and his co-workers,¹³⁻¹⁵ the immunological behavior of the human placenta is clearly related to that of gastric cancer tissue. Therefore, we have carried out the present work to see whether the human placenta itself contains such an anemia-inducing substance.

A number of fractions were obtained from the aqueous extracts of the placenta by fractionation with barium acetate, ethanol and ammonium sulfate in turn. Of these several fractions only the supernatant at 60% (v/v) ethanol concentration was obviously effective. The precipitate at full saturation with ammonium

sulfate of this fraction was further purified by gel-filtration with Sephadex G-150. The fraction obtained induced a marked anemia in rabbits even in a small dose of 0.02 mg per kg of body weight and was designated as P-62.

The electrophoretic examination revealed that P-62 was relatively homogeneous. Chemical analysis of P-62 indicated that it belonged to mucoprotein, and that the composition of P-62 was quantitatively very similar to that of the anemia-inducing substance from gastric juice of patients with gastric cancer (KIK factor II)² and from cancerous gastric tissue (MPRI).^{6,8}

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