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Pigeon milk: a new source of growth factor

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Abstract. Crude, partially purified and purified fractions of pigeon milk injected subcutaneously in newborn mice brought about precocious opening of eyelids by 2–3 days and eruption of incisors by 3–4 days. The biological activity of pigeon milk-derived growth factor (PMGF) compared well with that of mouse epidermal growth factor (mEGF).
Key words. Pigeon milk; growth factor; newborn mice.

Pigeons and doves are unique among the estimated 9,000 species of birds in the remarkable functional specialization of the crop in both sexes to synthesize and secrete a nutritive material, popularly called crop milk or pigeon milk (PM)[1]. PM is a high-protein (9–13%), high-lipid (9–11%) and low-carbohydrate (0.9–1.5%) juvenile diet with an energy content of 6.4 kcal/g[2–5]. The altricial baby pigeons (called squabs), fed with PM, increase in body weight by 22-fold in the first 3 post-hatching weeks[5]. Domestic chicks and weaned rats fed small quantities of PM also showed better growth[2,3]. Recently, we reported that PM is a rich source of trace minerals[6]. In spite of several studies on the chemical composition of PM[3–7], practically nothing is known about its growth-promoting properties or growth factors (GFs).

The speculation that PM could be a source of GF was based on: i) the phenomenal rate of growth of squabs; ii) the fact that the crop is close to the salivary glands, and that regurgitation of PM while the squabs are fed will lead to its being mixed with saliva (a source of epidermal growth factor (EGF) in mammals and possibly in birds)[8]; iii) induction of crop development by the synergistic action of prolactin, proinsulin and EGF[9]; iv) the analogy between PM and mammalian milk as a sole source of neonate nutrition, and v) the rapid growth of the small intestine in squabs[1]. In this paper, we report certain biological properties of PM-derived growth factor (abbreviated PMGF), and suggest that they compare well with those of the epidermal growth factor (EGF) isolated from mouse salivary gland[10,11] and from human milk[12]. As it is held that the most specific biological assay for EGF is its ability to cause precocious incisor eruption and eyelid opening in new-born mice[10–12], we mainly used this test in our study.

Materials and methods

PM was obtained from 1–5 day-old squabs. Because the parent pigeons do not store PM at any time in their crop cavity, the samples had to be extracted (once a day, normally between 11:00 and 12:00) from freshly-fed squabs. The squabs were anaesthesized with ether and their crops were slit open by a surgical incision one centimeter long. After squeezing out the semi-solid PM (a maximum of 4–6 g could be collected from each squab at one time), the slits were closed by double sutures and the squabs replaced in their nests for parental brooding and feeding. PM samples collected on different days were pooled and stored at −20 °C. Other details concerning PM and its collection were described by us recently[7].

A 5% (w/v) homogenate of PM was thrice extracted in distilled water and the combined filtrates were dialyzed (Mr cut-off 3500) against distilled water, and lyophilized. The crude aqueous extract made by dissolving the residue in phosphate buffered saline (PBS, pH 7.0) was tested in vivo by injecting subcutaneously 140 (n = 5), 210 (n = 6), 280 (n = 4) and 420 (n = 6) µg protein (in 10, 15, 20 and 30 µl respectively) per 1.4 g b wt per day into newborn mice for 12 days. A Sephadex G-25 fraction was obtained by passing 2 ml crude extract through a column of 1.0 × 50 cm. Another portion of the lyophilized dialyzate was reextracted from 25 mM ammonium acetate buffer (pH 4.6) and loaded onto a CM-cellulose column (1.5 × 35 cm) that had previously been equilibrated with the same buffer. Elution was performed with 60 ml of the sample buffer followed by a linear gradient elution in 550 ml of 25 mM ammonium acetate (pH 4.6) and 500 mM ammonium acetate (pH 7.6). Fractions (5 ml each) collected at the beginning of gradient elution, and those obtained later, were separately pooled and concentrated (ammonium acetate was removed by repeated lyophilization). The residue, dissolved in PBS, was used for bioassay. Mice (n = 12) were given s.c. injections of 68 µg protein (in 20 µl) per 1.4 g b wt per day from birth to 10 days. Another portion of the lyophilized material was reextracted in 20 mM ammonium acetate pH 5.6 and loaded onto a

DEAE-cellulose column (1.5 × 20 cm). The column was initially washed with 60 ml equilibrium buffer to improve the resolution, and this was followed by a 250 ml linear gradient elution in 20 mM ammonium acetate (pH 5.6) and 200 mM ammonium acetate pH 5.6. Fractions (6 ml each) were collected, concentrated, dissolved in PBS and subjected to bioassay in newborn mice (n = 16) by injecting s.c. 1 µg protein (in 10 µl) per 1.4 g b wt per day for 10 days. In all experiments the litter-mate controls were sham-injected with equivalent volumes of PBS. The significance of the differences was determined by Students 't' test.

Results and discussion

Results of PM bioassay on newborn mice given in the table indicate that when the dialyzed aqueous extracts of PM were injected, opening of eyelids and eruption of incisors took place in the experimental group earlier than in the sham-injected control group by 2 and 3 days respectively. The injected mice (280 µg group) weighed significantly ($p < 0.001$) less (3.1 g) than the controls (5.8 g) and there was delay in hair development by 2–3 days in the former; however, the ear opening was normal. Animals given lower doses (140 µg and 210 µg) weighted slightly more (4.2 g and 3.6 g respectively) and the delay in hair development was by 2–3 days. Administration of 420 µg of the sample led to the death of all the animals within 1–2 days. PM extract desalted through a Sephadex G-25 column elicited a response comparable to that of aqueous extract; however, the delay in hair development was only by 1–2 days. Injection of CM-cellulose and DEAE-cellulose PM fractions both resulted in eyelid opening and incisor eruption 3 and 4 days earlier than controls, respectively (fig.). In both cases, the delay in hair development was by 1–2 days and the growth impairment was less marked. There was little variation in the response among the controls of each group.

The probable reason for significant reduction in b.wt or concomitant death of mice injected with higher doses of PM crude extract is that the latter had some kind of toxic effect. It is relevant to mention in this context that although a crude homogenate of PM stimulates the in vitro growth of fibroblasts at concentrations up to 1% (v/v), beyond this concentration it has toxic effects and the cells undergo lysis and death[30]. In fact, in his pioneering experiments on the EGF of mouse submaxillary glands, Cohen[10] found a reduction in the body weight of mice when EGF was injected at high doses[10].

We have recently reported that aqueous extracts of PM exert stimulatory effects on growth in vivo and in vitro, and that the mitogenic activity of the PM extract is enhanced 4 and 14 fold after CM- and DEAE- cellulose chromatography respectively[13]. Further, we have also shown that the biologically active factor of PM coelutes from a DEAE-cellulose column and co-migrates on SDS-PAGE, with the EGF of mouse submaxillary

Table. Effect of pigeon milk fractions on eye-lid opening and incisor eruption in newborn mice

Treatment	Body Wt on 10th day (g)	Ear opening (d)	Incisor eruption (d)	Eye opening (d)	Hair growth (d)
Control	5.8 ± 0.4 (15)	3–4	11	13	4–5
Crude aqueous extract					
140 µg	4.2 ± 0.3 (5)	3–4	8	11–12	6–7
210 µg	3.6 ± 0.2 (6)	3–4	8	11	6–8
280 µg	3.1 ± 0.4 (4)	3–4	7	11	6–8
Sephadex G-25					
280 µg	3.8 ± 0.4 (4)	2–4	8	11	5–6
CM-Cellulose					
68 µg	4.40 ± 0.3 (12)	3–4	7	10	5–6
DEAE-Cellulose					
1 µg	4.8 ± 0.2 (16)	3–4	7	10	5–6

Effect of pigeon milk-derived growth factor (PMGF) on b. wt. gain, eye-lid opening, incisor eruption, ear opening and hair growth in newborn mice. Eye-lid opening and incisor eruption were determined according to Carpenter and Cohen[31], ear opening by visibility of the external acoustic duct and hair growth by the appearance of the down hair over the back and neck. The residue obtained after dialysis and lyophilization of crude extract of pigeon milk was injected as described under 'Materials and methods'. Biologically active fractions as verified by the in vivo bioassay procedure from Sephadex G-25, CM and DEAE-Cellulose columns were injected with 280 µg, 68 µg and 1 µg respectively in 20, 20 and 10 µl of PBS. Number of animals used for each bioassay is given in parentheses. The dosages mentioned were injected once daily for 10–12 days. Whereas animals[6] injected with 420 µg of pigeon milk crude extract in 30 µl of PBS died after 1–2 days, those in other groups did not show any mortality.

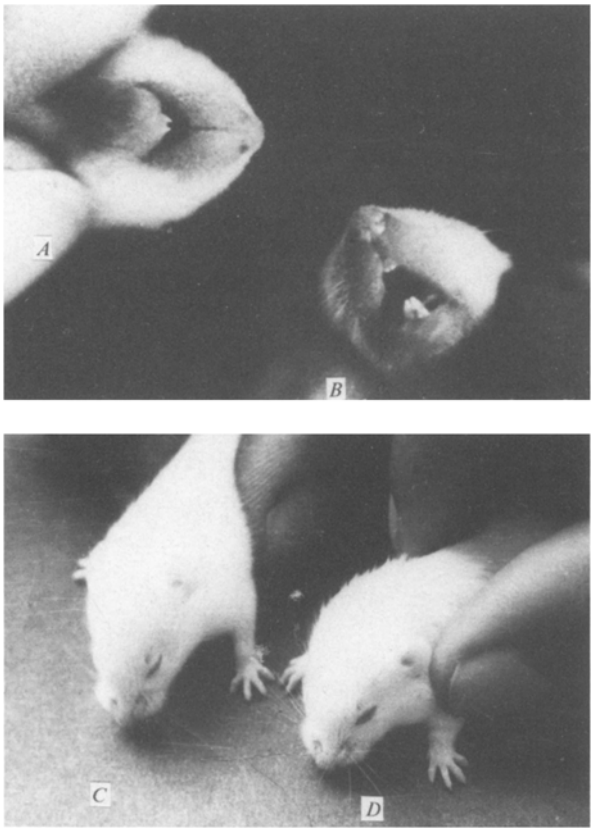

Figure. Effect of subcutaneous injection of pigeon milk-derived growth factor (PMGF), i.e. the DEAE-cellulose chromatography fraction (in PBS) on incisor eruption and eye-lid opening in newborn mice.
A and *B* show the effect of PMGF on incisor eruption in 10-day-old mice. [*A*, sham injected (10 µl PBS) control mouse; *B*, mouse injected with PMGF (1 µg in 10 µl PBS per 1.4 g b. wt per day). Photographs *C* and *D* show the extent of eyelid opening in 10-day-old mice [*C*, sham injected (10 µl PBS) control mouse; *D*, mouse injected with PMGF (1 µg in 10 µl PBS per 1.4 g b. wt per day).

gland. The chromatographic and electrophoretic profile of the PM factor is thus similar to that of mouse EGF (mEGF) under the conditions used. The active protein that we term pigeon milk-derived growth factor (PMGF) was homogeneous and appeared as a single band in SDS-PAGE[13]. Based on the results of SDS-PAGE and gel filtration (Sephadex G-100), its molecular weight was found to be ~6000[13]. Further immunochemical studies such as immunoblotting following SDS-PAGE or radio receptor assay would confirm the similarity of PMGF and mEGF, but were not feasible in our study. We therefore concentrated on using a bioassay to investigate whether PMGF has EGF-like properties.

Epidermal growth factor (EGF), a small anionic polypeptide mitogen (Mr ~ 6000) originally detected in the submaxillary glands of male mice by Cohen[10,11], was later isolated from the glands of rats[15] and shrews[16,17]. It is now recognized that EGF is present in the milk of humans[12,18], rats[19,20] and mice[21,22], and its occurrence in body fluids such as plasma, urine[23,24] and amniotic fluid[25] has also been reported. In ruminants (cow, goat and sheep) the mitogenic activity of colostrum was attributed to a growth factor that is distinct from EGF[18,26,27].

Cohen's report of EGF action on epithelial tissues in chick embryos[28] and the subsequent results of Santora et al.[29] on the presence of EGF receptor in chick tissues, suggested the possible role of EGF in the development of young birds. Kong et al.[8] found that the nest material of swiftlets, made predominantly of salivary secretions of the parent birds, has EGF-like activity. However, as far as we are aware, occurrence of an EGF-like molecule has not been previously reported in non-mammalian secretions of nutritional importance. The origin and source of PMGF is not known at the moment. Presumably, like EGF, it could be derived from the salivary glands, since the crop is an extension of the oesophagus and, as mentioned earlier, during regurgitation the PM is invariably mixed with a copious volume of saliva. Alternatively, the growth factor could also be synthesized in the crop mucosa per se, because the extract shows considerable mitogenic activity[30]. Serum of pigeons has high growth stimulatory effect on quiescent CHO and NIH/3T3 cells[30]. The pigeon crop glands proliferate as well as the mammary glands under the synergistic action of prolactin, EGF and proinsulin[9]. If it is ultimately established that the source of growth factor in PM is the salivary glands (there are 6–7 pairs in birds, but only 3 in mammals) or crop mucosa or both, it will be yet another example of parallel biochemical evolution in animals.

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