Inducement of blood vessel formation by ovarian extracts from mice injected with gonadotropins[1]

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Summary. Ovarian extracts prepared from immature mice injected with human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) were assayed for angiogenic activity. The assay method consisted of implanting a film coated with ovarian extracts to the lateral wall of the m. rectus abdominus of a mouse for 20 days and examining the site for vascularization. The higher angiogenic activity obtained with PMSG-treated extract may be related to its follicle stimulating activity.

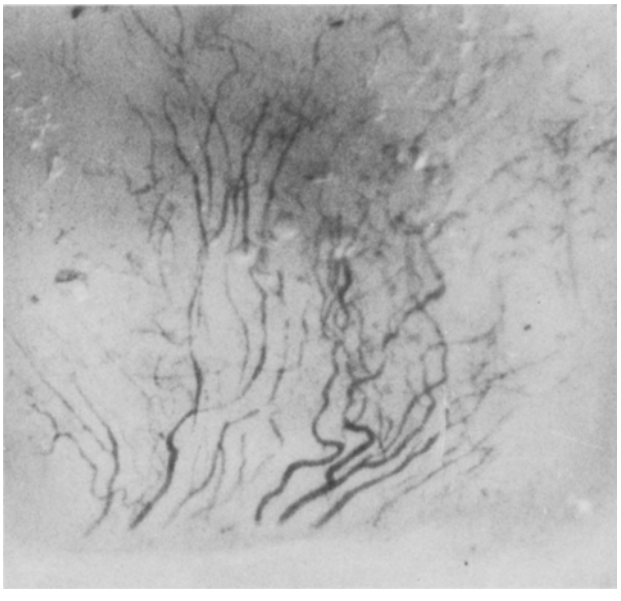
At the onset of puberty, follicular growth is accelerated which will impose a heavy demand on the supply of nutrients. To maintain this supply the blood vessels surrounding the follicles undergo a striking increase in number during follicular growth[2]. This increase in vascularization may be dependent upon the secretion of an angiogenic factor from the ovary. Since gonadotropins are known to induce rapid, striking follicular and vascular changes in the ovary, we examined ovarian extracts of mice injected with these hormones for angiogenic activity in the present study.

Materials and methods. Immature female mice (JCL-ICR strain) were injected s.c. with 5 IU of pregnant mare serum gonadotropin (PMSG) or human chorionic gonadotropin (hCG) dissolved in 0.2 ml of physiological saline. The ovaries were excised at 48 h after the injection, and the ovaries from non-treated mice were also removed as controls. The ovaries were homogenized in 0.5 M ammonium carbonate for 10 min. After freeze-thawing, the homogenates were centrifuged at 54,000 × g for 1 h and the supernatant was lyophilized.

A copolymer of ethylenevinyl acetate (Elwax 40) was washed extensively with ethanol and was dissolved in methylene chloride to a final concentration of 12% (w/v). The ovarian extracts were packed in polymer 'sandwiches' as described by Gospodarowicz et al.[3]. A drop of the copolymer solution (100 µl covering a surface area of 36 mm^2) was applied to a glass slide and dried under partial vacuum for 2 h, forming a thin, transparent film. A drop (100 µl) of a suspension of the 12% Elwax-methylene chloride solution containing either 8 or 24 mg of PMSG- or hCG-treated lyophilized ovarian extracts was then added on top of this film and dried. Finally, another drop (100 µl) of the 12% Elwax solution was applied to cover the film. The thin, transparent film of Elwax 40 containing lyophilized ovarian extract was cut into squares (3 × 3 mm) with a razor blade. Each square of this slow-releasing polymer sandwich thus contained 2 or 6 mg of extracts.

To determine any tissue response to the film, they were implanted into young adult mice (JCL-ICR strain). The mice were anesthetized with an i.p. injection of pentobarbital. A superficial incision was made in the abdominal wall and the implants, which consisted either of a slow-releasing form of Elwax 40 alone or with lyophilized ovarian extract, were inserted and placed on the lateral wall of the sheath of m. rectus abdominis. On the 20th day after implantation, the abdominal wall was examined for vascularization under a stereomicroscope. Tissues, including the films were excised, fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The sections were examined for inflammatory cells and capillary network.

A positive reaction is characterized by a visually distinct increase in capillary vasculature over the confine of the film. Control films without extract or protein will not induce vascularization or inflammatory reaction as determined by examination under the dissecting microscope and/or by histological sectioning. Grading of the angiogenic activity can be made and recorded as follows: 0, negative; +, a single ramified vessel; ++, 2 or 3 ramified vessels; +++, 4 or more ramified vessels; ++++, numerous ramified vessels and capillary network. An inflammatory reaction can be readily recognized by increased vasculature and hyperemic reaction at the peripheral border of the film. Films coated with 2 mg of bovine serum albumin induced an inflammatory reaction in 80% of the implanted specimens. In this study angiogenic activity was



Photograph of a film coated with ovarian extract prepared from a PMSG-treated mouse, excised from the rectus sheath 20 days after implantation. Note: Numerous blood vessels and capillary network overgrowing the surface of the film. × 40.

Vascularization induced by ovarian extracts prepared from gonadotropin-treated mice

Ovarian extracts	Dose of ovarian extracts (mg)	Total number of assays[e]	Positive neovascularization No.	Positive neovascularization %[a]
Control (untreated)	2	21	6	29.0 ± 4.8
	6	25	6	24.1 ± 1.8
PMSG-treated[d]	2	20	17	84.9 ± 16.2[c]
	6	26	20	77.3 ± 11.8[c]
hCG-treated[d]	2	28	9	31.7 ± 7.6
	6	20	12	60.3 ± 6.3[b]

Examined for neovascularization 20 days after implantation of films. [a] Values are means ± SD (n = 3); [b] $p < 0.02$, compared with control value; [c] $p < 0.01$, compared with control values; [d] ovarian extract prepared 48 h after the administration of hormone; [e] total of 3 separate experiments.

read as negative (0) or positive (+ to + + + +) as shown in the table.

Results. The Elwax 40 film itself was innocuous and did not incite any tissue reaction, nor induce neovascularization, while implants of films containing ovarian extracts induced neovascularization, characterized by capillary growth in the tissue surrounding the implants (fig.). Angiogenic activities of extracts prepared from the ovaries of untreated, hCG-treated and PMSG-treated mice are shown in the table. The control (untreated) extract elicited neovascularizing reaction in 24–29% of mice while those from hCG-treated and PMSG-treated mice induced a significantly higher percentage (32–60% and 77–85%, respectively). At equivalent dose, PMSG-treated extract had a greater angiogenic activity than extracts of hCG-treated mice.

Since neovascularization may occur in association with inflammation, the tissues were examined for signs of inflammatory reactions. Inflammatory cells were seen in some of the specimens. When the number of macrophages and other inflammatory cells around the film exceeded that observed with plain Elwax film (control), these specimens were recorded and considered as inflammation and not scored as neovascularization. Inflammatory reaction was observed in 2 out of 46 mice, 4 out of 50 mice and 5 out of 53 mice in the experiments with untreated, PMSG-treated, and hCG-treated ovarian extracts, respectively. Hence, inflammatory reaction was observed in 5–10% of the mice implanted with films containing ovarian extract.

Discussion. Capillary proliferation has been shown to be a general feature of actively growing tissue, such as the corpus luteum[4], salivary gland[5], granulation tissue[6] and tumor[7]. Neovascularization can be induced by extracts from tumors[8], by secretions from antigen- and phytohaemagglutin-stimulated lymph node cells[9], and by epidermal and fibroblast growth factors[3]. The present finding that ovarian extract from untreated mice showed angiogenic activity (24–29%, table) suggests that the factor is present in the immature ovary. The greater potency of ovarian extracts prepared from PMSG- and hCG-treated mice, indicate that the angiogenic activity was enhanced by hormone administration.

Although the mechanism of neovascularization is not known, it should be pointed out that this phenomenon is not a consequence of an inflammatory reaction. PMSG used in the present study can induce follicular growth since 5–10 IU of this hormone promotes development of mature follicles in mice[10]. Hence, the follicle stimulating activity and the angiogenic activity may be related. Ovarian extract prepared from hCG-treated mice induced neovascularization, albeit at a higher dose. It is well known that hCG induces luteinization of follicular cells and possesses intrinsic FSH activity[10–12]. These findings taken together suggest that gonadotropins may induce the formation of an angiogenic factor that stimulates proliferation of capillaries from the vascular wreath present in the theca layer and thereby promoting follicular development.

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The identification of ecdysterone (20-hydroxyecdysone) in 3 species of molluscs (Gastropoda: Pulmonata)[1]

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Summary. Ecdysterone has been identified in the schistosomiasis vector *Biomphalaria glabrata* Say and in *Helix aspersa* Müller as well as in *Lymmaea stagnalis* L. by chromatography, bioassay, radioimmunoassay, derivatization and by mass spectroscopy. Analysis of the food, faeces and hepatopancreas suggest that the sterol is derived from the diet. The probable function of ecdysterone in relation to calcification of the shell is discussed in this paper.

The procedure for extracting polar sterols from the bodies of the aquatic basommatophoran pulmonate *B. glabrata* (= *B. australorbis* Say) has been described[3], as has the method for rearing these molluscs. Previously a substance extracted from the same species of snail had been tentatively identified as ecdysone[4] but this finding was later questioned[5]. *Mytilus edulis*, the mussel, had been reported[6] to contain 0.2 pg g^{-1} b.wt of a substance active in the *Calliphora* moulting hormone bioassay. Horn[7], therefore suggested that calcification of the shell might be regulated by ecdysteroids in molluscs since this was demonstrable in the exoskeletons of crustacea[8] and the puparia of *Musca autumalis*[9]. Evidence to support this hypothesis was sought[3,10] before the investigation reported in this communication was completed. The support for the presence of an ecdysteroid in the hepatopancreas of *B. glabrata* and *H. aspersa* fed up to 4 days earlier on lettuce *(Lactuca sativa)* was as follows:

a) *Biomphalaria glabrata* (540 adults, 80.3 g) methanol extract after LC on silica gel[3] yielded a fraction eluted by 10% ethanol in chloroform (fraction S_1) with the following properties: 1. It absorbs in the UV (λ max 242 nm), suggesting a level of ecdysteroid of < 1 µg g^{-1} b.wt; 2. The R_f of the component was identical with ecdysterone; 3. The color reaction was olive with vanillin ethanol-H_2SO_4 spray[7]. 5. It was biologically active in a bioassay using ligatured abdomens of stage III *Sarcophaga peregrina* larvae[11], suggesting a level of 720 ng g^{-1} b.wt of moulting hormone; 5. The