Hyaluronic Acid-Like Substance from Mouse Ovaries with Angiogenic Activity

Eimei Sato, Hajime Miyamoto, and Samuel S. Koide*

Department of Animal Science, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
* Center for Biomedical Research, The Population Council, 1230 York Avenue, New York, N.Y. 10021, U.S.A.

Z. Naturforsch. **45c**, 873–880 (1990); received December 27, 1989

Neovascularization, Angiogenic Factor, Ovary, Glycosaminoglycans, Hyaluronic Acid

Glycosaminoglycans prepared from extracts of non-luteal mouse ovaries (JCL-ICR strain) were assayed for neovascularization by implanting Elvax films, impregnated with test samples, on the lateral wall of the sheath of m. rectus abdominis in adult mice of the same strain. Neovascularization occurred in a dose-dependent manner. When purified by chromatography on Dowex 1-x2 and DEAE Sephadex columns, fractions eluted with 0.5 M NaCl showed strong neovascularizing activity. On further purification by high performance liquid chromatography using TSK gel DEAE 2SW column, the fraction with a retention time nearly coincident with that of hyaluronic acid possessed high neovascularizing activity. The activity of this fraction was markedly reduced when treated with streptococcal hyaluronidase. The present results suggest that glycosaminoglycans, especially a hyaluronic acid-like substance, are involved in ovarian neovascularization.

Introduction

At the onset of puberty, follicular growth is accelerated in response to gonadotropin release. Although there is a striking difference in the distribution of blood vessels to individual follicles, the largest and presumably preovulatory follicles exhibit a high degree of vascularity and are supplied with numerous networks of vessels which project downward to the basement membrane surrounding the granulosa cells [1–3]. This increase in vascularization during follicular growth may be dependent upon the production of angiogenic factors by the ovary. Ovarian angiogenic factors have been detected in extracts prepared from porcine and bovine corpus lutea [4–7], non-luteal mouse ovaries [8], human follicular fluid [9], porcine and bovine follicles [10] and rat granulosa cells [11].

In the present study, glycosaminoglycans was prepared from non-luteal ovaries of mice treated with follicle-stimulating hormone (FSH) and assayed for angiogenic activity. Evidence will be presented showing that glycosaminoglycans, including a hyaluronic acid-like substance, are involved in neovascularization.

Reprint requests to Eimei Sato.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0700–0873 $ 01.30/0

Materials and Methods

Chemicals

Ethylenevinyl acetate copolymer (Elvax 40) was purchased from Aldrich Chemical Co.; follicle-stimulating hormone (FSH, from porcine pituitary), Dowex 1-x2 (200–400 mesh, Cl^- form), DEAE Sephadex A-25 (Cl^- form), streptococcal hyaluronidase, chondroitinase ABC (from *Proteus vulgaris*) from Sigma Chemical Co.; standard hyaluronic acid (from human umbilical cord) from Seikagaku Kogyo Co., Japan.

Preparation of ovarian extracts

Immature female mice (JCL-ICR strain, 4 weeks old, 20–22 g body weight) were injected with 1 i.u. of FSH dissolved in 0.3 ml of physiological saline. The injections were repeated after 24 h. The ovaries were excised 24 h after the second injection of FSH. The ovaries were homogenized in 0.5 M ammonium carbonate with an ultrasonicator. The homogenates were centrifuged at 54,000 × *g* for 1 h. The supernatant was collected, dialyzed against three changes of one liter distilled water, lyophilized and tested for angiogenic activity.

Extraction of glycosaminoglycans

Glycosaminoglycans were prepared from the ovaries according to the method described by

Schiller *et al.* [12] with slight modifications. Ovarian extracts were heated at 100 °C for 15 min and lyophilized. The lyophilized materials (100 mg) were suspended in 10 ml of 0.1 M acetate buffer, pH 5.8, containing 0.005 M cysteine HCl, 1.0 M NaCl, 0.005 M EDTA (disodium salt), activated crystalline papain (2 mg per 120 mg of the lyophilized extract) and incubated for 24 h at 60 °C.

The solution was dialyzed against distilled water for 24 h at 4 °C and the retentate centrifuged at $2000 \times g$ for 20 min. The protein in the supernatant was precipitated by adding trichloroacetic acid (TCA) solution to a final concentration of 10%. The TCA-soluble fraction was dialyzed against distilled water at 4 °C. Glycosaminoglycans were precipitated at 4 °C from the supernatant by the addition of three vol. of ethanol containing 2% sodium acetate (w/v). The precipitated glycosaminoglycans were washed with ethanol, redissolved in water, and fractionated by ion-exchange chromatography.

Chromatography of glycosaminoglycans

The crude glycosaminoglycans were chromatographed on an anion exchange resin, Dowex 1-x2 (200–400 mesh) in the chloride form, and on DEAE Sephadex A-25, Cl^- form. Columns, 0.9 cm in diameter, were packed with resins suspended in water (Dowex) or in a 0.1 M NaCl solution (DEAE Sephadex) to a height of 20 cm. A sample of 3 to 5 mg of glycosaminoglycans dissolved in water was applied. The column was washed with water (Dowex) or 0.1 M NaCl solution (DEAE Sephadex), and eluted stepwise with increasing concentrations of NaCl starting with 0.5 M. The amount of glycosaminoglycans in each fraction was determined by a modified method of Dische's carbazole reaction in the presence of borate as described by Bitter and Muir [13]. The fraction eluted from the Dowex column with 0.5 M NaCl possessed the highest potency and was further purified by high performance liquid chromatography (HPLC). The 0.5 M NaCl fraction was dialyzed against distilled water and concentrated to 0.1 ml containing 1 mg of glycosaminoglycans. Any insoluble residue was removed by centrifugation in a microfuge. The supernatant was injected into a TSK gel DEAE-2SW column (15 × 250 mm) (Toyosoda Ltd., Japan). The column was eluted with a linear gradient of water and 2 M NaCl at a flow rate of 1 ml/min. Fractions of 1 ml were collected, and glycosaminoglycans detected spectrophotometrically be measuring the absorbance at 260 nm. Each fraction was dialyzed against distilled water and lyophilized.

Degradation of glycosaminoglycans

HPLC-purified glycosaminoglycans with angiogenic activity were dialyzed against three changes of 1 l distilled water for 24 h, collected and placed in a boiling water bath for 5 min, and subsequently divided into 5 equal parts (400 µl) containing 200 µg of HPLC-purified glycosaminoglycans. Aliquots were incubated separately with 100 µg of streptococcal hyaluronidase, 100 µg of chondroitinase ABC dissolved in 100 µl enzyme buffer (0.1 M Na_2HPO_4/0.1 M NaH_2PO_4, pH 5.6) or 100 µl enzyme buffer alone.

The reaction mixtures were incubated at 37 °C for 3 h. The treated samples were boiled for 5 min and cooled, and cetylpyridinium chloride was added to a final concentration of 1% to precipitate the non-degraded glycosaminoglycans. The resulting precipitate was washed once in cold distilled water, and dialyzed against three changes of 1 l distilled water, and lyophilized.

Carrier and cetylpyridinium chloride were added to a fourth aliquot of HPLC-purified glycosaminoglycans with angiogenic activity. A fifth aliquot was subjected to nitrous acid degradation according to the method of Dische and Borenfreund [14] as modified by Kosher and Searls [15]. Briefly, an equal volume of 5% sodium nitrite and 33% acetic acid was added, the reaction mixture was shaken, stoppered, and left standing at ambient temperature for 90 min. Undegraded glycosaminoglycans were precipitated with 1% cetylpyridinium chloride and washed once by resuspending in cold distilled water, and dialyzed against three changes of 1 l distilled water at 4 °C, and lyophilized.

Bioassay for neovascularization

A copolymer of ethylenevinyl acetate (Elvax 40) was washed extensively with ethanol and dissolved in methylene chloride to a final concentration of 12% (w/v). The crude extract and partially purified fractions were embedded in the polymer as de-

scribed by Gospodarowicz *et al.* [16]. A drop of the copolymer solution was applied to a glass slide and dried under partial vacuum for 2 h forming a thin, transparent film. A drop (100 µl) of the 12% Elvax-methylene chloride solution containing the test extract was cut into squares (approximately 2 × 2 mm) with a razor blade. Tissue response to the film alone was determined by implanting films without the test extract into young adult mice (JCL–ICR strain). The tissue around the implanted film was examined by a histopathological technique. The mice were anesthetized by an intraperitoneal injection of pentobarbital. A superficial incision was made in the abdominal wall. The implants of Elvax 40 alone (control) or impregnated with the lyophilized material (test films) were exposed to ultra-violet light for 2 h and inserted on the lateral wall of the sheath of m. rectus abdominis. On the 15th day after implantation, the abdominal wall was examined for vascularization under a stereo- and inverted light microscope with phase contrast attachment. Positive reaction is characterized by a distinct increase in capillary vasculature over the confine of the film. Angiogenic activity is scored and recorded as follows: score 0, negative; score 1, ramified capillaries with diameters smaller than 10 µm; score 2, 1 or 2 ramified vessels with diameters greater than 10 µm and small capillaries; score 3, 3 or more ramified vessels with diameters greater than 10 µm and small capillaries; score 4, numerous ramified vessels with diameters exceeding 10 µm, numerous small capillaries and capillary networks. An inflammatory reaction can be readily recognized by increased vasculature and hyperemic reaction at the peripheral border of the film. P values were calculated by the student *t* test.

Morphological examination

For electron microscopy studies, tissue samples measuring 1–2 mm in length were fixed in 3.5% cold, phosphate-buffered glutaraldehyde (pH 7.5) for 2 h. After careful rinsing in buffer, samples were postfixed in 2% OsO_4 for 2 h, dehydrated in ethanol, and embedded in Luveak 812 (Nacalai Tesque Co. Ltd., Japan). Ultrathin sections were contrasted with uranyl acetate and lead citrate and viewed under a Hitachi transmission electron microscope Model H-300.

Results

Crude glycosaminoglycans prepared from ovarian extracts induced neovascularization in a dose-dependent manner (Table I). 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 films exceeded that observed with plain Elvax film (control), these specimens (about 3% of the films implanted in this series of experiments) were recorded and considered as inflammation and not scored as neovascularization. Crude glycosaminoglycans were separated into 4 fractions by chromatography on Dowex 1-x2 and DEAE-Sephadex A-25 columns eluted with increasing concentra-

Table I. Neovascularization induced by the ovarian glycosaminoglycan fraction prepared from ovaries of mice treated with FSH.

Dose	Total No. assays	Inflammation		Positive neovascularization		Score of neovascularization
[µg][a]		(No.)	[%]	(No.)[b]	[%]	(Mean ± S.D.)
0	63	2	3.2	2	3.2	0.1 ± 0.4
20	25	3	12.0	12	48.0	1.5 ± 1.0
50	27	3	11.1	14	51.9	1.7 ± 1.2
100	26	4	15.4	17	65.4	2.3 ± 1.1*
200	26	4	15.4	19	73.1	2.5 ± 1.2*

[a] Content of uronic acid [13] in a film.
[b] Excluded the number of films showing inflammation.
* Value is significantly different compared with control value ($P < 0.05$).

tions of NaCl. The fraction eluted with 0.5 M NaCl possessed neovascularizing activity (Table II). This fraction has been reported to contain hyaluronic acid and chondroitin [12]. The 0.5 M NaCl fraction obtained from the Dowex 1-x2 column was further purified by HPLC on a TSK gel DEAE-2SW column. Absorbance of the fraction was measured at 260 nm and the fractions comprising the peaks were pooled (Fig. 1). The highest biological activity was demonstrated with tubes No. 27–28. The peak value of reference marker, hyaluronic acid, was identified in tube No. 28. This fraction was effective in inducing neovascularization at a dose of 5 μg/implant (Fig. 2, 4). The biological activity of the HPLC-purified angiogen-

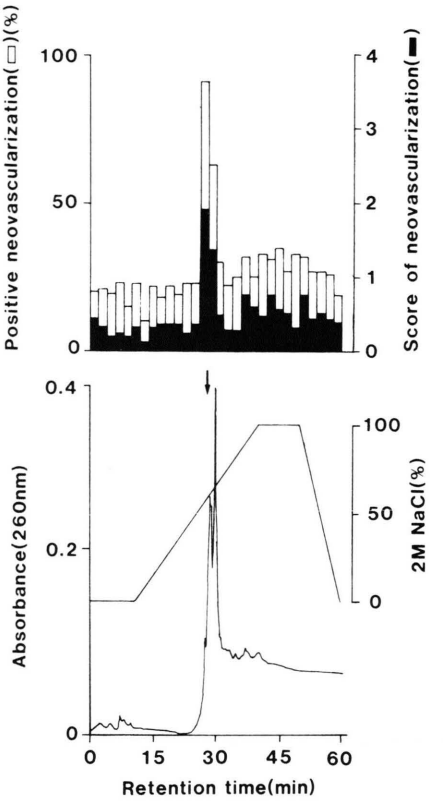

Fig. 1. Elution profile of ovarian extract separated by HPLC and angiogenic activity of the various fractions. The active fraction (1 mg) obtained from gel filtration on a column of Dowex 1-x2 using a NaCl gradient as shown (bottom). One minute fractions were collected, dialyzed and lyophilized. The lyophilized powder was dissolved in 0.1 ml of Elvax 40 solution. The mixture was dried and cut into 10 pieces of film and tested for biological activity (top) as described under the Method section. Note highest biological activity with tube No. 27–28. Arrows indicate elution position of reference hyaluronic acid.

Table II. Neovascularization induced by ovarian glycosaminoglycan fractions prepared from FSH-treated mice.

Chromatography of ovarian fraction	Eluting NaCl solution [M]	Dose (μg/implant)	Total assay (No.)	Positive activity (No.)	[%]	Score of neovascularization (Mean ± S.D.)
A. Dowex 1-x2 column	0 (control)	40*	18	9	50.0	0.5 ± 0.5
	0.5	40	19	18	94.7	1.9 ± 0.9[a]
	0.5	20	15	12	80.0	1.4 ± 0.8
	0.5	10	12	7	58.3	0.8 ± 0.7
	1.5	40	17	9	52.9	0.6 ± 0.6
	2.0	40	12	3	25.0	0.3 ± 0.6
B. DEAE Sephadex column	0.1	40	15	3	20.0	0.4 ± 0.8
	0.5	40	15	12	80.0	2.0 ± 1.4[b]
	1.5	40	15	3	20.0	0.4 ± 0.8
	2.0	40	18	9	50.0	0.8 ± 0.8

* Content of uronic acid in the implant determined by the carbazole reaction [13].
[a] $P < 0.05$, compared with the control value.
[b] $P < 0.05$, compared with the value of the 0.1 M NaCl fraction.

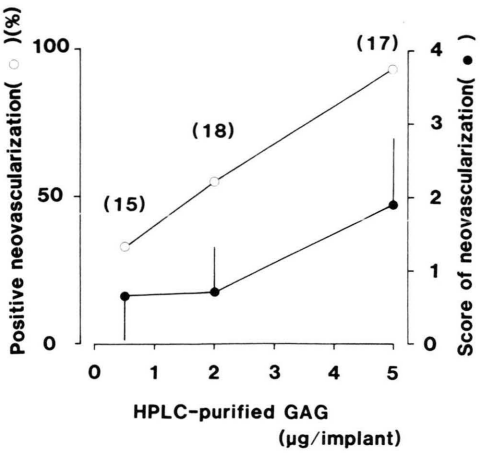

Fig. 2. Angiogenic activities at increasing doses of HPLC-purified mouse ovarian glycosaminoglycans. Fractions tube No. 27–28 depicted in Fig. 1 was tested. Neovascularization was determined by examining the tissues as described under the Method section. Number in parentheses indicates the total number of implanted films examined. Each point is mean ± S.D.

ic glycosaminoglycan was reduced on treatment with streptococcal hyaluronidase, chondroitinase ABC or nitrous acid. The greatest reduction occurred with hyaluronidase treatment (Fig. 3).

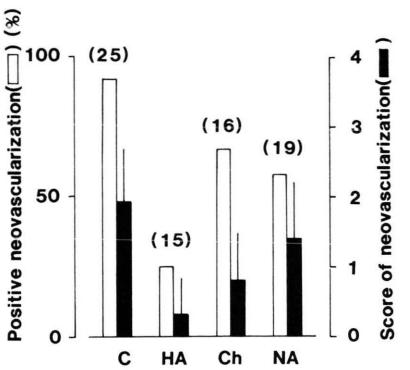

Fig. 3. Angiogenic activity of HPLC-purified glycosaminoglycans from mouse ovaries treated with hyaluronidase, chondroitinase ABC and nitrous acid. The HPLC-purified fraction (tube No. 27–28, Fig. 1) was tested. Samples of 200 μg of HPLC-purified glycosaminoglycans were treated with the enzymes, processed and tested as described under the Method section. Number in parentheses indicates the total number of implanted films examined. Three independent experiments were performed. Each point is mean ± S.D. C, implants containing HPLC-purified materials (control); HA, hyaluronidase; Ch, chondroitinase ABC; NA, nitrous acid.

Discussion

Capillary proliferation is characteristic of actively growing tissue, such as corpus lutea [4–7], ovarian follicles [8–11], salivary glands [17], granulation tissues [18], retina [19], adrenal glands [20], tumor [21], and aminochorion and placenta [22]. Capillary proliferation of angiogenesis involves the outgrowth of new capillaries directed toward a given stimulus. Microscopic examination of capillary growth revealed that endothelial cells migrate from the tip of the capillary before mitosis occurs [23], suggesting that the ability to promote directed migration of capillary endothelial cells is a prerequisite for angiogenesis [23]. These findings suggest that multiple factors are involved in the initiation, migration and proliferation of endothelial cells, and in the formation of the "three-dimensional" network of capillaries.

Several factors have been reported to induce neovascularization such as wound fluid [24], epidermal and fibroblast growth factors [16], tumor extracts [25, 26], secretions from antigen and phytohemagglutinin-stimulated lymph node cells [27] and activated macrophages [28], extracts of corpus luteum and non-luteal ovaries [4–11], ovarian follicular fluid [9], and conditioning medium of granulosa cells [11]. In these cases that angiogenic activity was assayed by *in vivo* and *in vitro* systems. Using the *in vitro* assay system, the process of proliferation and the migration of capillary endothelial cells can be measured [29]. Moreover, methods to examine the formation of extensive network of capillary-like structures from the endothelial cells derived from capillaries *in vitro* have been developed. *In vivo* assay system for angiogenic activity is based on the appearance of new capillaries on applying the test samples on the surface of the chorioallantoic membranes of chick embryo [30], by implanting films on the lateral wall of the sheath of m. rectus abdominis [8], or by placing the test samples in the corneal pockets [29]. Although the *in vivo* assays may have physiological relevance, angiogenic activity is difficult to quantify and the occurrence of artifactual neovascularization due to inflammatory reactions may be difficult to exclude.

Inflammation is the most potent inducer of angiogenesis and is a troublesome source of artifactual capillary proliferation [5, 29]. To exclude

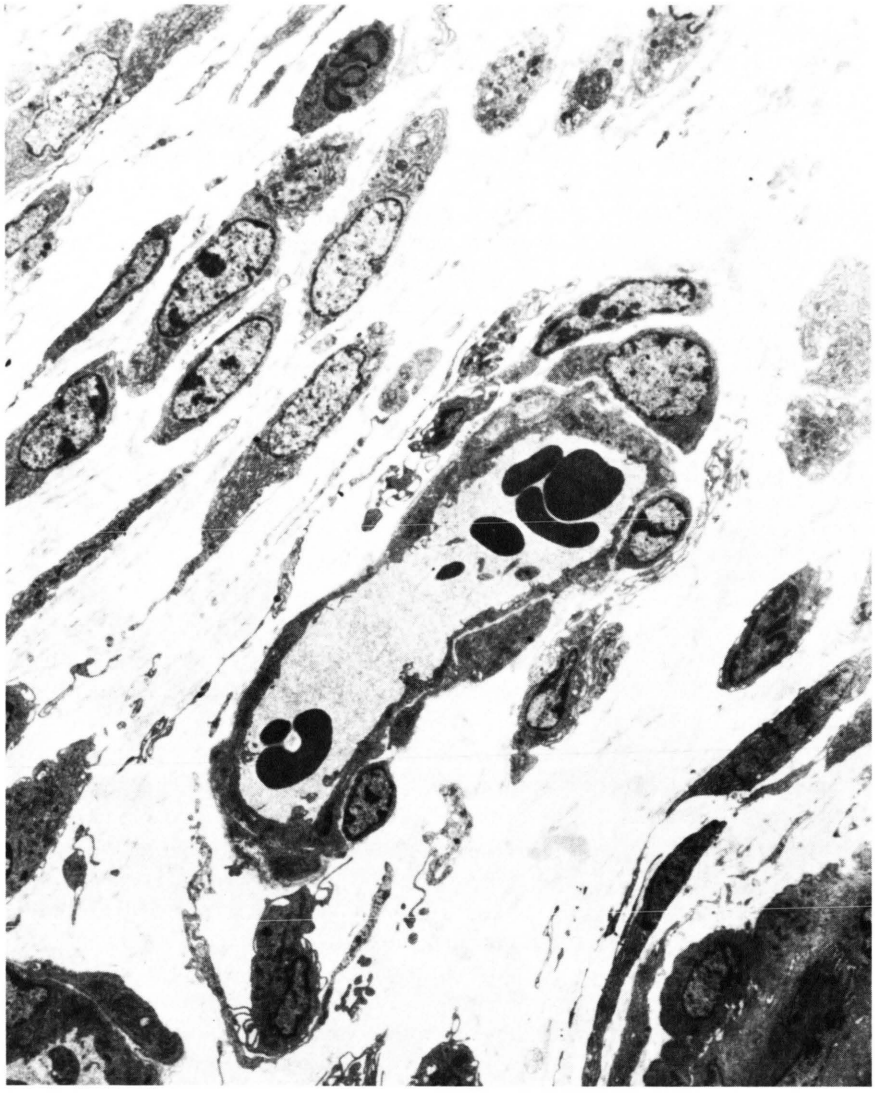

Fig. 4. Microphotograph of a newly formed small blood vessel in tissues around the implant. The implant contains 5 μg of HPLC-purified glycosaminoglycans and the tissue examined 15 days post-implantation (× 2000).

inflammation and to score as a true non-inflammatory angiogenic response to the extracts, there should be no edema or leukocytic infiltration about the implants. Neovascularization induced by certain compounds such as bovine serum albumin (BSA) is characterized by an inflammatory reaction associated with infiltration of leukocytes and macrophages into the subcutaneous tissues [8]. In the present study inflammation due to immunological reactions was avoided by utilizing inbred mice as the tissue source of the angiogenic factor and by using the same strain to bioassay for the activity. The films were sterilized by ultraviolet light treatment to avoid inflammation from bacterial infection. By using the sterile techniques outlined, the incidence of non-specific inflammation was minimized to about 3%.

In the present study, we have demonstrated that the glycosaminoglycans in the 0.5 M NaCl fraction of HPLC-purified ovarian extracts induced capillary proliferation. This fraction has been reported to contain hyaluronic acid and chondroitin [12]. The active factor was purified by HPLC and eluted in the fraction with a retention time nearly coincident with that of hyaluronic acid. Moreover, its biological activity is markedly reduced on treat-

ment with streptococcal hyaluronidase. Ausprunk [31] demonstrated that precise changes in glycosaminoglycan composition could be correlated with significant events in the development of the chick chorioallantoic membrane-blood vessels, and that hyaluronic acid might be involved in the formation, alignment, or migration of the capillary plexus of the chick chorioallantoic membrane.

Feinberg and Beebe [32] reported that vascular regions of the chick wing bud are rich in hyaluronic acid and that avascular zones can be induced in the wing bud mesoderm after implanting Elvax containing hyaluronic acid. On the other hand, West *et al.* [33] showed that partially degraded products of sodium hyaluronate hydrolyzed with testicular hyaluronidase induced angiogenesis (formation of new blood vessels) when applied to the chick chorioallantoic membrane, although the intact hyaluronate was inactive. Fractionation of the digested products showed that the activity was associated with the hyaluronate fragments containing 4 to 25 disaccharides in length [33]. We have shown that ovarian hyaluronic acid fraction possessed angiogenic activity when implanted on the lateral wall of the sheath of m. rectus abdominis, and the activity is markedly reduced on treatment with streptococcal hyaluronidase. The basis for the angiogenic activity demonstrated with the HPLC-purified hyaluronic acid from mouse ovary is not clear. It is suggested that the hyaluronic acid molecules in the ovaries might have been slightly modified to acquire angiogenic activity during ovarian follicular development or be a degradative form with angiogenic activity. The latter is possible since hyaluronic acid is taken up by endothelial cells and degraded to low-molecular weight products [33].

Glycosaminoglycans such as heparin potentiate ECGF activity [33, 34]. Heparin and ECGF can increase chemotaxis and chemokinesis of human endothelial cells [35]. Tumor ECGF has a strong affinity for heparin [36] and heparin stimulates the migration of endothelial cells [29, 37, 38]. Heparin in the ovarian extract should be eluted in the 2.0 M NaCl fraction of glycosaminoglycans. This fraction did not show significant angiogenic activity.

Follicular fluid contain about 0.2–0.3% (w/v) glycosaminoglycans [39, 40]. These substances are composed of a core protein of an estimated M_r of 400 kDa with an average of 20 dermatan sulfate chains and 350 sialic acid-containing oligosaccharides [41], and are produced by the follicular cells [42]. Yanagishita and Hascall [40] reported that 90% of the glycosaminoglycans secreted by rat granulosa cells are hydrolyzed by chondroitinase ABC, indicating that the major constituent is chondroitin sulfate. Nevertheless, hyaluronic acid is also present in mammalian ovaries [42]. During cumulus expansion initiated by FSH, hyaluronic acid is the major glycosaminoglycans produced by the mouse cumulus oocyte complexes [42]. Ball *et al.* [43] reported that massive amounts of glycosaminoglycans accumulated in the matrix enveloping the cumulus cells during the expansion of bovine cumulus-oocyte complexes induced with FSH or cyclic adenosine 3′,5′-monophosphate. When the intercellular matrix materials were isolated and subjected to electrophoretic analysis, the radiolabeled glycosaminoglycans co-migrated with reference hyaluronic acid [43] and hydrolyzed by hyaluronidase. However, it was resistant to chondroitinase ABC and nitrous acid degradation, indicating that the intercellular matrix produced by bovine cumulus-oocyte complexes is rich in hyaluronic acid [43].

Hyaluronic acid may be responsible for the angiogenesis observed in the ovary and regulated by gonadotropins. There is a general consensus that luteinizing hormone (LH) induces cumulus expansion *in vivo* [42]. *In vitro* study has established that FSH is responsible for hyaluronic acid production [44]. It is also noteworthy that FSH can induce cumulus expansion *in vitro* and stimulates the synthesis of glycosaminoglycans in the oocyte-cumulus cell complexes in the presence or absence of fetal calf serum (FCS) [45]. In the presence of FCS the glycosaminoglycans are retained within the complexes, while in the absence of FCS they are secreted into the culture medium [44, 45]. Taken together, it can be surmised that glycosaminoglycans, especially hyaluronic acid-like substance, are probably involved in the induction of angiogenesis in the ovary during follicular growth initiated by FSH.

Acknowledgements

This study was supported in part by grant GA PS 8712, 8732 and 8734 from the Rockefeller Foundation and grant 62615513 from the Ministry of Education, Science and Culture, Japan.

[1] H. Kanzaki, H. Okamura, Y. Okuda, A. Takenaka, K. Morimoto, and T. Nishimura, Acta Obstet. Gynecol. Jpn. **33,** 11–17 (1981).
[2] A. J. Zeleznik, H. M. Schuler, and L. E. Reichert, Jr., Endocrinology **109,** 356–362 (1981).
[3] R. D. Koos, and W. J. DeMaire, Factors Regulating Ovarian Function (G. S. Greenwald and P. F. Terranova, eds.), p. 191, Raven Press, New York 1983.
[4] D. Gospodarowicz and T. K. Thakral, Proc. Natl. Acad. Sci. U.S.A. **75,** 847–851 (1978).
[5] W. Jakob, K. D. Jentzsch, B. Mauersberger, and P. Oehme, Exp. Pathol. **13,** 231–236 (1977).
[6] G. Heder, W. Jakob, W. Halle, B. Mauersberger, G. Kambach, K. D. Jentzsch, and P. Oehme, Exp. Pathol. **17,** 493–497 (1979).
[7] D. Gospodarowicz, J. Cheng, G. M. Lui, A. Baird, F. Esch, and P. Bohlen, Endocrinology **117,** 2283–2291 (1985).
[8] E. Sato, T. Ishibashi, and S. S. Koide, Experientia **38,** 1248–1249 (1982).
[9] J. L. Frederick, T. Shimanuki, and G. S. DiZerega, Science **224,** 389–390 (1984).
[10] A. Makris, K. J. Ryan, T. Yasumizu, O. L. Hill, and B. Zetter, Endocrinology **15,** 1672–1677 (1984).
[11] R. D. Koos, Endocrinology **119,** 481–489 (1986).
[12] S. Schiller, G. A. Slover, and A. Dorfman, J. Biol. Chem. **236,** 983–987 (1961).
[13] T. Bitter and H. M. Muir, Anal. Biochem. **4,** 330–334 (1962).
[14] Z. Dische and E. Borenfreund, J. Biol. Chem. **184,** 517–522 (1950).
[15] R. A. Kosher and R. L. Searls, Dev. Biol. **32,** 50–68 (1973).
[16] D. Gospodarowicz, H. Bialecki, and T. K. Thakral, Exp. Eye Res. **28,** 501–514 (1979).
[17] H. Hoffman, B. McAustan, D. Robertson, and E. Burnett, Exp. Cell Res. **102,** 269–275 (1976).
[18] G. H. Algire and H. W. Chalkley, J. Natl. Cancer Inst. **6,** 73–85 (1965).
[19] P. A. D'Amore, B. M. Glaser, S. K. Brunson, and A. H. Fenselau, Proc. Natl. Acad. Sci. U.S.A. **78,** 3068–3072 (1981).
[20] D. Gospodarowicz, A. Baird, J. Cheng, G. M. Lui, F. Esch, and P. Bohlen, Endocrinology **118,** 82–90 (1986).
[21] M. A. Gimbrone, R. S. Cotran, S. B. Leapman, and J. Folkman, J. Natl. Cancer Inst. **52,** 413–427 (1974).
[22] H. Burgos, Eur. J. Clin. Invest. **13,** 289–296 (1983).
[23] D. H. Ausprunk and J. Folkman, Microvas. Res. **14,** 53–65 (1977).
[24] M. J. Banda, D. R. Knington, T. K. Hunt, and Z. Webb, Proc. Natl. Acad. Sci. U.S.A. **79,** 7773–7777 (1982).
[25] P. Phillips and S. Kumar, Int. J. Cancer **23,** 82–88 (1979).
[26] J. Folkman, E. Merler, C. Abernathy, and G. Williams, J. Exp. Med. **133,** 275–288 (1971).
[27] K. Nishioka and I. Katayama, J. Pathol. **126,** 63–69 (1978).
[28] P. Polveriri, R. S. Cotran, M. A. Gimbrone, and E. R. Unanue, Nature **269,** 804–806 (1977).
[29] J. Folkman, Ann. N.Y. Acad. Sci. **401,** 212–227 (1982).
[30] W. Jakob, K. D. Jentzsch, B. Mauersberger, and G. Heder, Exp. Pathol. Bd. **15,** 241–249 (1978).
[31] D. H. Ausprunk, Am. J. Anat. **177,** 313–331 (1986).
[32] R. N. Feinberg and D. C. Beebe, Science **220,** 1177–1179 (1983).
[33] D. C. West, I. N. H. Hampson, F. Arnold, and S. Kumar, Science **228,** 1324–1326 (1985).
[34] S. C. Thornton, S. N. Mueller, and E. M. Levine, Science **222,** 623–625 (1983).
[35] V. P. Terranova, R. Diflorio, R. M. Lyall, S. Hic, R. Freisel, and T. Maciag, J. Cell Biol. **101,** 2330–2334 (1985).
[36] Y. Shing, J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun, Science **223,** 1296–1299 (1984).
[37] S. Taylor and J. Folkman, Nature **297,** 307–312 (1982).
[38] R. Azizkhan, J. C. Azizkhan, B. R. Zetter, and J. Folkman, J. Exp. Med. **152,** 931–944 (1980).
[39] C. E. Jensen and F. Zachariae, Acta Endocrinol. **27,** 356–368 (1958).
[40] M. Yanagishita and V. C. Hascall, J. Biol. Chem. **254,** 12355–12364 (1979).
[41] M. Yanagishita, D. Rodbard, and V. C. Hascall, J. Biol. Chem. **254,** 911–920 (1979).
[42] E. Sato and S. S. Koide, Int. Rev. Cytol. **106,** 1–33 (1987).
[43] G. D. Ball, M. E. Bellin, R. L. Ax, and N. L. First, Mol. Cell. Endocrinol. **28,** 113–122 (1982).
[44] J. J. Eppig, Nature **281,** 483–484 (1979).
[45] J. J. Eppig, Biol. Reprod. **23,** 545–552 (1980).