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Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularization of the corpus luteum

(ovaries/follicles/growth factor/angiogenesis)

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ABSTRACT **Factors controlling the changes in the vascular pattern of the ovary that occur during the reproductive cycle have been investigated. By using the rabbit cornea, the abilities of ovarian corpus luteum and of follicles to induce neovascularization have been compared. While the corpus luteum is capable of inducing neovascularization, the follicles do not have this ability. It is therefore likely that the corpus luteum actively participates in its own neovascularization by secreting a factor that we have called "corpus luteum angiogenic factor" (CLAF).**

The factors controlling the changes in the vascular pattern of the ovary that occur during the normal reproductive cycle and during pregnancy have not been investigated. Nonetheless, the ovaries provide an excellent model for the study of the physiological adaptations of blood vessels in tissues that undergo rapid and repeated growth and involution. The rapidity of follicular growth and the rate of the rise and decline of the corpora lutea impose remarkable demands on the ability of blood vessels to proliferate and, subsequently, to regress. The cyclic changes also involve concomitant physiological adjustments in blood flow, capillary permeability, and other functional behavior.

Particularly impressive are the extremely rapid and radical vascular changes that take place in the capillary wreath surrounding the follicle at the time of ovulation (Fig. 1). From the endothelium of these vessels, capillary sprouts begin to grow into the granulosa cell layer and develop in 48 hours into a totally new, complex network of sinusoidal vessels, which invade the previously avascular granulosa cell layers, induce luteinization, and later nourish the parenchyma of the corpus luteum (Fig. 1).

The rapid proliferation of capillaries that takes place during the early phase of the development of the corpus luteum can be compared to the vascularization of solid tumors, which has been associated with the proliferation of capillaries in surrounding host tissues. Algire and Chalkley (1) appreciated early that growing tumors elicit capillary ingrowth from the host. They suggested that this might be an underlying factor responsible for the autonomous growth of these tumors. Greenblatt and Shubik (2) later demonstrated that the new capillary growth is elicited by some diffusible material, which later was partially purified by Folkman and his colleagues (3) and named "tumor angiogenesis factor" (TAF).

Because the early vascular changes taking place during the development of the corpus luteum are strikingly similar to the capillary proliferation induced by tumors, we have investigated the possibility that the early phase corpus luteum produces a diffusible substance similar to TAF.

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This report describes the host's angiogenic response to implants of follicles and corpus luteum in the cornea. As already pointed out by Gimbrone *et al.* (4), the cornea provides a transparent, avascular substratum in which the relationship between survival, differentiation, and growth of a given tissue and neovascularization can be continuously observed *in vivo*. Implantation at a distance from the circumferential vessels of the limbus produces an anatomic separation of the graft from responding host vessels. This arrangement allows independent observation of the behavior of both elements.

MATERIALS AND METHODS

Luteinizing hormone was purified from bovine pituitary gland as already described (5). Pregnant mare serum gonadotropin (2500 international units/mg) was obtained from Sigma. Female New Zealand White rabbits weighing 2–3 kg were used. The rabbits were injected intraperitoneally with 500 international units of pregnant mare serum gonadotropin dissolved in 0.1 ml of phosphate-buffered saline. Four days later the rabbits were anaesthetized and a unilateral oophorectomy was performed. The follicles were then dissected out (6, 7) and implanted in the cornea of the same rabbit (autologous implants) and in the cornea of other rabbits (homologous implants). The rest of the group was then injected intraperitoneally with 0.1 ml of a phosphate-buffered saline solution containing luteinizing hormone at 10 mg/ml. Four days later a unilateral oophorectomy was performed on one of the rabbits and the corpora lutea were dissected out (7) and implanted in the cornea of the same rabbit (autologous implants) and in the cornea of other rabbits (homologous implants) that had undergone the same hormonal treatment. Follicles obtained from normal rabbit ovaries were implanted in the cornea of the donor in some cases. At the time of autopsy, the ovaries were examined to evaluate their developmental stages.

The implantation technique was similar to that described by Gimbrone *et al.* (4). The rabbits were anaesthetized, and the eye was moved forward and secured in position by a fold clamped in the lower lid. With a scalpel a superficial incision 1.5 mm long was made in the corneal dome to one side of its center. The incision was then continued down into the cornea. A malleable iris spatula was inserted, and an oblong pocket was fashioned within the corneal stroma. Peripheral pockets ended 1–2 mm from the limbus; central pockets were positioned near the corneal apex (5–7 mm from the limbus). The corpus luteum or follicle was deposited in the bottom of each pocket, which then sealed spontaneously. Eyes with corneal implants were examined daily with the aid of a Zeiss slit lamp stereomicro-

Abbreviations: TAF, tumor angiogenic factor; CLAF, corpus luteum angiogenic factor.

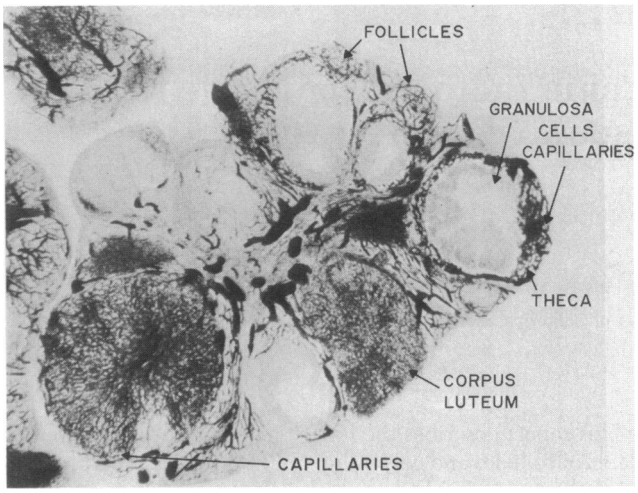

FIG. 1. A thick section of a rat ovary in which the blood vessels were perfused with carmine/gelatin. Notice the rich vasculature of the corpora lutea, while the granulosa cell layers within the follicles are avascular.

scope at ×10–40. Implant and new vessel growth were measured *en face* with an ocular micrometer at ×10. Empty corneal pockets fashioned at distances 1 mm from the limbus did not stimulate corneal neovascularization.

Entire eyes were excised and fixed by immersion in 10% buffered formalin. The eyes were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Crude extracts of corpus luteum follicles and ovarian stroma were prepared as already described for bovine and pituitary tissues (8, 9). The ability of the different fractions to stimulate DNA synthesis in 3T3 cells maintained in low serum concentration and to stimulate proliferation in vascular endothelial cell cultures was measured as already described (8, 9, 11–13).

RESULTS

Comparison of the Neovascularization Induced by Follicular and Corpus Luteum Implants. Of twelve follicular implants in the rabbit cornea, eight failed to stimulate neovascularization when transplanted into the cornea of untreated females or of females treated with PMSG. Of four follicles transplanted into pseudopregnant rabbits (i.e., female rabbits injected with gonadotropin followed by luteinizing hormone), all four induced neovascularization. Histological examination showed that these follicles had luteinized in the rabbit cornea (Table 1). This is in agreement with the results of others, who have shown that preovulatory follicles implanted under the kidney capsules of pseudopregnant animals luteinize spontaneously (10). Of fifteen corpus luteum implants, thirteen induced neovascularization (Table 2). In two ovarian stroma implants containing small follicles, but no corpus luteum, no neovascularization was observed. This demonstrates that early phase corpora lutea, unlike follicles, have the capacity to elicit sprouting and proliferation of capillaries from the host. This property was shared by follicular implants, which spontaneously luteinized when transplanted into the rabbit cornea of pseudopregnant rabbits.

Table 1. Effect of follicular implants on the growth of capillaries

Follicular implants	Luteinization	Vessel length, mm	Days
Normal female rabbit			
Autologous	−	—	16
Autologous multiple follicles	−	3.5*	16
Homologous	−	—	16
Autologous multiple follicles	−	—	16
Autologous	−	—	13
Autologous	−	—	13
Homologous multiple follicles	−	—	13
Homologous multiple follicles	−	—	13
Pseudopregnant female rabbit			
Homologous	+	7	19
Homologous	+	7	19
Homologous	+	3.5	19
Homologous	+	7.5	19

Inflammation was not observed for any implant.
* One vessel.

Table 2. Effect of corpus luteum implant on the growth of capillaries

Corpus luteum implants	Luteinization	Vessel length, mm	Days
Homologous	+	8*	20
Homologous	+	7*	20
Homologous	+	8*	20
Homologous	+	8*	20
Autologous	+	—	20
Autologous	+	—	20
Homologous	+	2	15
Homologous	+	3.5	15
Homologous	+	3.5	15
Homologous	+	2.5	15
Autologous	+	5.5	12
Autologous	+	3.5	12
Homologous	+	2.5	12
Homologous	+	3.5	12
Homologous	+	0.5	12

Inflammation was not observed for any implant.
* Because capillaries invaded the corpus luteum, the diameter of the implant (2 mm) was included in the total length of the vessels (Fig. 3*C'*).

Morphology of the Host's Neovascular Response. *Follicular implant.* When follicles were implanted in the rabbit eye, whether far from (4–6 mm) or near (1 mm) the limbus, no vascular response on the part of the host was observed, provided that the follicles did not luteinize. In a few cases a few delicate capillary loops originated from the limbus during the first 2 days, but they never grew, nor did they make contact with the follicular implant (Fig. 2*A*). By day 14 the follicles had col-

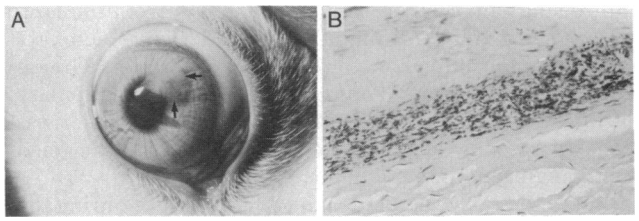

FIG. 2. Lack of proliferation of capillaries induced by implant of rabbit follicles in the rabbit cornea. (*A*) The follicular tissues, consisting of three follicles, have been implanted in the center of the eye (arrows). No capillary proliferation was observed 14 days later. (*B*) Histological section of the cornea stained with hematoxylin/eosin and containing the follicular implant. The cellular structures have disappeared. The nuclei exhibit nuclear condensation (pyknosis), and, although the tissues were clearly resorbing, no inflammatory cells or capillaries were observed near the implant. (×36.)

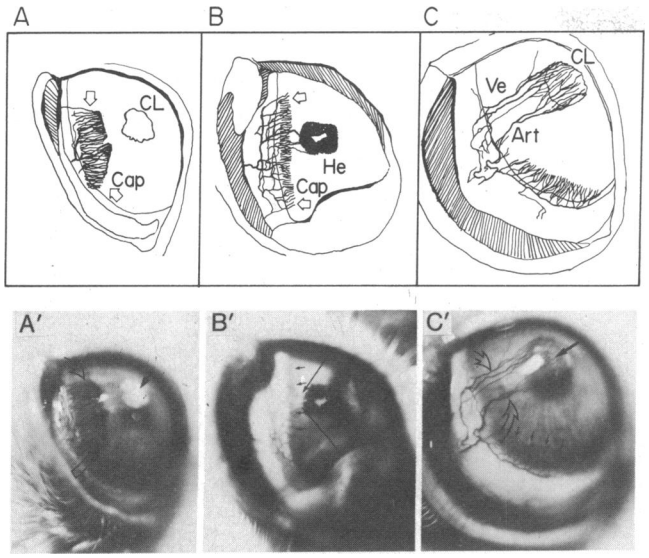

FIG. 3. Proliferation of capillaries induced by implant of rabbit corpus luteum in the rabbit cornea. (*A*) The corpus luteum (CL) has been implanted near the center of the eye. Six days following the implantation, capillaries (Cap) proliferate as a brush border from the limbus toward the implant. (*B*) Corpus luteum implanted near the limbus. By day 3, capillaries derived from the limbus have connected with the implant and anastomosed with luteal capillaries, thus leading to a hemorrhage (He) around the implant. (*C*) By day 14, the capillaries have invaded the corpus luteum and reorganization of the vascularization has taken place. The capillaries connecting with the corpus luteum have differentiated into arterioles (Art) and venules (Ve) forming three main vascular trunks. The capillaries not connecting are regressing, thereby leaving ghost vessels. *A*, *B*, and *C* are graphic illustrations of the eye in the photographs A', B', and C'.

lapsed and the cells exhibited pyknosis. No edema or inflammatory cells were observed (Fig. 2*B*).

Corpus luteum. When corpora lutea were implanted near the limbus (3 mm) (Fig. 3), after 24 hr newly formed vessels penetrated the cornea centripetally from the adjacent limbal area and grew toward the implant. Linear growth of the vessels initially proceeded at approximately 0.5 mm/day.

As soon as the capillaries reached the implant (by 4–5 days), hemorrhage was observed around it (Fig. 3*B*). This indicates that the preexisting vessels present in the corpus luteum did not disintegrate and reattached to the host vessels by anastomosis. The revascularization of corpora lutea implanted near the limbus results, then, from the capillary proliferation in the host as well as from the reattachment of the capillaries by anastomosis to preexisting corpus luteum capillaries. Following the neovascularization of the implant, capillaries that did not invade or contact the implant regressed, leaving ghost vessels in the cornea.

When the corpus luteum was implanted far away from the limbus (6–7 mm) (Fig. 3 *A* and *C*), newly formed capillaries, similar to those observed in previous experiments with corpora lutea implanted near the limbus, appeared within 48–72 hr and were fully developed within 4–5 days. Then secondary and tertiary branches rapidly developed, converting the initial sprouts into dense, vascular brushes (Fig. 3*A*). Vascular proliferation was greatest in the anterior layers of the corneal stroma but also occurred in deeper regions.

When the capillaries reached the corpus luteum (9–11 days), they invaded the tissue (Fig. 3*C*). Because by that time all of the preexisting capillaries of the corpus luteum had degenerated, its vascularization could only be accounted for by neovascularization dependent upon the host.

After neovascularization of the corpus luteum a spectacular rearrangement of the dense vascular network that proliferated in the cornea took place. All capillaries that did not connect to the corpus luteum regressed rapidly (Fig. 3*C*). Capillaries connecting with the implant started to differentiate into arterioles, while others simultaneously became venules, leaving two or three main vascular trunks reaching from the limbus to the

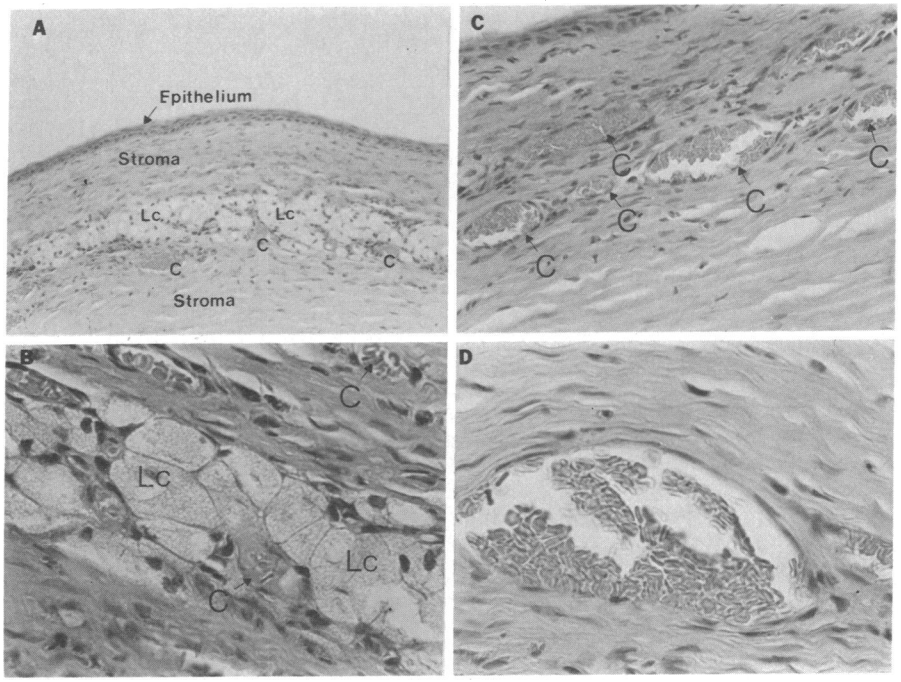

FIG. 4. Corpus luteum 15 days after implantation into the rabbit cornea. (*A*) The luteal cells (Lc) can be seen to be localized deep in the stroma. C, capillaries. (Hematoxylin/eosin staining, ×50.) (*B*) Capillaries growing deep in the stroma and invading the corpus luteum implant. (×200.) (*C*) Capillaries (arrows) growing deep in the stroma and full of erythrocytes. (×50). (*D*) Fully differentiated blood vessels filled with erythrocytes. (×200.)

corpus luteum and forming afferent and efferent vessels (Fig. 3C). Because it can be expected that the angiogenesis factor released by the corpus luteum was now carried off by the bloodstream and was no longer available for diffusion through the cornea, the gradient of angiogenesis factor that supported the capillary proliferation was probably no longer present. This could explain why all capillaries that do not infiltrate the corpus luteum regress.

Histological Study of the Corpus Luteum Implants. A histological section of a 15-day-old central implant of corpus luteum is shown in Fig. 4. Extensive luteinization could be observed through the corpus luteum (Fig. 4A). Large cells with light cytoplasm, when stained with hematoxylin and eosin, were clearly seen (Fig. 4B). Histological sections of growing capillaries approaching the corpus luteum implant showed that the surrounding stroma was essentially free of inflammatory cells (Fig. 4C); the vessels proper showed no margination or exudation of leukocytes. Numerous small capillaries were visible through the implant (Fig. 4B). Numerous differentiated blood vessels could be seen deep in the stroma (Fig. 4C). There was no sign of edema, no degeneration or necrosis of luteal cells was observed, and there was no accumulation of polymorphonuclear leukocytes (Fig. 4C).

Correlation between the Angiogenic Activities of the Corpus Luteum, Follicles, Ovarian Stroma, and Their Mitogenic Activity *In Vitro*. Because implanted corpora lutea were capable of stimulating the proliferation of capillaries in the rabbit cornea, while follicular implants were inactive, we have compared their relative mitotic activities *in vitro*. We have used the BALB/c 3T3 cell line and a bovine vascular endothelial cell strain as target cells (12–14). As shown in Fig. 5A, corpus luteum crude extracts were potent stimulators of the initiation of DNA synthesis; the minimal effective dose was found to be 200 ng/ml in two separate determinations and a plateau was observed at 1 μg/ml. Follicular extracts, on the other hand, were inactive when compared to the corpus luteum crude extracts. As shown in Fig. 5A, a marginal stimulation was observed at 2 μg/ml, while at 10 μg/ml the degree of stimulation was equivalent to that observed with corpus luteum crude extract at 300 ng/ml. Similar results were obtained whether rabbit ovarian tissues or bovine ovarian tissues were used. Crude extract of corpus luteum stimulated the proliferation of bovine aortic endothelial cells (Fig. 5B). Neither relaxin nor progesterone stimulated the initiation of DNA synthesis in BALB/c 3T3 cell cultures, nor did they stimulate the proliferation of vascular endothelial cell cultures.

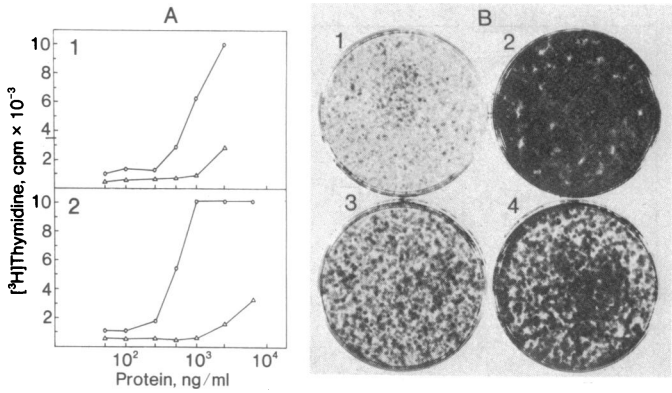

FIG. 5. Effect of crude extract of ovarian follicles and corpus luteum on the initiation of DNA synthesis in resting populations of BALB/c 3T3 cells and on the proliferation of vascular endothelial cells. (A) Increasing concentrations of crude extracts of ovarian follicles (△) or corpus luteum (○) were added to resting populations of BALB/c 3T3 cells. [^{3}H]Thymidine incorporation into DNA was determined as described in refs. 8, 9, 11. When increasing concentrations of ovarian stroma crude extract were used, the results were similar to those obtained with follicular crude extract. Graphs 1 and 2 show the results of two different experiments. (B) Effect of crude extract of corpus luteum on the proliferation of vascular endothelial cells. Bovine vascular endothelial cells (13, 14) were seeded at 20,000 cells per 6-cm dish in 5 ml of Dulbecco's modified Eagle's medium supplemented with 2.5% calf serum. (Plate 1) No additive, (2) fibroblast growth factor (100 ng/ml), or corpus luteum crude extract (3) (1 μg/ml) or (4) (10 μg/ml) was added every other day. On day 7 the plates were fixed and stained with 0.1% crystal violet. The cell number in absence of mitogenic addition was 61,000, while it was 1.2×10^6 cells for the cultures maintained in presence of 100 ng/ml of purified fibroblast growth factor and 550,000 and 860,000 cells, respectively, for cultures maintained in presence of 1 μg and 10 μg/ml of corpus luteum crude extract.

DISCUSSION

Our results demonstrate that ovarian corpus luteum, unlike follicles or ovarian stroma, is capable of inducing neovascularization. Using the rabbit cornea, which is a naturally transparent avascular structure, one can easily study the rates of new vessel formation and the parts played by the host and the ovarian implant in its vascularization. This model differed significantly from that of the ovarian implant but in the anterior chamber of the eye, for in the latter case the ovaries grafted onto the iris, which is a highly vascularized tissue (15). Thus, anastomosis between iris capillaries and ovarian capillaries resulted rather than a neovascularization of the implant by the host capillaries.

Although it has been reported (16) that adult tissue, with the possible exception of lymph nodes (17), does not stimulate capillary proliferation in the host, we find, using the corpus luteum, that a normal adult tissue does have this capacity. Although this does not necessarily mean that all adult tissues have the same capacity, it is probable that all adult tissues that are actively invaded by capillaries have this capacity at some stage of their development.

Corpora lutea implanted near the limbus behave similarly to an embryonic graft, because preexisting vessels do not disintegrate but are reattached by anastomosis to the host vessels. In contrast to the embryonic graft, however, in which there is either minimal or no vascularization on the part of the host vessels (16), corpora lutea are capable of inducing neovascularization, as is demonstrated by the distance that the capillaries grow through the cornea before reaching the implant.

In contrast, follicles did not stimulate neovascularization. This is in agreement with the *in vivo* situation, in which active capillary proliferation is observed *only* with corpus luteum, whereas follicles are an avascular structure. Because recent studies have shown that corneal tissue becomes vascularized as a consequence of leukocytic infiltration (18), it was of paramount importance to demonstrate that leukocytes did not invade the corpus luteum implant and that inflammation, which is known to be a potent inducer of corneal vascularization, did not develop. In our histological study we could not find any evidence of leukocytic infiltration in either early or late corpus luteum implants. Further proof that leukocytic infiltration is not a factor in inducing the vascularization observed with implants of corpus luteum is the observation that follicular implants did not induce neovascularization unless they luteinized. Because one would expect that infiltration of the follicle by leukocytes would stimulate the proliferation of blood vessels, the lack of angiogenesis following follicular implants demonstrates that such infiltration did not take place.

The observations that capillary budding always originated from a limited sector of the limbal plexus adjacent to the implant and developed into elongated hairpin loops directed toward the corpus luteum and that peripheral implants (1–2 mm from the limbus) elicited vessel formation as early as the first day, whereas centrally located implants elicited vessel formation after 3–4 days, demonstrate that the vascularization induced by the corpus luteum is mediated by diffusible substances.

Further proof that a stimulatory factor is being released from the corpus luteum is provided by the spectacular reorganization of vascularization once the capillaries have invaded the corpus luteum. What was before a very dense, reactive, cherry pannus with very little space between each blood vessel developed into two or three main arterioles and venules. All capillaries not contacting the corpus luteum regress, thus suggesting that the stimulating factor is now released into the bloodstream provided by the neovascularization and no longer diffuses through the cornea.

It may seem surprising that, besides synthesizing steroids, the corpus luteum should also be able to produce factors that stimulate vascular endothelial cell proliferation. However, the late corpus luteum of several species can synthesize and release the polypeptide relaxin (19). This indicates that the endocrine function of the corpus luteum is not strictly limited to the production of steroids.

If one speculates that corpora lutea, like tumor cells, contain a substance capable of stimulating the proliferation of capillaries, it may be possible to purify this substance by using as an assay system the growth of cultured vascular endothelial cells or that of BALB/c 3T3 cells. Using such a model, we have found that only the corpus luteum extract was capable of stimulating the proliferation of vascular endothelial cells maintained *in vitro*. Further support was thus lent to the hypothesis that corpora lutea can secrete an angiogenic factor which could be named "corpus luteum angiogenic factor" (CLAF).

One might wonder what the new situation is that causes the luteal cells to synthesize and release an angiogenic factor. As pointed out by Bassett (20), one of the main factors dictating the degree of vascularization of the various components of the ovaries is their metabolic requirement. This requirement is not peculiar to the ovaries, however, because capillary proliferation within a tissue seems, in most cases, to be correlative with an increased oxygen demand by that tissue. There is, for example, direct evidence suggesting that the initial stimulus attracting blood vessels in the fetal retina is an increased demand for O_2 arising from the inner layer of the retina which, in turn, directly or indirectly, stimulates capillary proliferation (21).

Considering the ovary in light of the retinal model, one notes that luteal cells secrete huge amounts of steroids, a process requiring great amounts of energy and, therefore, of O_2. This high requirement for energy is reflected by the high mitochondrial content observed in luteal cells. Granulosa cells, in contrast, are relatively dormant metabolically, and this dormancy is reflected by their low mitochondrial content. It is then reasonable that capillary proliferation should accompany the granulosa–luteal conversion as a response to the increased demand for oxygen by the luteal cells. An increased demand for O_2 could consequently stimulate the production of other factors such as TAF or CLAF which could, in turn, directly stimulate the proliferation of capillaries.

In conclusion, we would like to propose that the corpus luteum actively participates in its own neovascularization, which develops from capillaries sprouting from the vascular wreath present in the theca interna. While the initial stimulus is, in all likelihood, the increased demand for O_2 of the luteal cells caused by their high metabolic activity, the factor involved in inducing the proliferation of capillaries is a mitogenic factor (CLAF) produced by the corpus luteum.

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