Inhibition of Choriocapillaris Regeneration with Genistein

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Purpose. To test the effects of genistein on choriocapillaris regeneration and retinal pigment epithelial (RPE) wound healing in a surgical model in the rabbit.

Methods. Intravitreal injections of either 0.1 ml of a 90-JLMM concentration of genistein, dimethyl sulfoxide (DMSO; negative control), or 2 μg cycloheximide (positive control) were given 24 hours before surgical debridement of RPE in rabbits. Scanning electron microscopy (EM) of choriocapillaris vascular casts and the RPE wounds and histologic evaluation by light microscopy and EM of the disturbed areas were performed at days 1, 7, and 30 after surgery. Quantitative analysis of the area of the choriocapillaris bed and RPE was performed by automated image analysis, and the results were analyzed by paired Student's t test.

Results. Loss of RPE caused a rapid initial atrophy followed by slower subsequent revascularization of the choriocapillaris, which paralleled the RPE wound healing. Choriocapillaris regeneration appeared nearly normal by day 30 in the DMSO group. Inhibition of choriocapillaris revascularization by genistein was significant at day 30 when compared with the DMSO-treated negative control (P = 0.013). There was a strong trend toward inhibition in the cycloheximide-treated positive control group (P = 0.062), which reached significance at day 7 compared with the DMSO group (P = 0.02). RPE covered the wound area by day 7 in all groups.

Conclusions. Intravitreal injection of genistein was found to cause significant inhibition of choriocapillaris regeneration without apparent effect on RPE wound healing. Tyrosine kinase inhibitors such as genistein may be useful as a pharmacologic approach in the treatment of choroidal neovascularization. (Invest Ophthalmol Vis Sci. 1999;40:1477-1486)

S ubretinal neovascularization is an important cause of blindness that results from disorders including age-related macular degeneration, histoplasmosis, and pathologic myopia. Treatment with laser photocoagulation and different surgical methods has been reported to have varied success.1–5 However, both laser and surgically treated eyes have a high recurrence rate. Pharmacologic treatments to inhibit the growth of new vessels or to prevent recurrences should have beneficial effects on the disease.

The choriocapillaris is known to atrophy after retinal pigment epithelial loss. This has been clinically correlated in senile macular degeneration, retinitis pigmentosa, and thioridazine retinopathy.6–8 The relationship of retinal pigment epithelial loss with choriocapillaris atrophy has been studied in several experimental models.9–14 In these models, the retinal pigment epithelial loss is associated with choriocapillaris loss in the corresponding areas. After the recovery of the retinal pigment epithelium in these areas, the choriocapillaris also regenerates.

It is hypothesized that the retinal pigment epithelium releases vascular growth-modulating factors, that are diffusible and that locally modulate the choriocapillaris.9 In vitro cultures of retinal pigment epithelium release vascular mitogens including vascular growth factors.15–19 Pharmacologic agents that modulate the action of such factors may be useful in the modulation of the choriocapillaris and potentially in controlling choroidal neovascularization.

Genistein, a naturally occurring isoflavone isolated from soybean, has been shown to inhibit proliferation of vascular endothelial cells and tumor cell lines.20–26 Genistein has been shown to have varied tissue effects at different concentrations, including inhibition of angiogenesis, tyrosine kinase phosphorylation, DNA synthesis, and cell cycle arrest in the S phase.20–32 Thus, we studied this multifunctional compound as a potential candidate for inhibition of endothelial regeneration after RPE wounding. In this study we examined the effects of genistein on choriocapillaris regeneration after surgical RPE débridement in the rabbit model.

Materials and Methods

Eighty-one adult pigmented rabbits of 4 to 5 lb body weight were used in this study. All procedures conformed to the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research. The rabbits were equally grouped under the dimethyl sulfoxide (DMSO) group (hereafter referred to as the control group) and the genistein or cycloheximide group.
depending on the intravitreal drug injection. Nine normal fellow eyes were used to obtain normal values for comparison (hereafter referred to as preoperative values). Baseline color fundus photography and fluorescein angiography was performed in all rabbits.

All animals were anesthetized with ketamine (30 mg/kg body weight), xylazine (5 mg/kg body weight), and topical proparacaine hydrochloride for intravitreal injections and surgery. Pupils were dilated with 2.5% phenylephrine hydrochloride and 1% tropicamide.

**Intravitreal Injections**

Intravitreal injections of the drug (genistein, DMSO, or cycloheximide) were given 24 hours before the surgical débride ment of RPE. Animals were positioned under an operating microscope, the globe was exposed, and 0.1 ml of the solution containing the drug was injected into the vitreous cavity by a 30-gauge needle 3.5 mm from the limbus in the superotemporal quadrant. Adequate paracentesis was performed to prevent raised intraocular pressure, using venous pulsations as a guide.

**Genistein Group**

A 90-micromolar concentration of genistein was prepared using DMSO and lactated Ringer’s solution. The solution was protected from light during and after its constitution, because genistein is photosensitive. Intravitreal injection of 0.1 ml solution was given with a 30-gauge needle in all animals in the genistein group. Each time, fresh solution was prepared for injection.

**Control Group**

Dimethyl sulfoxide was chosen as a control treatment, because it was used as a buffer to dilute and preserve genistein. It was prepared in the same concentrations used for dilution of genistein, in lactated Ringer’s solution. Intravitreal injection of 0.1 ml was given as described above in all rabbits.

**Cycloheximide Group**

Cycloheximide, a known protein synthesis inhibitor and antiapoptotic compound was used as a positive control. Twenty micrograms per milliliter cycloheximide was constituted with animals, irrespective of the group. Animals were positioned produced in the area centralis with a jet of irrigating fluid irrigating solution. A vitrectomy was performed with a pneumatic cutter (Storz). A retinal detachment was produced in all rabbits, as described above.

**Surgical Procedure**

Similar surgical RPE débride ments were performed in all test animals, irrespective of the group. Animals were positioned under an operating microscope. The globe was exposed and a superior 180° conjunctival peritomy was performed. Two scleroto nies were made 3.5 mm from the limbus in the superotemporal and superonasal quadrants after applying diathermy and preplaced sutures with 6-0 Vicryl (Ethicon, Somerville, NJ) mattress sutures. Lactated Ringer’s solution was used as an irrigating solution. A vitrectomy was performed with a pneumatic cutter (Storz, St. Louis, MO). A retinal detachment was produced in the area centralis with a jet of irrigating fluid through a 30-gauge cannula, followed by a retinectomy over a 3-disc-diameter area and RPE débride ment with a 20-gauge silicone brush, taking care not to disturb the underlying Bruch’s membrane. The débrided RPE was aspirated from the vitreous cavity with the vitreous cutter. The sclerotomy ports were closed. Ointment with a combination of prednisolone acetate and gentamicin sulfate was applied. Rabbits were returned to their cages after gaining consciousness.

Surgical eyes were examined at days 1, 7, 14, and 30, until the rabbits were killed. Observations were documented by color fundus photography and fluorescein angiography. All animals were sedated and eyes dilated as described. Color fundus photography was performed using 100 ASA color film with a Topcon TRC-50F retinal camera (Tokyo Optical, Tokyo, Japan). Fluorescein angiography was performed using 400 ASA black-and-white film with the retinal camera, using barrier and exciter filters. A solution of 0.15 ml sodium fluorescein (10%) was injected into the ear vein, before photographs were obtained. Early and late phases were recorded up to 5 minutes after injection.

At days 1, 7, and 30 after surgery, animals were sedated as described and euthanatized using an intravenous overdose of pentobarbital sodium. Nine animals in each group were used at each specified interval. Three eyes in each group were used for vascular cast preparation to study the choroidal vascular changes, three eyes each for scanning electron microscopic (EM) study of RPE, and three eyes in each group for histologic evaluation by light microscopy and EM.

**Choroidal Vascular Cast Preparation**

Animals were anesthetized as described earlier. The carotid arteries were isolated on both sides and perfused with 500 ml of heparinized lactated Ringer’s solution. The animals were killed before injection of modified methyl methacrylate and benzoyl peroxide solution (Mercos; Ladd Research, Burlington, VT) through the isolated carotid arteries. The eyes were enucleated, the anterior segment separated, and the posterior segment bleached in 0.1 mol K2O. After the tissues were bleached, retinal vessels were separated from the choroidal vasculature. The choroidal vascular casts were rinsed in water and air dried. The casts were mounted on aluminum stubs (Ted Pella, Irvine, CA) and sputter coated with gold palladium before scanning EM (model JSM-840; JEOL, Peabody, MA). The wound area was recorded at X25 and X400 magnification. Three representative areas from the center of the wound in each specimen were recorded at X400 magnification for quantitative analysis of the choroidal vascular bed.

An image analysis program (Micro plan II; Laboratory Computer Systems, Cambridge, MA) was used for measuring the area of the choroidal vascular bed in each representative photograph. The choriocapillary area was measured by tracing all the capillaries in a given X400 photograph. The resultant values in each group at days 1, 7, and 30 were tabulated and analyzed by paired Student’s t-test in comparison with preoperative values (vascular cast from nonsurgical rabbit eyes) and among the groups and time intervals. P < 0.05 was regarded as significant.

**Scanning EM of RPE**

The eyes were enucleated and immersed overnight in 2% gluteraldehyde and 2% paraformaldehyde in 0.1 mol phosphate buffer at 4°C after dissecting the anterior segment. The posterior segment of the eye was trimmed down to the area of interest, postfix ed for 90 minutes in 1% osmium tetroxide in phosphate buffer, acetone dehydrated, and covered with tetramethylsilane. The tissues were mounted on
aluminum stubs and sputter coated with gold palladium before scanning EM. Quantitative analysis of the retinal pigment epithelium was performed using the image analysis program. The RPE cell density was analyzed from X400 photographs, from three areas in each specimen. The resultant values in each group at days 1, 7, and 30 were tabulated and analyzed by paired Student's t-test in comparison with preoperative values (specimens from nonsurgical rabbit eyes) and among the groups and time intervals. \( P < 0.05 \) was regarded as significant.

**Light Microscopy and EM**

The eyes were enucleated and immersed overnight in 2% gluteraldehyde and 2% paraformaldehyde in 0.1 molc phosphate buffer at 4°C after the anterior segment was dissected. The specimens were then dissected. Half of the wound area
RESULTS

Fundus Photography

Minimal exudation was observed on day 1, which cleared by day 7, with varying pigment epithelial regeneration from the periphery to the center of the debrided area. At day 30, the pigment epithelial regeneration was more complete with appearance of varying pigmentation and scarring. The degree of exudation, pigment epithelial regeneration and scar formation was not significantly different among the groups, judged by fundus photography (Fig. 1A, 1C, 1E, 1G).

Fluorescein Angiography

The wound area leaked fluorescein at day 1 in all groups. At day 7 no leak was observed (possibly because of retinal pigment epithelial regeneration), and none was observed at days 14 and day 30. Progressively blocked fluorescence from the periphery to the center was observed at each time point. No difference was observed among the groups. Clinically apparent choriocapillaris atrophy was observed in the genistein group at days 14 and day 30, when compared with control and cycloheximide groups (Fig. 1B, 1D, 1F, 1H).

Scanning EM of Choroidal Vascular Casts

Day 1. Scanning EM of choroidal vascular casts in the debrided area showed partial atrophy in all three groups. There was no difference among the groups. Compare the control group (Fig. 2A) with the genistein (Fig. 2B) and cycloheximide groups (Fig. 2C).

Day 7. The genistein (Fig. 2E) and cycloheximide (Fig. 2F) groups showed more atrophy compared with the control group (Fig. 2D), although all groups showed continued atrophy.

Day 30. The genistein group (Fig. 2H) continued to show severe capillary atrophy compared with the cycloheximide (Fig. 2I) and control groups. In the control group regeneration of choriocapillaris was near normal. The choriocapillaris was normal outside the wound area in all groups at all follow-ups, indicative of the nontoxic nature of the agents we used in this study.
Quantitative Analysis of the Choroidal Vascular Bed in the Wound Area

Choriocapillaris atrophy and regeneration in the wound area after RPE debridement was measured first in the DMSO control group. Significant choriocapillaris atrophy was observed in the control group at day 1 (P = 0.007) and day 7 (P = 0.0001) over the preoperative level. By day 30 there was no significant difference from the preoperative level (P = 0.07) signifying marked regeneration of the choriocapillaris after initial atrophy (Table 1).

We analyzed choriocapillaris regeneration in the wound area after genistein injection compared with the control and cycloheximide groups. There was significant inhibition of regeneration with genistein at day 30 compared with control (P = 0.012) and nearly so when compared with the cycloheximide group (P = 0.062). Inhibition with genistein was also seen at day 7 when compared with the control group (P = 0.02) (Fig. 3). Inhibition of choriocapillaris regeneration was significant with cycloheximide at day 7 compared with the control group (P = 0.039); however, it was not significant at day 30 (P = 0.24). At day 1 the control group showed significant choriocapillaris atrophy compared with the genistein group (P = 0.016), and the difference in area was not significant in the cycloheximide group compared with the genistein (P = 0.18) and control groups (P = 0.34). Sparing of atrophy was present in the cycloheximide group but was not statistically significant (Table 1).

Scanning EM of RPE Wound Healing

Day 1. Scanning EM of the RPE at day 1 did not show any residual or regenerating RPE. Bruch's membrane was intact and was covered by transudate in some specimens.
FIGURE 4. Scanning EM of retinal pigment epithelium at day 1: (A) control, (B) genistein, and (C) cycloheximide, showing smooth surface of Bruch's membrane in all groups. At day 7 the pigment epithelium covered the wound area in all groups: (D) control, (E) genistein, and (F) cycloheximide. At day 30 (G, H, I) pigment epithelium was more mature and hexagonal from periphery to the center. The RPE was polymegathous in the center of the wound. The genistein group (H) showed more immature RPE than the control (G) and cycloheximide groups (I). Bar, 100 μm.

Day 7. Retinal pigment epithelium covered the whole wound area by day 7 in all groups, although in the genistein group (Fig. 4E) the RPE appeared flattened and less hexagonal compared with the control group (Fig. 4D). The cycloheximide group showed small, round, and unevenly distributed RPE compared with the control group (Fig. 4F).

Day 30. Retinal pigment epithelium in the control group appeared nearly normal compared with both the genistein and cycloheximide groups, though all groups showed centrally a number of immature RPE cells compared with the intermediate and marginal areas (Fig. 4G, 4H, 4I).

Quantitative Analysis of the Retinal Pigment Epithelium

The retinal pigment epithelial débridement was complete at day 1. At day 7 the RPE cell density in the control group were significantly less compared with the preoperative cell density \( (P = 0.059) \). At 1 month the RPE cell density increased to a statistically insignificant level compared with the preoperative cell density \( (P = 0.176; \text{Table 2}) \).

We analyzed the retinal pigment epithelial cell density in the genistein group compared with the control and cycloheximide groups. The RPE cell density was not statistically significant between the genistein group and the control \( (P = 0.938) \) and cycloheximide groups \( (P = 0.54) \) at day 7; however, RPE cell density was significantly lower in the genistein group compared with the control group \( (P = 0.001) \) at day 30. The RPE cell density was significantly lower in the cycloheximide group compared with the genistein group \( (P = 0.057) \) at day 30. When we compared the increase in RPE cell density in individual groups, the control \( (P = 0.17) \) and genistein groups \( (P = 0.51) \) showed an increase or stabilization in RPE cell

| Table 2. Summary of Quantitative Analysis of Retinal Pigment Epithelium |
|---------------------------|------------------|------------------|------------------|------------------|
|                           | Preop            | Day 1            | Day 7            | Day 30           |
| Control                   | 255 ± 23         | 0.33 ± 0.58      | 189 ± 93         | 247 ± 16         |
| Genistein                 | 255 ± 23         | 0.33 ± 0.58      | 186 ± 65         | 172 ± 45*        |
| Cycloheximide             | 255 ± 23         | 1.53 ± 1.52      | 217 ± 101        | 119 ± 40*        |

Data are expressed as average cells per x400 field ± SD. \( n = 9 \) in all groups. Preop, normal values.

\* \( P < 0.05 \) between groups.
Light Microscopy and Transmission EM

Day 1. Light microscopy revealed that pigment epithelial remnants, transudate, and a few red blood cells were present in specimens from all the groups. Bruch’s membrane was intact in all specimens, and RPE debridement appeared complete (Fig. 5A, 5B, 5C). The choriocapillaris showed endothelial fenestrations on the RPE side with good apposition of basement membrane on transmission EM. Choroidal capillary histology was comparable with the nonsurgical eye. The pigment epithelial debridement appeared complete with intact Bruch’s membrane in all specimens (Fig. 6A, 6B, 6C).

Day 7. Pigment epithelium covered the wound area in all the specimens on light microscopy (Fig. 5D, 5E, 5F). On transmission EM early changes of maturation were evident (i.e., apical pigment granules, basal villi, and zonulae occludens); however, multiple layers of RPE were observed. The choriocapillaris lumen appeared narrow with fewer vascular fenestrations and loose basement membranes, indicating choriocapillaris atrophy in all groups (Fig. 6D, 6E, 6F).

Day 30. Pigment epithelium appeared to be multilayered with areas of atrophy in all specimens (Fig. 5G, 5H, 5I). On transmission EM changes of maturation were similar to day 7 specimens, with areas of acinar formation of RPE. In the DMSO (Fig. 6G) and cycloheximide (Fig. 6I) groups choriocapillaris appeared to be regenerating, with new basement membrane formation and good vascular cavities. Open cavities were strikingly absent in the genistein group (Fig. 6H).

DISCUSSION

The choriocapillaris is known to atrophy after loss of the overlying retinal pigment epithelium. The relationship of RPE and choriocapillaris atrophy has been well studied in various experiments in rabbits and rats in iodate-induced retinopathy.10,11 Similar observations have been made in surgical models of RPE débridement.12-14 In surgical models the choriocapillaris has been shown to degenerate for the first 7 days. As RPE regenerates, choriocapillaris regeneration starts and reaches its near-normal level (preoperative level) by 1 month. Choriocapillaris regeneration curve in the control group in this study correlated well with the previous studies.

Retinal pigment epithelial cells have been shown to produce vascular mitogenic factors in vitro.15-19 Schweigerer et al.17 have shown that RPE cells are capable of producing basic fibroblast growth factor. It was hypothesized that vascular growth-modulating factors produced by RPE play an important role in choriocapillaris atrophy and subsequent regeneration along with regeneration of retinal pigment epithelium.9

Genistein has been shown to inhibit tyrosine kinase activity induced by exogenously applied growth factors in vitro
FIGURE 6. Electron microscopic photographs showing intact Bruch's membrane (BM) with complete RPE débridement at day 1: (A) control, (B) genistein, and (C) cycloheximide. Choriocapillaries (cc) are well opposed with Bruch's membrane, and vascular fenestrations (small arrows) are evident in all groups. At day 7 resurfacing of RPE was complete. Note the RPE with dividing nuclei (F), zonulae occludens, and basal villi: (D) control, (E) genistein, and (F) cycloheximide. Regenerating capillaries with open cavities were observed at day 30 in the control (G) and cycloheximide groups (I). Compare with closed cavities (E, curved thick arrow) seen at day 7. Continued atrophy of the choriocapillaris was seen in the genistein group at day 30 (H). Bar, 1 μm.

to inhibit vascular endothelial cell proliferation.21,25 We therefore hypothesized that genistein might inhibit choriocapillaris regeneration after RPE débridement-induced atrophy. To test our hypothesis, we examined the effects of genistein on choriocapillaris regeneration and compared the effects with DMSO as a negative control and a protein synthesis inhibitor (cycloheximide) as positive control.

Cycloheximide, a potent protein synthesis inhibitor, acts on protein kinases and phosphotases.33,34 Cycloheximide has been shown to be antiapoptotic, presumably by inhibition of protein synthesis required for apoptosis.35-37 It has also been shown to be neuroprotective and inhibits antigen-induced histamine release by similar effects.38-40 In this study cycloheximide was used as positive control for its nonspecific and generalized effect on protein synthesis.

At day 1, the control group showed more severe atrophy compared with the genistein and cycloheximide groups (although cycloheximide group was not statistically significant). This points to a possible role of apoptosis in the choriocapillary bed in early stages after RPE débridement. Because cycloheximide has been shown to have antiapoptotic properties, blocking apoptosis could explain the sparing of atrophy at day 1.35,36,45,46 It is possible that genistein has some effect on apoptosis. Inhibition of factors involved in apoptosis through protein synthesis inhibition by both cycloheximide and genistein might be considered in future experiments.

At day 7, the area of the choriocapillaris vascular bed was significantly less in the genistein and cycloheximide groups compared with the DMSO control group and at day 30 in the genistein group compared with the DMSO control group. We speculate that the inhibitory effects on choriocapillaris regeneration by genistein could be caused by inhibition of growth factor action, as is suggested by Morse et al. and Schweigerer et al.16,17 The difference in the inhibitory effects of genistein and cycloheximide could be related to the pharmacodynamics of the drugs, or inhibition of protein synthesis may not be the only mechanism of inhibition of choriocapillary regeneration by genistein.

We used a 90-micromole concentration of genistein for the intravitreal injection in this study. This concentration is well below the safety range of intravitreal genistein in rabbits. At a 500-micromole concentration, genistein did not show any toxic effects, either on histologic or electrophysiological examination of the retina (data not shown). At a 90-micromole concentration, genistein was expected to have antiangiogenic action.21 At this level it may act also through inhibition of tyrosine kinase phosphorylation, topoisomerization, or cell-cycle arrest in the S phase.21-31 As discussed, the genistein
effects on choriocapillaris may be antiapoptotic on day 1 and involve tyrosine kinase inhibition and antiangiogenic effects at days 7 and 30. However, other mechanisms cannot be ruled out. Biochemical aspects were not examined in this study but should be examined in future.

The histologic observations correlated well with the vascular cast results. However, it would have been difficult to compare the degree of inhibition of pharmacologic agents based only on histologic evaluation of choriocapillaris. We believe the quantitative analysis of the area of the choriocapillar bed in the wound area allowed us to measure the degree of the drug effects more precisely.

The retinal pigment epithelium appeared to cover the wound area by day 7 in all groups, although more unevenly in cycloheximide and genistein groups. This correlated well with previous studies on RPE débridement.\(^{12-14}\) Initially the RPE was nonpigmented, and pigmentation appeared first at the margins of the wound and then progressed to the center on histology. This was also evident on fundus photography and fluorescein angiography. Two mechanisms were explained for RPE resurfacing the wound area: RPE sliding and true RPE hyperplasia.\(^{43}\) The sliding mechanism was thought to be the main mechanism of RPE resurfacing the débrided area.\(^{45}\) However, scanning photographs and mitotic figures in some electron microscopic photographs in this study suggest that hyperplasia may also contribute to a significant extent.

Retinal pigment epithelial resurfacing was not affected in any of the groups in this study at day 7, shown by quantitative analysis of RPE cell density (Table 2). However, the RPE cell density was significantly less in the genistein and cycloheximide groups compared with the control group at day 30. When we compared the cell density between day 7 and day 30, there was no significant change in cell density in the control (\(P = 0.17\)) and genistein groups (\(P = 0.51\)). In the cycloheximide group there was a significant decrease in cell density at day 30. This can be partially attributed to inhibition of choriocapillaris regeneration; the RPE cell density was significantly less in both the genistein group and cycloheximide group compared with the control group. However, if it was a main factor, the genistein group should have also shown a marked decrease in RPE cell density, but this did not happen. We cannot rule out the role of other biochemical factors involved in RPE choriocapillaris complex. The difference in alteration of RPE cell density and inhibition of choriocapillaris regeneration between genistein and cycloheximide suggest factors through which these two agents acted were different.

In this study intravitreal genistein injection was found to cause significant inhibition of choriocapillaris regeneration without affecting the retinal pigment epithelial resurfacing or adjacent normal choriocapillaris. It would appear to us that this class of drugs (tyrosine kinase Inhibitors) could be agents in pharmacologic treatment of choroidal neovascularization in conditions associated with age-related macular degeneration, histoplasmosis, and pathologic myopia.

### References


