Inhibition of Oxygen-induced Retinal Neovascularization in the Neonatal Rat by Green Tea Extract

Masayuki Minami,¹ Yasuko Hasebe,¹ Takako Nakanishi-Ueda,^{2,*} Sinichi Iwai,² Toshihiko Ueda,¹ C. Kathleen Dorey,³ Katsuji Oguchi,² Hajime Yasuhara,² and Ryohei Koide¹

¹Department of Ophthalmology, School of Medicine, Showa University, Tokyo 142–8555, Japan

²Department of Pharmacology, School of Medicine, Showa University, Tokyo 142–8555, Japan

³R & D Consulting, 15 Draper Avenue, Arlington, Massachusetts 02474, USA

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Summary To investigate the effect of green tea extract (GTE) on oxygen-induced neovascularization (NV) in neonatal rat model, NV was induced by maintaining neonatal rats in 80% oxygen on a cycle of 23.5 h oxygen/0.5 h room air until postnatal day 12 (P12), when the rats were placed in room air. The control rats were maintained in room air. From P6 to P17, rats were treated once daily by oral administration of saline (50 μ l/10 g body weight) that contained 25% GTE, 12.5% GTE, or nothing. On P18, the rats were sacrificed and the retinal samples were collected. Retinal NV was scored and avascular areas (AVAs) were measured in ADPase stained retinas. The retinal vascular endothelial cell growth factor (VEGF) contents were measured with immunoassay kit, and matrix metalloproteinase-2 (MMP-2) activity was determined by gelatin zymography. GTE (25%) treatment suppressed NV and slightly increased AVAs in oxygen-induced NV model compared with the control. VEGF contents in retina significantly increased on P13 and P15, but GTE treatment did not prevent the increase of VEGF contents. Oxygen-induced increases in MMP-2 activity on P13 and P15 were suppressed by GTE treatment. These results suggest that GTE suppressed oxygen-induced NV in the neonatal rat, possibly through inhibition of MMP-2 activity. It also suggests that orally administered green tea has the potential to inhibit neovascular disease.

Key Words: neovascularization, oxygen-induced retinopathy, green tea, matrix metalloproteinase, vascular endothelial cell growth factor

Introduction

Retinal neovascularization (NV) is the final common pathway to sight threatening disease including retinopathy of prematurity, diabetic retinopathy and age related macular degeneration [1, 2]. Oxygen-induced retinopathy (OIR) of rodents provides a

good model system for studying retinal neovascular disease [3]. The underlying mechanism of OIR is not fully understood. Angiogenic stimulators such as vascular endothelial growth factor (VEGF), insulin-like growth factor 1 and/or retinal hypoxia have been found to play a role in oxygen-induced retinal NV [4–6]. Green tea is widely consumed in the eastern world, especially in Japan and China, where it has also been utilized as an herbal medicine. Epidemiologic studies have suggested that consumption of green tea may help prevent some types of cancer [7,

*Correspondence to: T. Nakanishi-Ueda

Tel: +81–3–3784–8128 Fax: +81–3–3784–8129

E-mail: tachueda@med.showa-u.ac.jp.

8], but not others [9, 10]. The major components in green tea are polyphenols, known as catechins, caffeine and flavonols. The predominant catechin, (–)-epigallocatechin gallate (EGCG) is an effective antioxidant, reacting with most reactive oxygen species [11, 12]. Several lines of evidence suggest that green tea and/or EGCG have anti-angiogenic properties. Cao and Cao [13] demonstrated that EGCG inhibited angiogenesis in chick chorioallantoic model and that drinking tea inhibited VEGF-induced corneal NV in mice. Garbisa *et al.* [14, 15] showed that EGCG suppressed the activity of matrix metalloproteinases (MMPs), and blunted tumor invasion. Furthermore, Bertolini *et al.* [16] reported that green tea inhibited angiogenesis and induced endothelial and tumor cell apoptosis in animal model of human high-grade non-Hodgkin's lymphoma. Recently, Sartippour *et al.* [17, 18] reported that EGCG inhibited protein and mRNA expression of VEGF, and fibroblast growth factors 1 and 2 by human breast cancer cells and by human umbilical vein endothelial cells *in vitro*.

In this study, we investigated whether orally administered green tea extract (GTE) or EGCG could inhibit oxygen-induced retinal NV in neonatal rats and whether NV correlated with levels of VEGF and MMPs.

Material and Methods

All experiments were performed in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the institutional committee of animal care and use at our institution.

Animal model

Sprague-Dawley neonatal rats (Clea Japan Co., Tokyo, Japan) were used. For each experiment, 12 or 13 neonatal rats were kept with a mother rat in each cage. Mother rats were rotated between the oxygen and room air environments every two days within

the experimental period. NV was induced in neonatal Sprague-Dawley rats by our standard protocol [3, 19]. Briefly, neonates were exposed from birth to day 12 (P12) to daily cycles of 80% oxygen (20.5 h), room air (0.5 h) and progressive return to 80% oxygen (3 h); on P12, the rats were placed in room air until P18. For each experiment, 12 or 13 neonates were caged with each mother rat in oxygen or room air.

Treatment schedule

GTE (12.5% or 25%) in saline, EGCG (2 mg/50 μ l) in saline, or saline alone was given orally to animals in a constant volume of 50 μ l/10 g body weight once daily from P6 to P17.

Preparation of green tea extracts

Organic green tea leaves with no preservation (Mouriseicha, Kyoto, Japan) were obtained commercially. GTE were prepared as previously described [20] by flooding green tea leaves with saline (12.5 or 25 g tea leaves in 100 ml saline, pH 6.3) for 3 h at room temperature, and then filtering the sediments out.

EGCG

EGCG (Sigma, St. Louis, MO, USA) was prepared at 40 mg/ml in saline, a concentration that corresponds to 25% (25 g/100 ml) GTE.

Retinal processing and analysis

On P18, the rats were sacrificed by *i.p.* injection of 0.3 ml of sodium pentobarbital (50 mg/ml) and the retinal samples were fixed, stained for adenosine diphosphatase (ADPase), and flatmounted as previously described [3]. Retinal NV was scored using the method of Hasebe *et al.* [19] (Table 1). Avascular areas (AVAs) were measured in ADPase stained retinas. Digital images of the mounted ADPase stained retinas were obtained using a camera and scanner (Nikon, Tokyo, Japan). Avascular and total retinal areas were measured using NIH image software

Table 1. Criteria for neovascular score (NV Score).

Score (In each quadrant)	Characteristics of neovascularization
0	None observed
1	Less than five glomerular buds
2	Five or more buds or a frond
3	Short ridge (neovascular ridges extending less than halfway across a quadrant)
4	Long ridge (neovascular ridges extending at least halfway across a quadrant)

(National Institutes of Health, Bethesda, MD, USA). The mean percentages of AVAs in the total retinal area (%AVAs) were calculated.

Quantification of VEGF protein

On P12, P13, P15, and P18, retinal samples were collected and stored at -70°C till analysis. The sample and 100 μl H_2O were submerged in crushed ice, sonicated with 5 s bursts at 40 W (Branson Sonifier Cell Disruptor 200, Branson, Rochester Hills, MI, USA), and centrifuged at 3,000 rpm for 10 min. VEGF in the supernatant was assayed by using a Quantikine immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA). Protein concentrations were determined by the method of Lowry *et al.* [21] with bovine serum albumin as standard.

Measurement of MMP activities

MMP-2 and -9 were measured by gelatin zymography on 10% polyacrylamide gels containing 1 mg/ml gelatin in a polymerization mixture of SDS. The frozen retinas were sonicated and centrifuged as described above. Samples containing equal protein concentrations (15 μg) were mixed with saline. For detection of proteinases, SDS was washed out of the gels with 2.5% triton X-100 for 30 min at room temperature before incubation at 37°C for 1 day in a

solution containing 50 mM Tris-HCl, pH 7.5; 5 mM CaCl_2 ; and 0.02% sodium azide. Gels were stained with 0.1% Coomassie blue in 10% acetic acid and 50% methanol for 30 min, and then de-stained in 10% acetic acid and 50% methanol. Clear areas indicated proteolytic activity [22]. Molecular weight standards (Sigma) were included to calibrate the gels. The 72 kDa purified human proenzyme MMP-2 (pro-form; pro-MMP-2), the 62 kDa purified proenzyme MMP-2 (active-form; active-MMP-2) and the 92 kDa purified proenzyme MMP-9 were used as a positive control and standard for the gels (Oncogene Research Products, San Diego, CA, USA). The zymography bands were digitized using Gel Doc/Chemic Doc Imaging System (Bio Rad, Hercules, CA, USA) and the data analyzed using the Quantify One analysis software program (Bio Rad). Total protein was measured in the retina samples using the BCA Assay (Pierce Chemical Co., Rockford, IL, USA) with bovine serum albumin as the standard.

Statistics

Statistical analysis was performed by using ANOVA or Mann-Whitney U-Test. All data are expressed as a mean $\pm$ standard error (SE) and differences were considered significant when $p < 0.05$.

Fig. 1. ADPase stained retina. (A) control group. NV score is 8 %AVAs is 14.6%. (B) 12.5% GTE treated group. NV score is 5 %AVAs is 12.9%. (C) 25% GTE treated group. NV score is 4 %AVAs is 5.0%.

Results

Rats treated with either GTE or EGCG did not show growth retardation compared with controls in room air or 80% oxygen exposure. Normal retinal vasculature was seen in the animals raised in room-air. Oxygen-exposed retinas exhibited neovascular changes and retained avascular areas, as seen in Fig. 1.

The NV scores and %AVAs in the EGCG-treated and control groups were not significantly different from the saline treated control (Fig. 2). In EGCG treated and controls, NV scores were 4.6 ± 1.1 and 4.2 ± 0.7 , respectively, and %AVAs were $25.2 \pm 4.7\%$ and $17.8 \pm 4.0\%$, respectively.

In contrast, animals treated with the 25% GTE had NV scores that were 39% lower than those in the untreated controls (Fig. 3). NV scores in the 25%

Fig. 2. Effect of EGCG treatment on the oxygen-induced retinal NV in the neonatal rat.

(A) NV score, (B) %AVAs. Data represent mean $\pm$ SE for control group ($n=10$) and EGCG treated group ($n=10$). The significant differences were not observed between control and EGCG treated groups.

Fig. 3. Effect of GTE treatment on oxygen-induced retinal NV in the neonatal rat. (A) NV score, (B) %AVAs. Data represent mean $\pm$ SE. In GTE (25%)-treated groups, NV score is significantly lower than saline controls ($*p=0.05$; ANOVA).

Fig 4. Effect of GTE treatment on VEGF contents in retina after oxygen exposure. Data represent mean $\pm$ SE (pg/mg protein, $n=3$ for each point). The content of VEGF significantly increased at day 13 in each group ($\square$, control; $\blacksquare$, 12.5% GTE; $\blacksquare$, 25% GTE; $p=0.05$, Mann-Whitney U-test) and at day 15 in 12.5% and 25% GTE compared with day 12.

Table 2. Effect of GTE treatment on MMP-2 activities in retina.

	O ₂ treatment			Room air			
	Saline	12.50%	25%	Saline	12.50%	25%	None
P13							
pro-	181	151	149	105	107	101	100
active-	177	83	119	79	79	83	100
P15							
pro-	145	98	91	90	90	95	100
active-	192	161	143	66	60	64	100

Relative activity of MMP-2 normalized to room air vehicle controls.

GTE treated group were significantly lower than those treated with the 12.5% ($p=0.05$; ANOVA). The %AVAs was also substantially higher in these animals than in the controls ($25.0 \pm 6.1\%$ vs. $15.0 \pm 1.9\%$) but the differences were not significant. Treatment with the 12.5% GTE had no significant effect on NV scores or on %AVAs.

The quantity of VEGF protein in the oxygen-treated retinas increased significantly on P13 *i.e.* one day after removal from oxygen ($p=0.05$; Mann-Whitney U-Test), then declined gradually from P13 through P18. Higher quantities of VEGF were observed in the GTE-treated retinas on P12 and P13, but the differences did not achieve significance between control and GTE-treated groups (Fig. 4).

Figure 5 shows zymography of P13, 15, and 18 day old retinas. Pro-MMP-2 and active-MMP-2

were higher in oxygen-exposed retinas than in controls on P13 (A, 181%, and 177% of control, respectively) and on P15 (B, 144% and 192% of control, respectively), but not on P18. These increases on P13 and P15 were suppressed by GTE treatment (Table 2). MMP-9 was not clearly observed in these experiments.

Discussion

Here we showed that orally administered GTE suppressed NV in oxygen-exposed neonatal rats. In addition, we found that purified EGCG, the major tea catechin, had no effect on NV.

Pierce *et al.* [23] showed that VEGF protein and its mRNA increased between 6 and 12 h of relative retinal hypoxia and remained elevated during the

Fig. 5. Gelatin zymogram of retina, illustrating the elevated production of MMP-2 on P13 (A) and P15 (B) in oxygen-exposed animals. However, by P18 the MMP-2 production returned to levels seen in room air animals. The elevated production of MMP-2 was suppressed by GTE treatment on P13 and P15.

development of NV in murine OIR. Kondo *et al.* [24] has shown that tea catechins inhibit human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding and Lamy *et al.* [25] further found that catechins suppress VEGF receptor phosphorylation. Similarly, we found that VEGF protein was elevated by oxygen exposure, and decreased following removal from oxygen. The higher levels of VEGF found in animals treated with 25% GTE was consistent with the larger AVAs observed in them. The effect of GTE on %AVAs and on NV scores could both be explained by GTE-inhibition of the VEGF receptor activity [24, 25]. A drug that inhibited VEGF receptor

kinases, protein kinase C and other kinases inhibited both retinal vascular development and NV in murine OIR [26]. Potent inhibition of VEGF receptor-2 phosphorylation by GTE, catechins or other polyphenols could explain reduced NV scores in animals treated with 25% GTE. In dogs [27] and in mice [28], VEGF receptor-2 is preferentially associated with NV, and NV can be suppressed by inhibiting VEGF receptor-2. The larger AVAs observed in these animals suggest that the catechins may also suppress activity of VEGF receptor-1, which is expressed in normal retinal vessels [29].

It is possible that complex formation between EGCG and other components in decaffeinated

green tea (DGT) may increase the absorption of EGCG. Chen *et al.* [30] reported that when administered orally EGCG in DGT showed a 3.6-fold higher absorption rate constant than pure EGCG in male Sprague-Dawley rats. And the $C_{\max}$ were 16.3 ± 6.5 ng/ml ($0.036 \mu\text{M}$) and 19.8 ± 3.5 ng/ml ($0.043 \mu\text{M}$), after EGCG (14.6 mg/kg) in DGT (200 mg/kg) and pure EGCG (75 mg/kg) oral administration. Following their report, we could calculate that the $C_{\max}$ of EGCG in our oxygen-induced neonatal rat models; 223.3 ng/ml ($0.487 \mu\text{M}$) and 53.5 ng/ml ($0.116 \mu\text{M}$) after 25% GTE or 40 mg/ml EGCG treatment, respectively. Therefore, treatment with a comparable dose of EGCG (40 mg/ml) did not prevent NV or alter AVAs. On the other hand, Cao and Cao [13] reported that orally administered tea inhibited VEGF-stimulated NV in the mouse corneal model. The blood concentration of EGCG in the mouse corneal model ($0.1\text{--}0.3 \mu\text{M}$) was similar to our results. There were also reported that epigallocatechin (EGC), which is contained in GTE, reduced IL-8 production and inhibited tube formation *in vitro* using a model in which human microvascular endothelial cells [31]. It may be one of the inhibition mechanisms in this OIR model. The quantity of green tea administered in the 25% GTE treatments would correspond to a person drinking 1.5 liters of tea/day. Thus identification of the factor present in green tea would greatly facilitate further exploration of their therapeutic effect on NV, and provide a means to deliver effective doses in human subjects.

It has been firmly established that MMPs are required for angiogenesis and tumorigenesis [32, 33]. When endothelial cells are cultured on matrigel, the formation of tubular networks is increased by the addition of recombinant MMP-2 and decreased when neutralizing antibody or tissue inhibitor of MMP-2 (TIMP-2) is added [34]. MMP-2 deficient mice are reported to have reduced tumor-induced and corneal angiogenesis [35, 36].

Garbisa *et al.* [14, 15] showed that EGCG directly suppressed the activity of MMP-2 and MMP-9. Annabi *et al.* [37] reported EGCG inhibited MMP-2 secretion. Demeule *et al.* [38] reported that green tea polyphenol, and more particularly EGCG, inhibited the activity of various MMPs as well as the activation of pro-MMP-2 activation. On the other hand, EGCG inhibits membrane-type 1 MMP (MT1-MMP) activity [39] or urokinase type plasminogen activator (uPA) activity [40], those are

important factors of pro-MMP-2 activation, leading to decrease of active-MMP-2. Therefore, these data suggest that GTE-treatment suppressed the oxygen-induced increases in pro-MMP-2 and active-MMP-2 by through the inhibition of MMP-2 secretion, and active-MMP-2 formation. Our data propose that green tea may also inhibit NV by reducing MMP-2 activity.

We cannot exclude the possibility that the GTE may have acted through its antioxidant activity [41, 42], quenching reactive oxygen intermediates that increase VEGF expression *in vitro* and during the reperfusion of ischemic retina *in vivo* [43]. Our group has reported that lipid hydroperoxide-induced NV in rabbit cornea could be inhibited by both a combination of antioxidants (EPC-K1; vitamins E and C compound) [44], and by Saiei-to, a compound with antioxidant activity *in vitro* [45].

In conclusion, more than one component of GTE may influence oxygen-induced retinal NV. These results suggest that orally administered green tea has the potential to slow neovascular disease such as the diabetic retinopathy and the retinopathy of prematurity.

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