Subconjunctival Doxifluridine Administration Suppresses Rat Choroidal Neovascularization through Activated Thymidine Phosphorylase

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PURPOSE. Doxifluridine (5′-deoxy-5-fluorouridine) is an oral anticancer drug with antiangiogenic effects, with vasoclastic action that is enhanced by a major member of the pyrimidine phosphorylases, thymidine phosphorylase (TP). Previous studies have demonstrated that TP is upregulated in the lesions where pathologic angiogenesis occurs and TP itself promotes angiogenesis. To investigate the possible role of TP and doxifluridine in choroidal neovascularization (CNV), the expression level of TP was measured and the effect of doxifluridine was investigated in rat eyes with experimental CNV.

METHODS. CNV was induced in rat eyes by diode laser photocoagulation. The expression level of TP in the laser-treated and control eyes was examined with high-performance liquid chromatography (HPLC). For the evaluation of CNV activity, the intensity of fluorescein leakage from the photocoagulated lesions was scored, and the areas of CNV lesions were measured histologically in the control eyes and eyes treated with a subconjunctival injection of doxifluridine 14 days after photocoagulation.

RESULTS. The expression level of TP was higher in the laser-treated eyes than in the control eyes. Fluorescein leakage from the CNV lesions significantly decreased in the eyes given a subconjunctival injection of doxifluridine compared with the control. Histologic analysis demonstrated that both the areas of CNV lesions and the degree of vascular formation in the subretinal membrane were reduced in the doxifluridine-treated eyes compared with the control eyes.

CONCLUSIONS. TP may be involved in the formation of CNV. Subconjunctival injection of doxifluridine significantly reduced experimental CNV activity without apparent adverse effects. These results suggest the possibility that doxifluridine can be beneficial in treating CNV. (Invest Ophthalmol Vis Sci. 2005; 44:751–754) DOI:10.1167/iovs.02-0222

Exudative age-related macular degeneration (AMD), characterized by choroidal neovascularization (CNV), is a major cause of visual loss in developed countries. Because surgical therapy,2,3 as well as photocoagulation therapy,4–7 inevitably affects not only the CNV itself but also the healthy retina, development of treatments for CNV with minimal damage to the healthy retina, such as photodynamic therapy8 and transpupillary thermotherapy,9 is under way. In addition, compounds with antiangiogenic properties are under intensive study for possible clinical applications.10–12 To develop a pharmacologic therapy for CNV, it would be helpful if a drug already approved for clinical use could suppress the condition.

The cytostatic drug doxifluridine (5′-deoxy-5-fluorouridine; 5′-dFUr; Furtulon; Nippon Roche K. K., Kamakura, Japan) is an oral anticancer agent that generates 5′-fluorouracil (5′-FU) selectively in tumors.13,14 A study in vitro15 has demonstrated that doxifluridine is a potent inhibitor of microvesSEL formation and that the vasoclastic activity of the drug is enhanced by thymidine phosphorylase (TP), which is an essential enzyme for the conversion of doxifluridine to 5′-FU.15,14 TP is a major pyrimidine nucleoside phosphorylase that catalyzes the reversible phosphorylorysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphatase. Several lines of evidence suggest that TP is upregulated in the area where pathologic angiogenesis occurs. Studies in vivo have demonstrated that TP activity is increased in various human tumor tissues compared with normal adjacent tissues and that the expression level of thymidine phosphorylase in malignant tumor cells is associated with an increase in microvesSEL density in patients with ovarian cancer,16 non–small-cell lung cancer,17 gastric carcinoma,18 colon carcinoma,19 and tubulointerstitial injuries in scarred kidneys secondary to urinary tract diseases.20 Studies in vitro have demonstrated that the expression level of TP is upregulated by several inflammatory cytokines21 and hypoxia22 through a similar transcriptional regulatory mechanism to vascular endothelial growth factor (VEGF).23 TP itself is identical with platelet-derived endothelial growth factor.24 Recent studies in vitro and in vivo demonstrated that TP itself, different from the other PyNpase, uridine phosphorylase (UP), is an angiogenic factor.25 These results indicate that TP is likely to be involved in pathologic angiogenesis.

These observations raised the possibility that the expression of TP is upregulated in CNV. If so, it is possible that doxifluridine would suppress CNV. Thus, we analyzed the expression level of PyNpase and the effects of doxifluridine in a laser-induced CNV model in rats, to explore the possible functions of TP and doxifluridine in the development of CNV.

MATERIALS AND METHODS

Animals

Female Brown Norway (BN) rats weighing between 200 and 250 g were obtained from CLEA Japan (Tokyo, Japan). All experiments were conducted in accordance with the Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental CNV

General anesthesia was induced by an intraperitoneal injection of a 1000 μg/kg mixture (5:1) of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal; Bayer, Tokyo, Japan).
PyNpase in Laser-Treated Eyes

To examine the expression level of PyNpase in the laser-treated eyes, 100 applications of laser photocoagulation were delivered to the right eyes of 10 BN rats. Fourteen days after laser photocoagulation, the eyes were enucleated and immediately homogenized and centrifuged. The eyes were subjected to analysis of the expression of TP and UP. The expression level of the PyNpase was examined by high-performance liquid chromatography (HPLC), as previously described.55 The left eyes served as the non-laser-treated control.

Effect of Doxifluridine on Experimental CNV

To study the effects of doxifluridine on experimental CNV by fluorescein angiography (FA), six applications of laser photocoagulation were delivered to both eyes of 19 BN rats. Rats that underwent laser photocoagulation received 50 mg of doxifluridine diluted in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 4.5 mM NaHPO₄, and 1.5 mM KH₂PO₄, [pH 7.5]) per eye (n = 4) or PBS alone as a control (n = 7) by subconjunctival injection using a 30-gauge needle under a microscope (SZ1045; Olympus, Tokyo, Japan). When the drug or PBS was delivered subconjunctivally, it was administered only to the left eye, to examine whether the subconjunctival administration affects the activity for CNV of the contralateral eye by comparing the FA leakage between the PBS-injected eyes, doxifluridine-treated eyes, contralateral eyes, and control eyes from nontreated rats. The results revealed that subconjunctival doxifluridine exerts no significant effect on the CNV in contralateral eyes. In addition, PBS alone has no significant effect on CNV (data not shown). To examine the effect of oral doxifluridine, rats that underwent laser photocoagulation received 100 mg of doxifluridine orally in 1% methylcellulose (n = 4) or 1% methylcellulose alone as a control (n = 4) and both eyes were subjected to FA analysis. In both the subconjunctival and oral administration groups, each drug was administered just after the photocoagulation (on day 0) and given once daily thereafter.

Another round of photocoagulation was performed for the histologic analysis of the CNV lesion after doxifluridine administered just after the photocoagulation (on day 0) and given once daily thereafter. The CNV lesions were analyzed by quantitative morphometric analysis on day 14. As shown in Table 1, the size of the CNV lesions was reduced significantly in the doxifluridine-treated eyes compared with the control eyes (1.557 ± 0.234 mm² and 0.395 ± 0.059 mm² in the control and doxifluridine-treated groups, respectively; P = 0.045). Moreover, the doxifluridine-treated lesions contained fewer vascular channels and stromal cells (Fig. 2).

Histologic Analysis of CNV Lesions

On day 14, after the rats were killed with an overdose of pentobarbital sodium, the eyes were immediately enucleated and prepared for light microscopy by immersing them in PBS containing 4% paraformaldehyde for 12 hours. Eyes were then transferred into 70% ethanol and processed for paraffin embedding. Once embedded, 4.0-μm sections of the tissues were prepared for staining with hematoxylin and eosin. For measurements of CNV lesions, a lesion that exhibited its largest area in consecutive serial sections was chosen in each sample, similar to a previous study.26 To determine the size of the lesion, microscopic images were imported into a computer using a software system (Studio Lite; Immervision International, Marseilles, France), and area measurements of CNVs were performed on computer (Macintosh; Apple Computer, Cupertino, CA) using NIH Image (http://rsb.info.nih.gov/nih-image/provided in the public domain by the National Institutes of Health, Bethesda, MD) with the observer masked as to the doxifluridine treatment. In each group, 42 photocoagulated lesions from seven mice were analyzed.

TABLE 1. Expression of PyNpase in Experimental CNV

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<th>Control Eyes</th>
<th>Laser-Treated Eyes</th>
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<td>TP</td>
<td>1.258 ± 0.158</td>
<td>1.557 ± 0.234</td>
<td>0.0036</td>
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<tr>
<td>UP</td>
<td>0.37 ± 0.046</td>
<td>0.395 ± 0.059</td>
<td>0.3012</td>
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Data are the mean ± SEM (nmol/min per milligram protein). n = 10 in both groups.

RESULTS

With the use of a diode laser, under our experimental conditions, approximately 90% of photocoagulation sites reproducibly developed CNV, as characterized by leakage detected with FA and by histologic analysis 14 days after photocoagulation.

Expression Level of PyNpase in Laser-Treated Eyes

As shown in Table 1, the expression level of TP was significantly higher in the lasered eyes than in the control eyes (1.557 ± 0.234 nmol/min per milligram protein in the lasered eyes and 1.258 ± 0.158 nmol/min per milligram protein in the control eyes; P = 0.0036). In contrast, no significant change in the expression level of UP protein was observed between the lasered eyes and the control eyes (0.359 ± 0.058 nmol/min per milligram protein in the lasered eyes and 0.37 ± 0.045 nmol/min per milligram protein in the control eyes; P = 0.3012).

Effect of Oral or Subconjunctival Administration of Doxifluridine on Experimental CNV

Rats treated with subconjunctival injections of doxifluridine showed a significant decrease in leakage as judged by leakage score (Fig. 1A) compared with control rats, suggesting the subconjunctival administration of doxifluridine effectively suppresses leakage of CNV. When taken orally, doxifluridine also had a tendency to suppress leakage of CNV on day 14 (Fig. 1B), although the differences were not statistically significant. FA revealed no apparent abnormalities in normal retinal blood vessels (data not shown). No systemic adverse effect such as hair loss was observed.
Discussion

The pathogenesis of the laser-induced experimental CNV is different from that of natural CNV in humans in some respects. However, the essential processes (i.e., the breakup of the basement membrane, endothelial cell proliferation, and migration, and tubular formation) are similar. In our results the expression level of TP protein was upregulated in laser-treated eyes compared with that in the normal eyes. Although we measured the expression level in the laser-treated eyes and not in the CNVs directly, it is assumed that the upregulation of TP protein occurred in the retina and choroid, because diode-laser photocoagulation produced CNVs without any apparent change in other ocular structures. The upregulated expression of several inflammatory cytokines and the overexpression of growth factors, such as basic fibroblast growth factor (bFGF), is reported in human CNV as well as experimental CNV in rats. Considering that such inflammatory cytokines and growth factors are capable of inducing expression of the TP gene, the change in the expression of these cytokines in CNV might trigger upregulation of the expression of TP. Similarly, it is possible that hypoxia, which is also demonstrated to induce expression, may have upregulated expression, although further study is needed to determine whether hypoxia is relevant to CNV. Several lines of evidence suggest that TP promotes pathologic angiogenesis. Studies in vivo have demonstrated that TP, through the thymidine metabolite, 2-deoxy-D-ribose, enhances endothelial cell migration and proliferation. Studies in vivo have demonstrated that the expression of TP is upregulated in solid tumors in humans and the expression level of TP correlates with tumor vascularization in solid tumors. Our finding may support the idea that TP is also involved in the pathogenesis of CNV.

Our results demonstrate that doxifuridine inhibited development of CNV in vivo. A study in vitro demonstrated that doxifuridine reduced the size and number of microvessels in a three-dimensional model of angiogenesis in which cultured segments of rat aorta were used and that doxifuridine is

\[ \text{TABLE 2. The Size of CNV Lesions} \]

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<th>Control Eyes</th>
<th>Doxifuridine-Treated Eyes</th>
<th>( P )</th>
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<tr>
<td>CNV area (mm(^2))</td>
<td>0.0148 ± 0.0036</td>
<td>0.0051 ± 0.0007</td>
<td>0.0042</td>
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Data are the mean ± SEM. \( n = 7 \) in both groups.
potentially vasoclastic and its vasoclastic effect is enhanced by TP. Consistent with this, our results demonstrated that doxifluridine suppressed leakage from CNV as analyzed by FA and reduced the size of CNV lesions, with a concomitant increase in the expression level of TP. To our knowledge, this is the first report demonstrating the effect of doxifluridine to suppress pathologic neovascularization in vivo except for the neovascularization in solid tumors. Our results demonstrated the suppressive effect on CNV by subconjunctival, not oral, administration of doxifluridine, presumably because the drug concentration in the choroid was significantly higher in the subconjunctival group compared with the oral-intake group. On the contrary, it is likely that the serum concentration of this drug would be lower in the subconjunctival than oral intake group. In ophthalmic practice, 5'-FU is administered subconjunctivally after trabeculectomy. Because the cytotoxic effect of 5'-FU is not cell-type-selective, numerous reports of adverse effects of this adjuvant therapy have been documented, such as persistent corneal epithelial defect. Because doxifluridine is a produg of 5'-FU and TP is necessary to convert doxifluridine to 5'-FU, a cytotoxic effect of 5'-FU through administration of doxifluridine is exerted in the cells overexpressing TP, with minimal damage to normal cells. In fact, on histologic analysis and FA analysis, no abnormalities were detected in the retina outside the CNV lesions and no corneal damage was apparent (data not shown) after subconjunctival administration of doxifluridine. Because only local adverse effects such as persistent corneal defect have been reported in the literature when 5'-FU is administered subconjunctivally as adjuvant therapy of trabeculectomy, and no systemic adverse effect was seen in our experiments, we believe that doxifluridine would produce less severe side effects and could be safely used in treatment of CNV when administered subconjunctivally.

References