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Inhibition of protein kinase CK2 suppresses angiogenesis and hematopoietic stem cell recruitment to retinal neovascularization sites

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Abstract

Ubiquitous protein kinase CK2 participates in a variety of key cellular functions. We have explored CK2 involvement in angiogenesis. As shown previously, CK2 inhibition reduced endothelial cell proliferation, survival and migration, tube formation, and secondary sprouting on Matrigel. Intraperitoneally administered CK2 inhibitors significantly reduced preretinal neovascularization in a mouse model of proliferative retinopathy. In this model, CK2 inhibitors

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had an additive effect with somatostatin analog, octreotide, resulting in marked dose reduction for the drug to achieve the same effect. CK2 inhibitors may thus emerge as potent future drugs aimed at inhibiting pathological angiogenesis. Immunostaining of the retina revealed predominant CK2 expression in astrocytes. In human diabetic retinas, mRNA levels of all CK2 subunits decreased, consistent with increased apoptosis. Importantly, a specific CK2 inhibitor prevented recruitment of bone marrow-derived hematopoietic stem cells to areas of retinal neovascularization. This may provide a novel mechanism of action of CK2 inhibitors on newly forming vessels.

Keywords

CK2; Retina; Endothelial; Astrocyte; Diabetes; Neovascularization; Hematopoietic stem cell; TBB; TBCA; Emodin; DRB

Introduction

Protein kinase CK2 (CK2) is a ubiquitous serine/threonine protein kinase that phosphorylates more than 300 substrates in the cell and is involved in a variety of biological processes, including cell proliferation, migration, differentiation, apoptosis, circadian rhythms, and angiogenesis [1–7]. In cell culture, different chemical and antisense inhibitors of CK2 were shown to induce apoptosis of various cells [8–11]. In vivo, antisense oligonucleotide to CK2 α catalytic subunit was able to inhibit growth of xenografted tumors [3]. We have examined the involvement of CK2 in angiogenic process in vitro and in vivo. In addition, the effect of CK2 inhibition on endothelial precursor cell contribution to neovascularization was analyzed. The available evidence suggests that CK2 is intimately involved in angiogenic processes and that its inhibitors may emerge as promising anti-angiogenic therapeutics.

Methods

Human tissue

Diabetic and non-diabetic autopsy human eyes were purchased from the National Disease Research Interchange (NDRI, Philadelphia, PA). NDRI has a human tissue collection protocol approved by a managerial committee and subject to National Institutes of Health oversight.

Isolation and culture of bovine retinal microvascular endothelial cells

This was done as previously described [12, modified in 13,14]. Briefly, bovine retinal endothelial cells (BREC) were isolated from fresh bovine eyes (Sierra for Medical Science, Santa Fe Springs, CA). Retinas were dissected free, passed through sterile 45- μ m nylon mesh (Tetko Inc., San Antonio, TX), and washed with 50% fetal calf serum (FCS, Omega Scientific Inc., Tarzana, CA) in Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). The enriched retinal vessels were digested with collagenase (Worthington Biochemical Corp., Lakewood, NJ) for 30 min at room temperature in Ca⁺⁺/Mg⁺⁺-free PBS (Invitrogen). The digest was resuspended in 50% F-12/50% low-glucose DMEM with antibiotics/antimycotics (Invitrogen) and 10% FCS and centrifuged at 400g for 5 min. The pellet was resuspended in 50% F-12/50% low-glucose DMEM with antibiotics/antimycotics (Invitrogen) and 20% FCS, supplemented with ITS (insulin/transferrin/selenium), and ECGS (endothelial cell growth supplement), all from Sigma-Aldrich Co. (St. Louis, MO). After the first passage cells were cultured in the same medium but with 10% FCS. Experiments were done with BREC at passages 3–7. Cultures were checked for purity by immunostaining with a polyclonal antibody to von Willebrand factor (Sigma-Aldrich).

Wound migration assay

Cells in 24-well plates were grown to confluence and serum-starved overnight in medium with 0.5% FCS. Cultures were wounded with a single sterile wood stick of constant diameter [13]. Cells were rinsed with medium and incubated with combinations of human growth factors at 10 ng/ml each. The following growth factors (R&D Systems Inc., Minneapolis, MN) were used: insulin-like growth factor-I (IGF-I), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and placenta growth factor (PIGF). On day seven, cells were rinsed with PBS, fixed with methanol for 15 min, rehydrated with water, and stained with Meyer's hematoxylin for 5 min, followed by destaining with water. Some cells received CK2 inhibitors, emodin (1,3,8-trihydroxy-6-methylantr-aquinone; Sigma-Aldrich) or DRB (5,6-dichloro-1- β -*o*-ribofuranosyl benzimidazole; BIOMOL, Plymouth Meeting, PA). Cells were photographed with a 4 $\times$ or 10 $\times$ objective using a Kodak MDS 100 digital camera attached to a Leitz DM IL inverted microscope. Counting of migrating cells was done using special software as described [13–15].

Bone marrow isolation and HSC enrichment

Animals were treated in accordance with The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80–23), and the Association for Research in Vision and Ophthalmology “Statement for the Use of Animals in Ophthalmic and Vision Research.” All protocols were approved by the University of Florida's Institutional Animal Care and Use Committee. TgN(GFPU)5Nagy mice (The Jackson Laboratory, Bar Harbor, ME) are heterozygous for green fluorescent protein (gfp) expression under the control of chicken β -actin promoter with a cytomegalovirus (CMV) intermediate early enhancer. Mice were bred to obtain GFP homozygous mice (GFP+/+). Hematopoietic stem cell (HSC) sorting is accomplished based on the presence of the markers Sca-1 (stem cell antigen) and c-kit. Cells that were isolated from donor mice were GFP+, Sca-1+, and c-kit+.

To obtain HSC, GFP+/+ mice were anesthetized by intraperitoneal injection of xylazine (30 mg/ml) and ketamine (14 mg/ml) mixture at 5 μ l per 20 g of body weight, sacrificed by cervical dislocation, and their bone marrow isolated. Tibias and fibulas were removed and placed on ice. Both ends of the bones were removed, flushed with ice-cold PBS, and cells were collected by centrifugation. Cells were incubated with Sca-1-phycoerythrin antibody (Sca-1-PE, Beckton Dickinson Pharmingen, San Diego, CA) and c-kit-allophycocyanate antibody (c-kit-APC, Beckton Dickinson Pharmingen) at 4°C for 15 min to allow the antibodies to react. Then PBS was added to controls (no antibody) and samples, and the tubes were centrifuged. The supernatant was discarded and cell pellets were resuspended in PBS and filtered using polystyrene tubes with cell strainer caps (Becton Dickinson). The bone marrow cell population was then enriched for HSC by fluorescence-activated cell sorting (FACS). Only the cells that had both antibodies bound to them were selected and placed in PBS with 30% FCS on ice until needed. The cells were then centrifuged and resuspended in a volume adequate to yield 10,000 cells per 1 μ l of sterile PBS for intraocular injection.

Proliferative oxygen-induced retinopathy (OIR)

Proliferative oxygen-induced retinopathy (OIR) was induced in heterozygous C57BL/6J neonatal mice by a standard protocol [16]. Briefly, 7-day-old mouse pups and their moms were kept for five days in hyperoxia (75% oxygen), which arrested retinal vascular development. Pups and their moms were brought to room air for 5 days, their retinas became hypoxic under these conditions and neovascularization occurred and peaked at post-natal (P) day P17.

CK2 inhibition in vivo

For neovascularization inhibition studies, 12-day-old C57BL mouse pups were removed from hyperoxic chamber (see above) and were treated with inhibitor or vehicle. For HSC engraftment studies, 12-day-old pups received one intravitreal injection into both eyes of 5,000–10,000 Sca-1+, c-kit+, gfp+ HSC. On days 12–17, all pups received intraperitoneal injections of CK2 inhibitors or vehicle twice daily. Either emodin, DRB, 3,3',4',5,7-pentahydroxyflavone (quercetin; Sigma-Aldrich), 4,5,6,7-tetrabromobenzotriazole (TBBt), 4,5,6,7-tetrabromobenzimidazole (TBBz) [17]), dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT/K25), or tetrabromocinnamic acid (TBCA; EMD Biosciences, San Diego, CA) were used at 10–100 mg/kg/day. Control mice received the same volume of vehicle (20% PEG 400 +2% Tween-80 in PBS, pH 7.4). On day 17, mice were euthanized, eyes enucleated, and incubated in 3% para-formaldehyde for 60 min and then in PBS for at least 30 min. Eyes were embedded in paraffin, sections (10–30 per eye) were cut on a microtome and preretinal nuclei (as a measure of preretinal neovascularization) counted after hematoxylin-eosin staining. In recent experiments, neural retinas dissected from the posterior cups were incubated overnight at 4°C in 0.2% Triton X-100 and 0.2% BSA in HEPES-buffered saline with 0.1% rhodamine-conjugated *Ricinus communis* agglutinin (Vector Laboratories, Burlingame, CA) to reveal blood vessels (with or without 0.02% anti-gfp-FITC to reveal HSC). They were washed for 24 h at 4°C in HEPES-buffered saline, flat-mounted with four to seven radial cuts, and examined by confocal microscopy. With this method, neovascular tufts are seen as discrete spots of hyperfluorescence and as more diffuse fluorescent regions. The neovascular tufts were counted and their total number was determined in both the mid-peripheral and peripheral regions of all retinas. The central retinas were not analyzed due to potential interference from remnants of the hyaloid vasculature. This method may provide a more objective measure of neovascularization than counting preretinal nuclei on sections because it allows detecting all neovascular tufts.

Western blot analysis of retinal lysates, indirect immunofluorescence, and quantitative real-time RT-PCR

Western blot analysis of retinal lysates, indirect immunofluorescence, and quantitative real-time RT-PCR were all performed exactly as described previously [18], using the same antibodies and primers. For quantitative RT-PCR four to five samples were pooled in the normal and diabetic retinopathy groups, whereas individual samples were analyzed on Western blots.

Statistical analysis

In vitro data were analyzed for two groups by the paired Student's *t*-test, and for several groups, by non-parametric one-way ANOVA test using GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA). In animal studies, data from various groups were compared using one-way ANOVA with Bonferroni post-test.

Results

Effects of CK2 inhibitors on cultured endothelial cells

The action of several CK2 inhibitors, quercetin, apigenin, chrysin, and the more specific ones, DRB and emodin [15], was tested in cultures of low-passage BRECs. All CK2 inhibitors effectively decreased BREC tube formation when added at the time of seeding, but stabilized the preformed tubes 24 h after cell seeding on Matrigel. The agents also inhibited secondary sprouting on Matrigel [13], cell proliferation in 10% FCS, and reduced cell viability in 0.5% FCS, consistent with anti-apoptotic functions of CK2. We have previously shown that BREC migration into the wound in 0.5% serum was highly and

synergistically stimulated by a combination of four angiogenic growth factors (VEGF, FGF-2, IGF-I, and PlGF) [13]. Both DRB and emodin significantly inhibited growth-factor-induced BREC migration (Fig. 1; [15]). All inhibitors produced similar concentration-dependent effects, with apigenin, DRB, and emodin being the most active on a molar basis (10–25 μ M).

Reduction of retinal neovascularization by CK2 inhibitors in vivo

We next tested whether CK2 inhibition would block retinal neovascularization in a well-established model of proliferative oxygen-induced retinopathy (OIR) in the mouse. Retinal vasculature development continues after birth in the mouse and artificial hyperoxia (5 days in 75% oxygen) stops this process. When mice are brought back to room air (21% oxygen) from hyperoxia, the retinas become severely hypoxic and ischemic and respond in 5 days by a florid compensatory neovascularization [16].

Intraperitoneal administration of several CK2 inhibitors (quercetin, DRB, emodin, and TBBt) over a period of 5–6 days after hyperoxia significantly reduced the extent of preretinal neovascularization (Fig. 2; [15]). The effective doses ranged from 10 to 100 mg/kg body weight/day. Surprisingly, a very potent and safe CK2 inhibitor, DMAT/K25 [19,20], did not exert an effect on neovascularization at 40 mg/kg/day with intraperitoneal administration (data not shown). Recent experiments performed with 100 mg/kg body weight/day TBBz and TBCA confirmed that both inhibitors were able to reduce both mid-peripheral and peripheral retinal neovascularization similar to TBBt (Fig. 3). TBCA was somewhat more potent than TBBt. Because of very high TBCA specificity towards CK2 [21], the observed effects were likely due to CK2 block rather than inhibition of other kinases [19,21,22].

Combined effects of CK2 inhibitors and octreotide on retinal neovascularization

Angiogenesis appears to be a multifactorial process requiring involvement and interactions of many growth factors and their respective signaling pathways (Fig. 4; [23]). Therefore, it is possible that a combination of various anti-angiogenic agents would provide a greater effect than each agent alone. This hypothesis was tested using CK2 inhibitors, emodin and TBBt, and a somatostatin analog, octreotide [18]. CK2 and somatostatin have some similarities in signaling pathways that they affect but also some differences that could be expected to result in additive or even synergistic effect. Specifically, CK2 modulates Raf-ERK-S6K, p38 MAPK, and Akt pathways, whereas octreotide can modulate p27, STAT3, and PKA signaling (Fig. 4).

Octreotide reduced preretinal neovascularization by 67% at the dose of 5 mg/kg/day (Fig. 5a). At 1 mg/kg/day octreotide produced 50% inhibition, and emodin at 30 mg/kg/day showed 57% inhibition. When 1 mg/kg/day octreotide was combined with 30 mg/kg/day emodin, the same inhibitory effect was observed as with 5 mg/kg/day octreotide (Fig. 5a). Combination of 60 mg/kg/day TBBt and 1 mg/kg/day octreotide produced 61% inhibition, significantly higher than TBBt (46%) or octreotide alone (Fig. 5a). All inhibitors reduced preretinal pathological neovascularization without noticeable effect on main vascular tree (Figs. 2, 5b; [18]). Therefore, different anti-angiogenic compounds may exert an increased reduction of neovascularization when applied in combination, which may be important for future clinical applications.

Retinal expression of CK2

Using quantitative RT-PCR and immunohistochemistry with a panel of antibodies, it was shown that all three CK2 subunits were present in the mouse and human retina. Surprisingly, despite the presence of CK2 in every cell, only astrocytes of the inner retina stained positive

[18]. Mild treatment of sections with 0.05% SDS revealed the staining of retinal vessels as well for all three subunits (Fig. 6). However, the monoclonal antibody 1AD9 used in most human experiments [18] failed to stain endothelial cells even after epitope unmasking (data not shown). Other retinal cell types in normal and diabetic human retinas remained negative.

Retina in advanced diabetes is typically hypoxic with increased apoptosis [24], suggestive of reduced CK2 expression and/or activity. Therefore, it was tested whether CK2 expression was changed in diabetic retinas. By quantitative RT-PCR, mRNA levels of all three subunits were downregulated in retinas from patients with diabetic retinopathy (Fig. 7a). CK2 α subunit was the most affected, with about 65% reduction in mRNA level compared to non-diabetic retinas. However, semi-quantitative Western blots normalized for β -tubulin failed to reveal the same change of CK2 α at the protein level (Fig. 7b). It could be attributed to a known post-translational increase in stability of some mRNAs in hypoxic conditions [25]. The preferential astrocytic immunolocalization of CK2 also did not change in human diabetic retinas [17].

Effects of CK2 inhibition on HSC engraftment into retinal neovasculture

In the mouse OIR model, intravitreally injected HSC were incorporated to the sites of neovascularization as revealed by the co-localization of gfp+ cells with some of the rhodamine-agglutinin-stained retinal neovascular tufts (Fig. 8). The retinal neovessel formation and HSC incorporation were suppressed by a specific CK2 inhibitor, TBBt (Fig. 9). TBBt did not abolish neovascular formation completely (Fig. 10) but the remaining tufts were devoid of gfp+ HSC. TBBt administration did not result in HSC emigration from the vitreous/retina and return to the bone marrow, because, by flow cytometry using Sca-1, c-kit, and gfp markers, only negligible amounts of positive cells were found in the bone marrow of either vehicle or TBBt-treated mice (data not shown).

Discussion

Protein kinase CK2 is a multifunctional regulatory molecule that participates in a wide variety of cellular events by phosphorylating and/or interacting with key signaling molecules, structural proteins, and transcription factors [1,7]. Angiogenesis is a complex phenomenon generally thought to be mediated by distinct growth factors and their combinations [23,26–28]. In recent years it was found that several key elements of angiogenic growth factor signaling cascades were substrates for CK2 (Fig. 4). Therefore, CK2 could be an important mediator of angiogenesis.

Our tissue culture studies have shown that many endothelial cell responses important for the angiogenic process could be significantly downregulated by CK2 inhibitors. This prompted further analysis of CK2 involvement in angiogenesis using an *in vivo* model of retinal neovascularization [15]. Indeed, a number of intraperitoneally administered CK2 inhibitors significantly reduced retinal neovascularization in a well-established model of proliferative retinopathy. It should be noted, that the specificity of these inhibitors is of some concern. Most of them including quercetin, emodin, DRB, TBBt, and TBBz can act not only on CK2 but also on other kinases. The last two inhibitors are potent blockers of DYRK1A kinase beside CK2 [19,21,22]. However, TBCA seems to be a really selective CK2 inhibitor that does not block DYRK1A [21]. Because its activity against retinal neovascularization was even slightly higher than that of TBBt, the observed effect of CK2 inhibitors on retinal angiogenesis was most likely due to blocking the activity of CK2 but not of other kinases including DYRK1A.

Many agents and their combinations are being tested for inhibition of pathological angiogenesis and some of them are in clinical trials or have been already approved for

human use [18,29–33]. We have obtained proof of principle that a combination of a CK2 inhibitor with another anti-angiogenic compound, octreotide, would inhibit retinal neovascularization stronger than any of these agents alone. Choosing a combination of inhibitors that would affect differing pathways could provide a more pronounced effect [30]. At the same time CK2, as a “master regulator” of many critical signaling pathways in angiogenesis, may be a key component to target to fight unwanted angiogenesis. Small molecule or antisense drugs based on CK2 inhibition may be promising future therapeutics of cancer and proliferative retinopathies [3,6,18,34].

The results presented here also show that bone marrow-derived HSC locally injected into the vitreous of neonatal mice can incorporate into the retinal neovasculature in the OIR model. Systemically administered potent CK2 inhibitor, TBBt, significantly reduced this incorporation. Therefore, interfering with HSC recruitment during angiogenesis may be an important novel mechanism of action of CK2 inhibitors. Possibly, other anti-angiogenic drugs may act by this mechanism as well.

There may be several explanations of the molecular events in this inhibitory process. First, it is known that CK2 inhibition can cause cell apoptosis [2,3,35]; interestingly, fast dividing tumor cells were significantly more susceptible to the action of this inhibition than normal cells [3]. Therefore, apoptosis by CK2 inhibition may also be more pronounced in proliferating cells such as HSC compared to slowly proliferating or non-proliferating cells. This could also explain preferential affect of CK2 inhibitors on newly forming vessels as compared to established vasculature.

Another possibility is the action of CK2 on cell migration. Movement of progenitor cells to sites of injury is thought to be necessary for their repair of the damaged tissue. We have shown that CK2 inhibition blocks growth-factor-induced migration of cultured retinal endothelial cells [15]. Our data on cultured human astrocytes suggest that CK2 may be closely associated with the cytoskeleton including GFAP-containing intermediate filaments [16]. Possible CK2-mediated phosphorylation of cytoskeletal proteins in retinal astrocytes and/or endothelial cells may cause changes in cell shape and motility. It is thus possible that CK2 may promote endothelial and HSC migration. Its inhibition by chemical agents would block endothelial migration during neovascularization as well as HSC migration into the retina and their participation in retinal angiogenesis in the OIR model. Further experiments are needed to verify these hypotheses.

It should be mentioned that the experiments described here concern HSC that represent early stem cells capable of differentiating into many cell types. It is possible, that sites of vascular injury or neovascularization would attract not only HSC but also (and may be primarily) endothelial progenitor cells (EPC) that would be committed to endothelial differentiation. Experiments are underway to compare HSC with the more differentiated EPC in the same setting in terms of the magnitude of incorporation into retinal neovessels and the effects of anti-angiogenic drugs on this process.

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Fig. 1.

Effect of CK2 inhibitors on growth factor-induced BREC migration. Confluent BREC were wounded and cultured for 7 days in 0.5% serum-containing medium with IGF-I + FGF-2 + VEGF + PlGF (four GFs) at 10 ng/ml each $\pm$ CK2 inhibitors, emodin (10 μ M) and DRB (15 μ M). Cell migration into the wound was counted using the AAB software. Error bars correspond to mean $\pm$ standard error of mean of at least three individual experiments. * $P < 0.005$ of CK2 inhibitor versus four GFs. Both inhibitors significantly reduced cell migration. Modified from [15]

Fig. 2.

CK2 inhibitors reduce retinal neovascularization in the OIR mouse model. Upper left pair, fluorescein-dextran perfusion of retinas from mice treated intraperitoneally with solvent (vehicle) or inhibitor (TBBt). Note reduction of brightly fluorescent vessel tufts (arrows) after TBBt treatment; upper right pair, hematoxylin-eosin-stained sections of retinas from mice treated with vehicle or TBBt. Arrow, neovascular tuft in vehicle-treated retinas; after treatment, the retina appears normal. Bar = 30 μ m; lower graph, counts of preretinal nuclei in various groups of mice. Neovascularization is significantly decreased in all inhibitor-treated groups. Quercetin was used at 10 or 100 mg/kg/day with similar results. Emodin and DRB were used at 30 or 60 mg/kg/day with somewhat stronger inhibition at a higher dose, and TBBt, at 60 mg/kg/day. *N*, number of animals in a group. Five to ten sections per eye from each mouse were counted. * $P < 0.001$ versus untreated or vehicle. Error bars correspond to mean $\pm$ standard error of mean of at least three individual experiments. Adapted with modifications from [15]

Fig. 3.

Effects of specific CK2 inhibitors on retinal neovascularization. Following the OIR model, retinal flatmounts were stained with endothelium-specific rhodamine-conjugated *Ricinus communis* agglutinin and neovascular tufts were counted. The counts in the inhibitor-treated retinas are shown as percentage of those in vehicle-treated retinas taken as 100%. All three inhibitors, TBBt, TBBz, and TBCA, significantly reduced the number of neovascular tufts at 100 mg/kg/day. *N*, number of eyes in a group. * $P < 0.01$ versus vehicle. Error bars correspond to mean $\pm$ standard deviation

Fig. 4. Schematic of intracellular signaling from angiogenic growth factors, somatostatin, insulin, and ECM. Somatostatin (SST; octreotide is its analog) and CK2 modulate several major signaling pathways involving growth factor receptor tyrosine kinases, G-protein coupled receptors (SSTR), and extracellular matrix (ECM) receptors, integrins. Along with many similarities in the action of these major effectors, there are notable differences. CK2 modulates Raf-ERK-S6K, p38 MAPK, and Akt pathways, whereas, octreotide can influence p27, STAT3, and PKA signaling. Together, SST/octreotide and CK2 can influence most major signaling pathways. This makes them good candidates for combination therapy for abnormal retinal angiogenesis

Fig. 5.

Combination therapy for retinal neovascularization. (a) Octreotide reduced preretinal neovascularization by 67% at 5 mg/kg/day and by 50% at 1 mg/kg/day. Emodin at 30 mg/kg/day showed 57% inhibition. When 1 mg/kg/day octreotide was combined with 30 mg/kg/day emodin, the same inhibitory effect was observed as with 5 mg/kg/day octreotide (69%). Combination of 30 mg/kg/day TBBt with 1 mg/kg/day octreotide produced 61% inhibition compared to 46% by TBBt alone. Five mice were used per each group in three independent experiments. Counts of preretinal nuclei as a measure of neovascularization in various groups of mice are shown. Vehicle represents emodin solvent because octreotide solvent was just PBS. Error bars correspond to mean $\pm$ standard deviation. * $P < 0.001$ versus vehicle, ** $P < 0.05$ versus single drug or vehicle. Combination of emodin or TBBt with octreotide more efficiently reduces mouse retinal neovascularization than any single drug alone; (b) representative cryostat sections of control and treated retinas. Immunofluorescent staining was performed with anti-perlecan (green color) to reveal the blood vessels and inner limiting membrane (ILM; located at the boundary of the retina and vitreous body), with counterstaining of nuclei with DAPI (blue color). Prominent preretinal vessels in the vehicle group (arrowheads) disappear after the treatments but much less change is seen in intraretinal vasculature. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar = 60 μ m. Adapted with modifications from [18] with permission from the American Society for Investigative Pathology

Fig. 6.

Positive CK2 staining of retinal vessels after epitope unmasking. (a) Double staining of human retinal section for CK2 β (red color) and perlecan (green color). There is co-distribution of both labels in retinal capillaries (right panel, yellow color). In this particular small area astrocytes that are also positive for CK2 were virtually absent. ILM, inner limiting membrane. (b) Staining of serial human retinal sections for different subunits of CK2. A large vessel is positive (red color) along with astrocytes in the inner retina. CK2 α and β subunits give identical staining, but α' subunit has less apical and more basal staining in the vessel wall. Nuclei were counterstained with DAPI (blue color)

Fig. 7.

Expression of CK2 subunits in normal and diabetic human retina. (a) Real-time RT-PCR of different CK2 subunits. Note decreased levels of all messages in the diabetic retinopathy (DR) group compared to age-matched non-diabetic group (N). β -micro-globulin was used as a normalizing housekeeping gene [18]. (b) Western blot of individual retinal samples including seven non-diabetic (N) and seven with diabetic retinopathy (DR). The membrane was probed with anti-CK2 α antibody. Lane loading was normalized by β -tubulin. There are no significant differences in the amount of CK2 α between diabetic and non-diabetic samples. Markers in kD are at the left

Fig. 8. HSC incorporation into sites of retinal neovascularization in the mouse OIR model. (a and b) Mouse retinal flat mount double stained with rhodamine-agglutinin (Rh-agglutinin) to reveal blood vessels and anti-gfp to reveal gfp+ c-kit+, Sca-1+ HSC (arrowheads) injected intravitreally. Arrows point to neovascular tufts in the peripheral retina. Bar = 100 μ m; (c) superimposition of pictures in a and b at higher magnification. Several neovascular tufts (arrows) contain gfp + HSC. These appear in yellow color. Bar = 50 μ m

Fig. 9.

Effect of TBBt on HSC incorporation into mouse retinal neovasculature. Vehicle, double-stained retinal flat mount of vehicle-treated retina. Note several neovascular tufts revealed by rhodamine-agglutinin; two of them contain gfp+ HSC (arrow); TBBt, no tufts and no gfp + HSC are visible after TBBt treatment (5 days at 100 mg/kg/day). Bar = 50 µm

Fig. 10.

Lack of HSC incorporation into neovasculature upon TBBt treatment. Vehicle, vehicle-treated retina at high magnification. A rhodamine-agglutinin positive (red color) neovascular tuft (arrow) contains several GFP+ HSC (green-yellow color); TBBt, two neovascular tufts (arrows) remaining after TBBt treatment are shown; they are both devoid of GFP+ HSC. Bar = 20 μm