Novel therapeutic modalities for the treatment of ophthalmic diseases

Ph.D. Thesis

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LIST OF PUBLICATIONS

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In extenso cikkek és absztraktok összesített impakt faktora: 7,489

ABBREVIATIONS

Bach 1	leucin β-zipper transcription protein
cDNA	c deoxyribonucleic acid
CO	carbon monoxide
COHb	carbonmonoxy-hemoglobin A
HBP 23	heme-binding protein, also termed peroxiredoxin (Prx) I or heme oxygenase 1 (HO-1)
HIV	human immunodeficiency virus
HO	heme oxygenase
ICAM	intercellular adhesion molecule-1
iNOS	inhibition of purified macrophage nitric-oxide synthase
MARE	Maf-recognition element
NO	nitric oxide
Nrf2	nuclear factor erythroid 2-related factor 2
TNF	tumor necrosis factor
sGC	soluble guanylyl cyclase
VCAM-1	vascular cell adhesion molecule
VSV	*vesicular stomatitis virus*

INTRODUCTION

An understanding of the molecular mechanisms implicated in diseases of the eye could could permit an outline of new therapeutic strategies and it is therefore now a topic of great interest in ophthalmology.

A large body of experimental evidence has shown that cellular death is involved in various pathologic processes affecting the eye, including glaucoma, retinitis pigmentosa, ischemic retinopathy, corneal and conjunctival reparative processes, cataract and retinoblastoma.

It has also been revealed that both extracellular and intracellular stresses can induce apoptotosis or non-apoptotic cell death, including necrosis, autophagy, a mitotic catastrophe, senescence and anoikis. The pathogenic mechanisms of various diseases may involve unique combinations of the different forms of cell death.

Apoptotic cell demise is a mode of cellular death genetically programmed to maintain the homeostasis of tissues (1-14). Depending on the origin of the death-inducing signal, the biochemical steps of apoptosis can be mediated by intrinsic and extrinsic pathways. The apoptotic response can be divided into three phases: *(i)* initiation, *(ii)* effector phase and *(iii)* degradation. In the eye, apoptosis seems to play a role starting from embryogenesis to diseases of all its components. In specific pathologic circumstances, the death program may be activated by various environmental factors, such as exposure to toxic substances or bacteria or deprivation of nutrients. From this point of view, apoptosis can be considered the final event in several pathologies. Mostly studied in the retina, apoptosis is also extensively involved in the anterior segment, and especially the ocular surface. Any epithelial aggression stimulates a series of mechanisms leading to the death of deep keratocytes. The conjunctiva is a major site at which inflammation and apoptosis are combined. Apoptosis plays a pivotal role in the pathogenic mechanisms of several other ophthalmic diseases.

Medical interventions inhibiting the apoptotic pathways may provide useful therapeutic modalities for the effective treatment of these conditions, such as *ischemic retinopathy.* The identification new compounds that can protect against multiple inducers of cellular death is likely to have the greatest therapeutic potential. In contrast, the apoptosis induction exploited by some novel therapeutic agents may be a powerful alternative modality for the treatment of ophthalmic malignancies, such as *squamous conjunctival cell carcinoma.*

I. MODULATING THE APOPTOTIC PATHWAYS IN ISCHEMIC RETINOPATHY

I.1. Arterial obstructive disease of the eye

Central retinal obstruction, one of the most sudden and dramatic events seen by the ophthalmologist, was described as early as 1859. While there have been numerous clinical and experimental studies of the pathophysiology of central retinal artery obstruction in recent years, and while these studies were augmented by the introduction of intravenous fluorescein angiography in the 1960s, the disease still remains one that carries a relatively poor visual prognosis (15).

The clinical picture is typically striking. Patients present with a sudden painless loss of vision. The appearance of a cherry-red spot in the fundus is characteristic. The cherry-red spot appears because, soon after obstruction of the blood flow to the inner retina, the normally transparent retina becomes opaque and blocks the brown-red color from the underlying choroid, which is still supplied by blood. However, as the retina overlying the foveola is relatively thin, the normal color of the choroid is still visible in this area. While characteristic, the cherry-red spot is not pathognomonic for a central retina artery obstruction. Sometimes the characteristic cherry-red spot does not develop and there may be only a slight accentuation of the brown-red color in the foveola. It is not known how long it takes for this cherry-red spot to emerge, but in a primate model it has appeared as early as 30 minutes after obstruction.

Intravenous fluorescein angiography is very useful in revealing the details of the abnormal circulation of a central retinal artery obstruction. The principal abnormality is the delay in the appearance of the dye in the central retinal artery and its branches.

In about 10% of eyes with a central retinal artery obstruction, there are abnormal choroidal filling defects reflecting a posterior circulation obstruction. These defects occur even in patients who appear to have a typical central retinal artery obstruction.

The electroretinogram in a subject with a central retinal artery obstruction is generally abnormal. The electro-oculogram may be either normal or abnormal. Visual field defects are usually profound, but occasionally a small portion of the temporal peripheral visual field will remain or the patient will be left with a large central scotoma.

The onset of obstruction occurs most often between midnight and 6 a.m., with the second most common period being between 6 a.m. and noon.

Most patients with a central retinal artery obstruction complain of a sudden loss of visual acuity in the affected eye. The majority of such patients present with a visual acuity ranging from finger-counting to light perception. Only about 5% of patients have a visual acuity of no light perception, and most of these patients display involvement of the posterior ciliary circulation. With or without treatment, most eyes retain a visual acuity of finger-counting to light perception although about 35% have vision of 20/200 or better, and about 20% have vision better than 20/40. Patients with a spared cilioretinal artery do not seem to have a follow-up visual acuity that is significantly different from those without a spared cilioretinal artery (15).

Central retinal artery obstruction is due to the disruption or cessation of blood flow to the central retinal artery, its branches and the retinal layers supplied by those vessels. The most common site of obstruction of the central retinal artery is at the level of the lamina cribrosa.

The most clinically relevant finding of experimental studies is that there is a period of time in which a total lack of blood flow (ischemia) to the inner retina can be tolerated; that is, if the obstruction does not extend beyond a certain period, visual function may return to normal. This critical time in the cat eye is 90 minutes, and in the rhesus monkey it is 100 minutes. After this lapse time of total obstruction, the histologic changes are irreversible and the animal suffers permanent visual loss.

On average, a central retinal artery obstruction occurs in patients who are in the fifth and the sixth decades of life, with a reported age range varying between 17 and 84 years. Fewer than 10% of patients will be younger than 30 years of age.

The cause of a central retinal artery obstruction is in some cases relatively clear, such as when it is due to a visible embolus. However, in other cases, especially when there is an association with a systemic disorder such as diabetes, the association is less clear. Arteriosclerosis is probably the most commonly associated systemic condition. The next most common systemic condition associated with a central retinal artery obstruction is probably arterial hypertension, which can be found in as many as two-thirds of all patients with a central retinal artery obstruction. Carotid artery disease has been found in about 45% of patients with a central retinal artery obstruction and is probably the third most commonly associated condition. Hemodynamically significant stenosis occurs in about 20% of patients. Diabetes mellitus and valvular heart disease are probably the next two most commonly associated conditions (15).

The etiology of a central retinal artery obstruction depends to some extent on the age of the patient presenting with an obstruction. A central retinal artery obstruction in patients 30 years of age or younger tends to be associated with migraine, coagulant disorders, intraocular abnormalities, and trauma. Systemic diseases such as systemic arterial hypertension, atherosclerotic disease and diabetes mellitus are not found in this younger age group.

I.1.1. Treatment

The results of the treatment of a central retinal artery obstruction are generally unsatisfactory. The treatment goal is always to restore the retinal blood flow as soon as possible. Lowering the intraocular pressure is one of the principal methods of treatment because, by lowering the intraocular pressure, the weak pressure load may perfuse the retina or dislodge an embolus.

Paracentesis may be the fastest way to achieve this aim. A reduction of the intraocular pressure can also be achieved through the use of intravenous acetazolamide or topical ß-blockers. Some investigators have advocated continuation of the acetazolamide for up to 2 weeks.

A variety of gases have been suggested for use in the treatment of a central retinal artery obstruction. These gases include oxygen, either delivered at standard atmospheric pressure or hyperbaric oxygen, and a mixture of 95% oxygen and 5% carbon dioxide. Theoretically, it may be possible to supply enough oxygen to the outer retina of a patient with a central retinal artery obstruction by delivering 100% oxygen at atmospheric pressure. However, it has been found that the administration of 100% oxygen can lead to vasoconstriction and a decrease in blood flow. The rationale for the addition of carbon dioxide to the oxygen is that carbon dioxide leads to vasodilation and the combination might allow delivery of the oxygen to the retina without vasoconstriction (15).

For the treatment and prevention of ocular diseases, and particularly those that involve the loss of nerve cells, *flavonoids* have multiple properties that are potentially of benefit.

I.2. Flavonoids

Flavonoids are polyphenolic substances with a flavan nucleus consisting of 15 carbon atoms arranged in three rings (C6–C3–C6), which generally occur as glycosylated derivatives (17).

Only plants and micro-organisms are capable of the biological synthesis of the aromatic nucleus, which is the basic structure of plant phenolic compounds. Six subclasses of

flavonoids are known, depending on the to positions of the hydroxy groups: flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanidins.

A large number of antimicrobial substances, called phytoalexins (including flavonoids), that are found in nature, make up a variable group of compounds that play important roles in the natural defense of certain organisms. The bioavailability of different polyphenols can vary greatly. The plasma concentrations of flavonoids with the best bioavailability profiles are about 5 μmol/l, whereas the corresponding values for polyphenols with poor bioavailability profiles (monomeric flavonols, flavones and flavanols) are usually less than 1 μmol/l.

However, it is possible that some phenolic compounds accumulate inside specific target tissues causing a higher local concentration than the plasma concentration. As the elimination of polyphenols from the body is generally quite fast, they must be consumed daily in order to maintain high concentrations.

The effects of flavonols and flavones on the enzymes regulating cell division and proliferation, platelet aggregation, detoxification and the inflammatory and immune responses are well documented. Phenolic compounds can interfere with the initiation and promotion of tumor growth, and some of the molecular mechanisms responsible for their cancer-prevention properties. A number of naturally occurring and also synthetic flavonoids, have potent anti-HIV activity *in vitro*. Quercetin, a widely studied flavonoid, is reported to prevent lung cancer. Due to their phytoestrogenic properties, flavonoids and other plant phenolic compounds are able to prevent menopausal symptoms, osteoporosis, breast and ovarian cancer and heart disease, and epidemiological studies have actually demonstrated a beneficial role of flavonoids as regards the risk of coronary heart disease. Recently, the polyphenols in tea have been shown to inactivate *Chlamydia pneumoniae in vitro*, and the antichlamydial activity of at least one flavonoid (luteolin) has also been demonstrated *in vivo*. In the past, the rather poor bioavailability of phenolic compounds has complicated their antichlamydial efficacy studies in humans, since it is not easy to obtain adequate amounts of these compounds from the normal diet.

A number of physiologic benefits that have been attributed to flavonoids are thought to stem from their potent antioxidant and free radical-scavenging properties (18). Flavonoids have been shown to reduce the growth of malignant carcinomas and to inhibit tumor angiogenesis, and are believed to be responsible for the low rates of cancer and atherosclerosis in people who consume soy-based diets.

Flavonoids can protect central nervous system-derived nerve cells from oxidative stess-induced death, which has been linked to the nerve cell loss seen in multiple ocular

diseases. Several flavonoids that are particularly effective at protecting the retinal ganglion cells from oxidative stress-induced death are found in relatively high quantities in specific fruits and vegetables. Studies with human volunteers have demonstrated that a single meal of fried onions can significally increase the plasma levels of quercetin, which is then slowly eliminated over the next 17 hours. Thus, the repeated intake of onions and other foods containing quercetin would lead to a build-up in the plasma concentration. In cattle, quercetin has also been observed to accumulate in the retina. These results suggest that the consumption of a diet rich in these fruits and vegetables could have beneficial effects on the eye in pathologic conditions and in normal aging (18).

The results of research toward the long-term goal of identifying a neuroprotective compound that could be used clinically for the treatment of multiple retinal disorders indicate that specific flavonoids can protect retinal ganglion cells from oxidative stress-induced death with high levels of potency and low toxicity. Furthermore, as compared with traditional antioxidants, flavonoids have unique advantages that make them especially attractive for clinical use. In particular, some flavonoids induce the expression of antioxidant proteins that can help protect cells from oxidative stress, thereby potentially providing long-term protection of the eye (18).

Several studies have shown that certain flavonoids can induce the activity and expression of phase-2 detoxification proteins. The phase-2 detoxification proteins include enzymes associated with glutathione biosynthesis and metabolism and redox-sensitive proteins such as *heme-oxygenase 1 (HO-1)*. The transcriptional activation of these and other genes encoding phase-2 detoxification proteins is mediated by a *cis*-acting enhancer termed the antioxidant response element. By inducing the expression of antioxidant defense enzymes, these flavonoids have the potential to exert long-lasting effects on the cellular function. This, in turn, could be highly beneficial to cells exposed to chronic oxidative stress. Moreover, flavonoids have been demonstrated to induce neurite outgrowth, reduce inflammation and inhibit endothelial cell proliferation, all properties which could be of additional benefit in the treatment of ocular diseases. Finally, in animal studies, flavonoids have generally displayed low levels of toxicity over a wide range of doses (18-19).

I.3. Biological functions of heme oxygenases

I.3.1. Production of carbon monoxide, biliverdin/bilirubin, and ferrous iron

HO forms complexes with NADPH-dependent flavoprotein reductase (cytochrome P450 reductase) and biliverdin reductase (a cytosolic enzyme) on the endoplasmic reticulum. In the presence of functional HO, the porphyrin ring of heme (ferroprotoporphyrin IX) is broken and oxidized at the α-methene bridge, producing equimolar amounts of CO, ferrous iron and biliverdin. The heme-CO metabolism pathway also requires the participation of NADPH and O_2. The presence of O_2 is therefore required for HO activity, but even during severe hypoxia HO continues to produce CO. Cytochrome P450 reductase transfers electrons to the HO-heme complex. The process of endogenous CO production displays a wide color spectrum. Black heme breaks down to green biliverdin and colorless CO. Biliverdin complexes with iron until its final release. Yellowish bilirubin is generated from biliverdin, catalyzed by biliverdin reductase (20).

Biliverdin and bilirubin are believed to be the most powerful endogenous antioxidants, efficiently scavenging peroxy radicals and inhibiting lipid peroxidation. Bilirubin protects cells from a 10,000-fold excess of hydrogen peroxide (H_2O_2). The overproduction of bilirubin leads to jaundice, a clinical syndrome of hyperbilirubinemia. Besides its antioxidant capability, biliverdin is also an endogenous inhibitor of sGC. In the micromolar range, biliverdin, but not bilirubin, inhibits the basal and NO-stimulated activity of the recombinant α(1)β(1) isoform of sGC without affecting the affinity of sGC for GTP.

Heme catabolism-generated ferrous iron stimulates the synthesis of ferritin through its regulatory protein binding and activation of iron response elements. Ferritin, an intracellular iron repository, allows the safe sequestration of unbound iron liberated from heme degradation. In this way, ferritin possesses additional antioxidant capabilities. It has been estimated that each apo-ferritin molecule (450 kD) can sequester about 4500 iron atoms. The modulation of intracellular iron stores and an increased efflux of uncomplexed iron has recently been suggested as a mechanism for the cytoprotective effects of HO-1. In this activity, HO-1 cooperates with a recently identified Fe-ATP pump. Once HO-1 has been knocked out, iron accumulates in the cells. HO-1 knockout mice exhibit hepatosplenomegaly, lymphadenopathy and leukocytosis. The massive iron overload in the liver and kidneys in these mice leads to death at a young age. Iron accumulation in the absence of HO-1 makes these animals much more susceptible to oxidative stress damage. The transfection of cells

derived from HO-1 knockout animals with HO-1 cDNA restores the normal cellular iron levels in these cells. Iron accumulation has also been documented in the liver and kidney of human HO-deficiency case. Finally, a feedback mechanism between iron and HO-1 exists. Iron has been shown to regulate the transcriptional expression of HO-1 and iNOS.

I.3.2. Heme metabolism

Regulation of the cellular heme level is another important function of HO. After completing their life cycle of 120 days, red blood cells release hemoglobin into the circulation. Haptoglobin captures free hemoglobin and transports it to the reticuloendothelial system in the spleen, liver and bone marrow. The rapid transformation of hemoglobin to methemoglobin also occurs, leading to the release of the incorporated heme. The free heme is then carried by hemopexin or albumin to the reticuloendothelial system, where HO functions as the rate-limiting enzyme in further heme degradation. The HO activity in different types of cells is also largely responsible for the degradation of heme derived from denatured heme proteins other than hemoglobin (20).

Heme consists of ferrous iron complexed with four porphyrin groups. The organic synthesis of heme was achieved as early as 1927 in the laboratory of Hans Fischer. The full elucidation of all of the enzymes involved in heme synthesis in mammalian cells was not available until about 25 years later. We now know that heme is synthesized in all human nucleated cells through the use of glycine and succinyl CoA as the precursors. Involving 8 different enzymes, heme synthesis starts in the mitochondria, continues in the cytosol, and is completed in the mitochondria. The iron is eventually inserted by ferrochelatase. Heme can also be derived from the degradation of hemoproteins. This recycling process would be energy-efficient without the need to start over again from glycine and succinyl-CoA.

The turnover of heme is rapid. For instance, cultured cerebellar granule cells consume 17% of the total heme pool to produce about 1 to 5 μmol CO in 5 hours. The physiological level of free heme in normal cells is below 1 μmol. At this concentration range, free heme down-regulates δ-aminolevulinate synthase and reduces the expression of Bach1. The latter lifts the inhibition of HO-1 gene expression.

Extracellular heme is transported into the cells via a heme transporter. Due to its lipophilic nature, heme readily moves around among different organelles and interacts with many cellular membranes and organelles, including lipid bilayers, mitochondria, the

cytoskeleton, nuclei and several intracellular enzymes. Free heme at high concentrations catalyzes oxidative reactions to generate reactive oxygen species, mainly due to the catalytic effect of heme. This would explain the increased expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin on endothelial cells in the presence of high levels of heme. The pro-oxidant effect of free heme is not due to the release of iron from heme molecule since heme induces more peroxidation of rat liver microsomal lipids in the presence of H_2O_2 than iron and iron release is very low under the conditions employed. Increased oxidative stress affects a variety of substrates, including lipids, thiols, proteins, carbohydrates, and nucleic acids. Normal cellular functions would be disturbed and pathological cellular injuries exacerbated. An abnormally high heme level is correlated with many diseases, such as nephrotoxin-induced renal injury. The elevated circulatory heme level results either from excessive filtration of heme proteins, as would occur in rhabdomyolysis, or from the destabilization of intracellular heme proteins (e.g. cytochromes) in ischemia-reperfusion and nephrotoxin-induced renal injury. When the level of free heme exceeds the physiological range, its cytotoxic role predominates over its constitutive role in heme protein formation. Heme-responsive genes remain repressed when heme at low concentrations binds to Bach1 with MARE sequences. However, at higher concentrations, heme inactivates the binding of Bach1, allowing the access of transcription factors such as Nrf2 to interact with the MARE sequences. This in turn activates the heme-responsive gene (20).

I.3.3. Heme-containing protein and heme-binding protein

Most mammalian cells contain a "free" heme pool, i.e. notprotein-bound heme, providing heme for the synthesis of heme-containing proteins and for CO production. As the prosthetic moiety for heme proteins, the availability of heme influences the metabolism of hemoglobin, myoglobin, cytochromes, prostaglandin endoperoxide synthase, NOS, catalase, peroxidases, respiratory burst oxidase, guanylyl cyclase, tryptophan dioxygenase, pyrrolases and many others. These heme proteins play important roles in regulating cellular functions, from oxygen delivery and mitochondrial respiration to signal transduction. The heme proteins include cyclooxygenase isoforms that need the heme prosthetic group for their catalytic activity (20).

The function of HO is linked to another class of proteins, i.e. heme-binding proteins. These proteins, without heme in their molecular structure, have marked affinities for it. As such, they regulate the availability of heme for the catalytic activity of HO and CO

production. Examples include HBP23, and the glutathione *S*-transferases. Hemopexin, which has the highest affinity for circulatory heme of all heme-binding proteins, completely inhibits heme-catalyzed lipid peroxidation at concentrations slightly higher than that of heme, suggesting a unique role for this acute phase protein in antioxidant defense mechanisms. The protein itself is not oxidized, presumably because the putative bis-histidyl heme-hemopexin complex cannot interact with H_2O_2. Rat and human albumin and rat glutathione *S*-transferases, proteins with moderate affinities for heme, decrease heme-catalyzed lipid peroxidation in a dose-dependent manner, but are subject to oxidation. Bovine albumin and rat liver fatty acid-binding protein have lower affinities for heme. These proteins enhance, instead of inhibiting, lipid peroxidation. In short, heme-binding proteins may enhance, decrease, or completely inhibit heme-catalyzed oxidations, and in doing so the proteins themselves may be oxidized, depending upon their relative affinities for heme, the nature of the amino acids in the vicinity of the bound catalyst, and the availability of a free coordination site on the iron (20).

I.4. Carbon monoxide

At temperatures above –190 °C, CO is a colorless and odorless gas. Its specific gravity is 0.967 relative to air, and its density at standard temperature and pressure is 1.25 g/l. CO is a chemically stable molecule. Chemical reduction of CO requires temperatures well above 100 °C. The aqueous solubility of CO at standard temperature and pressure is very low (354 ml/dl; 44.3 ppm by mass). CO cannot react with water without a substantial energy input. Even for molecular oxygen (O_2), the reaction rate of CO is slow and needs a high activation energy (213 kJ/mol). Theoretically speaking, CO can be involved in redox reactions. Free CO does not readily react with reducing agents, including hydrogen. Coordinated CO has a greater reactivity that than of the free gas, and the reduction of CO can be greatly facilitated by transition metals. Once formed, metal carbonyls are relatively stable until the CO is displaced, e.g. by molecular oxygen ($O_{2)}$.

I.4.1. Endogenous Production of carbon monoxide

As long ago as the 1850s, the French physiologist Claude Bernard recognized that the reversible binding of CO with hemoglobin was a potent chemical reaction that could cause asphyxia (Bernard, 1857). A later study, in 1895, revealed the antagonistic effect of a high partial pressure of O_2 on CO binding to hemoglobin. The first indication of endogenous CO production was discovered by Saint-Martin and Nicloux in 1898. In 1930 Warburg reported,

that CO could inhibit respiration in yeast in a light-sensitive manner, extending the original discovery that COHb could be dissociated by exposure to light of appropriate wavelengths. These pioneer studies initiated a century of investigations of the biological actions of CO. In the early 1950s, Sjöstrand provided the first actual experimental evidence for the existence of CO in the human body. He observed that the decomposition of hemoglobin *in vivo* produces CO. The endogenous production of CO can be regulated. Increased heme levels after erythrocyte destruction increase endogenous CO production, as reflected by the elevated COHb level. When the heme metabolism is abnormally increased, as in hemolysis, the rate of production of CO in the body can increase tremendously. In the late 1960s, Tenhunen and colleagues ascribed the driving force for the endogenous source of CO production to heme oxygenase (HO). The inducible HO isoform, HO-1, was identified in 1974 in two different laboratories. In 1986 the Maines laboratory identified the constitutive HO isoform, HO-2, from rat liver microsomes. About 10 years later, the same laboratory identified the third HO isoform, HO-3 (20).

The rate of production of CO in the human body is 16.4 μmol/h, and the daily production of CO is substantial, reaching more than 12 ml (500 μmol). The average physiological concentrations of CO in tissues are rather low, in the nanomolar range if based on normal levels of COHb of 1 to 2%. However, the accuracy of this estimation is questionable, since CO generated in living cells would first be scavenged in the cytosol before being released into the bloodstream where COHb is formed. Previous studies have the shown poor correlation of COHb levels the with biological changes induced by CO and the residual effects of CO after COHb elimination.

I.4.2 Physiological functions of carbon monoxide

While investigations on the endogenous production of CO and its regulation had progressed continuously, especially as regards the expression and biological implications of HO, the questions of how the body uses, or why it needs, endogenous CO were not answered by the conventional understanding of this gas as an endogenous waste or by-product of the heme metabolism. It was not until little more than a decade ago that an appreciation of the biological and physiological functions of endogenous CO, and the role of CO in the beneficial effects of HO, was made. The break-through discovery of HO opened the way to further research on membrane/receptor-independent signaling gas molecules. In 1991, Marks and colleagues predicted that there was a metabolic reason and a physiological meaning for the

production of CO in the body. This pioneering reasoning stimulated the resurgence of CO as a physiological signaling molecule. Two years later, the research team of Snyder provided the first comprehensive evidence for the role of CO as an endogenous neural messenger, based on the effects of HO inhibitors and the histological location of HO (20).

The relaxation of the pulmonary vasculature induced by exogenous CO under normoxic conditions was reported as early as the late 1970s. However, cardiovascular researchers struggled for many years to find evidence for the vasoactive effect of endogenous CO. The inhibition of HO activity with zinc protoporphyrin-IX (ZnPP) provided the evidence that ZnPP treatment reduces endogenous CO generation and increases the vascular resistance in the rat liver. Other laboratories at the same time argued that the vascular effect of ZnPP might not be related to the inhibition of HO. Ensuing studies in which a HO substrate was applied and/or the expression of HO-1 was upregulated, confirmed the vasorelaxant effect of endogenous CO.

The identification of the physiological role of endogenous CO was greatly facilitated by the use of gene knockout or gene overexpression techniques. The first HO-2 null mutant mouse was produced in 1995. Poss and Tonegawa (1997) first generated mice deficient in HO-1 by targeted deletion of a 3.7-kb region including exons 3 and 4 and a portion of exon 5 of the mouse HO-1 gene. The direct relevance of the HO/CO pathway to human health was indicated by the first reported human case of HO-1 deficiency in Japan in 1999. This HO-1-deficient patient died at the age of 6, showing growth retardation, anemia, thrombocytosis, hyperlipidemia, leukocytosis, elevated serum levels of ferritin and heme, and lower serum levels of bilirubin (20).

The effects of the HO/CO system on apoptosis have already been studied extensively. The overexpression of HO-1 exerts either a pro-apoptotic or an anti-apoptotic effect, depending on the histological type of cells used. HO-1 stimulates an apoptotic response of smooth muscle cells that involves the action of transcription factor p53. In contrast, HO-1 overexpression inhibites the apoptotic cell death of several other cell types, including endothelial cells, epithelial cells and fibroblasts. A number of studies have shown that CO modulates apoptosis in a cell type- and inducer-specific manner. CO stimulates apoptosis in endothelial cells and thymocytes, while smooth muscle cells, pancreatic β cells and hepatocytes are protected from death in its presence. It has been revealed that, in Jurkat cells, the Fas/CD95-induced extrinsic death pathway is augmented by CO, while CO inhibites the

apoptotic events elicited by TNF-α or etoposide. Thus, the HO/CO system has the capability to regulate the apoptotic response in a highly intricate way. However, further experimental work is required to determine the *in vivo* role of the HO/CO-mediated modulation of apoptosis in ophthalmic diseases and to elucidate the underlying molecular mechanisms.

I.4.3. Heme-dependent and -independent endogenous production of carbon monoxide

Upon the action of hydrogen peroxide (H_2O_2) or ascorbic acid, heme methylene bridges can be broken and CO released. Cytochrome P450 can be inactivated by free NADPH oxidation, NADPH-dependent monooxygenase reactions and lipid peroxidation. This self-inactivation also leads to breakage of the bond between heme and apoenzyme and to heme degradation.

The majority of CO in the body is produced by the enzymatic heme metabolism. This metabolism is catalyzed by HO, mainly occurring in the reticuloendothelial system of the spleen and the liver. There are three isoforms of HO. HO-1 is the inducible isoform. An increased cellular stress level is one common denominator for most of the stimuli to upregulate the *de novo* transcription of HO-1. HO-2 is constitutively expressed in many mammalian cells. HO-3 is also a constitutive isoform of HO. One theory suggests that HO-3 may be derived from the retrotransposition of the HO-2 gene since the HO-3 gene does not contain introns (20).

In summary, the levels and activities of heme, HO, and CO are closely interrelated. HO-2 may function as a physiological regulator of cellular functions via control of the size of the free heme pool and the provision of physiologically important heme metabolites. The elevated heme level itself presents a pro-oxidant threat. The cell copes with this threat by upregulating HO-1 expression. The latter degrades heme to increase the production of CO. The role of HO-3 in heme degradation is very limited. It may function as a heme-sensing or heme-binding protein. Thus, an appropriate heme level is reached and cellular homeostasis is maintained.

II. TRIGGERING THE APOPTOTIC RESPONSE OF MALIGNANT CELLS BY AN ONCOLYTIC VIRUS

In spite of the use of a wide array of diagnostic and therapeutic approaches, tumors continue to pose a serious problem in ophthalmology. Deregulated and impaired apoptotic

pathways have shown shown to play pivotal roles in tumor transformation, progression and metastasis, as well as in the emergence of multidrug resistance during cancer therapy. The use of viruses, endowed with apoptogenic capabilities to destroy and to eliminate malignant cells offers a novel method for the more effective treatment of tumors and thereby extends the therapeutic arsenal of ophthalmologists.

II.1 .Epithelial tumors of the eye

The following definitions are necessary for an understanding of epithelial tumors (16):

Pseudocancerous lesions: lesions that may resemble cancer clinically or microscopically, but that do not develop into cancer (pseudo-epitheliomatous hyperplasia, keratoacanthoma).

Cancerous lesions: lesions that have the capacity to invade and destroy adjacent tissue and to metastasize.

Acanthosis: a thickening of the squamous layer of the epithelium.

Keratosis: abnormal keratinization of the epithelium.

Hyperkeratosis: a thickening of the keratin layer (since keratinization does not normally occur in the conjunctiva, any keratin formation is hyperkeratosis).

Parakeratosis: the retention of nuclei in the keratin layer.

Dyskeratosis: premature keratinization of individual epithelial cells before they reach the surface.

Papillomatosis: a papillary configuration of acanthotic epithelium permeated by fibrovascular stromal cords.

Leukoplakia: a clinical term for a white plaque-like lesion on the mucous membrane (several benign and malignant lesions can have this clinical appearance).

Bowen's disease: a specific form of an *in situ* carcinoma of the skin (this term should not be applied to lesions on mucous membranes such as the conjunctiva).

Carcinoma in situ: a lesion that has all the histologic characteristics of malignancy except the invasion of the underlying stroma (if these lesions are left untreated, many will become invasive).

Dysplasia or atypical epithelial hyperplasia: a lesion in which there is a disturbance in the normal maturation of the surface epithelium, associated with some criterion of malignancy (cellular atypism and loss of polarity); however, these abnormalities do not involve the entire thickness of the epithelium; some such lesions in the conjunctiva may develop into malignant neoplasms.

Conjunctival intraepithelial neoplasia: a term used to classify both benign (conjunctival dysplasia or atypical epithelial hyperplasia) and malignant (carcinoma *in situ*) *in situ* neoplasms. Since both clinical and histologic differentiation between these benign and malignant *in situ* neoplasms may be extremely difficult, and since the prognosis and treatment of both benign and malignant tumors are similar, this classification is acceptable.

II.1.1 .Squamous cell carcinoma

*Squamous cell carcinoma*s and sebaceous cell carcinomas are common conjunctival tumors. Basal cell carcinomas do not arise in the conjunctiva; rarely, a basal cell carcinoma from the skin of the eyelid extends into the conjunctiva, particularly if an adjacent lid transfer reconstructive procedure is performed after incomplete excision of a basal cell tumor.

Carcinoma *in situ* and invasive carcinomas of the conjunctiva occur most frequently at the limbus in the area of exposed conjunctiva between the eyelids; they may also occur on the palpebral conjunctiva and as isolated lesions in the cornea. Most have a gelatinous surface, but some are covered with keratin, producing a leukoplakic lesion. Usually, fibrovascular cores permeate the lesion, giving it a papillomatous appearance, and invasion is slow unless the tumor is the rare spindle cell (pseudosarcomatous) or mucoepidermoid variety. Metastases and death from conjunctival carcinomas are very rare. Recurrences are common (16).

II.1.1.1. Treatment

Complete surgical excision is the treatment of choice; even large lesions can be locally excised, but those involving over 50% of the limbus and cornea heal poorly. The use of autologous limbal transplants can rehabilitate some of these eyes. Tumors that involve the cornea usually do not extend through Bowman's layer and therefore they can be "peeled" off the cornea. It is important to preserve Bowman's layer since this layer seems to act as a barrier to stromal invasion by recurrent lesions. The use of cryotherapy after surgical excision may help to decrease the incidence of recurrences. Recurrences should be locally excised; more extensive surgery is seldom necessary.

II.2. Virotherapy

The use of viruses as oncolytic agents has recently emerged as a viable and useful antitumor strategy. Oncolytic viruses have the ability to kill cancer cells directly and the potential to stimulate the production of cytokines with anticancer activity. Further, the ability to alter virus vectors enetically provides the opportunity to tailor these viruses to specific aspects of tumor development or tumor biology. A variety of different viruses have been

tested as oncolytic agents, including DNA viruses such as adenovirus and herpes simplex virus, and RNA viruses such as Newcastle disease virus, reovirus, influenza virus and ***vesicular stomatitis virus (VSV)***. One or more of these agents may have the potential for the treatment tumors with extensive hypoxic areas that are resistant to other therapies.

Previous studies have shown that VSV can infect and kill a diverse set of cancer cells. The advantages of this virus are that oncolytic strains do not appear to be pathogenic in humans, replicate entirely in the cytoplasm, and have no known transforming abilities. VSV has been shown to infect and kill cancer cells while sparing nontransformed cells in tissue culture because of defects in the antiviral responses in the tumor cells. This oncolytic behavior has also been demonstrated *in vivo*, where VSV injection affects the growth of tumors in both xenograft and syngeneic tumor models following intratumoral or systemic administration.

II.2.1. Rhabdoviridae-Vesicular stomatitis virus

Viruses of the family *Rhabdoviridae* (in the order *Mononegavirales*) are perhaps more widely distributed in nature than those of any other virus family. Rhabdoviruses infect vertebrates and invertebrates, as well as many species of plants. The rhabdoviruses that cause rabies and economically important diseases of fish appear to have life cycles confined to vertebrate species. All other rhabdoviruses are thought to be transmitted to vertebrates and plants by infected arthropods, which may be the original hosts from which all rhabdoviruses evolved.

The viruses of the family *Rhabdoviridae,* known to infect mammals including humans, have been classified into two genera: the *Vesiculovirus* genus stemming from VSV and the *Lyssavirus* genus, otherwise known as the rabies and rabies-like viruses.

Among the animal rhabdoviruses, many of those belonging in the genus V*esiculovirus* infect insects, and perhaps other arthropods, but it is uncertain whether they transmit infection to vertebrates; however, identical VSV-New Jersey viruses were recovered from black flies and diseased horses during the 1982 epizootic of vesicular stomatitis in Colorado. VSV, which can be divided into two antigenetically distinct serotypes called VSV-Indiana and VSV-New Jersey, appears to infect insects and mammals. These viruses generally cause nonfatal disease of significant economic importance in cattle and swine. Rare vesicular stomatitis infections in humans have been observed and result in influenza-like symptoms. These infections have occurred in the laboratory or after exposure to infected animal carcasses.

II.2.1.1. Virion and genome structure

A helical nucleocapsid can be seen in the bullet-shaped VSV particles. The particle size and shape, 180 nm long and 75 nm wide, are typical of all rhabdoviruses except certain plant virions, which are bacilliform in shape and almost twice the length.

Rhabdovirus virions are all composed of two major structural components: a nucleocapsid or ribonucleoprotein (RNP) core and an envelope in the form of a lipoprotein bilayer membrane closely surrounding the RNP core. The virion RNP core in the infectious form of VSV is tightly wound into 35 coils. Extending from the outer surface of the envelope is an array of spike-like projections.

The infectious component of VSV and all rhabdoviruses is the RNP core. VSV serves as an excellent experimental system for studying viral replication and cytopathology because of the relative simplicity of its structure, genetics and physiology. The VSV-Indiana genome is an unsegmented single strand of RNA containing 11,161 nucleotides. The genomic RNA is the negative-sense strand and therefore requires its own endogenous RNA polymerase for transcription of plus sense messenger RNA. The RNA genome is tightly encased by the nucleocapsid (N) protein, consisting of 422 amino acids, to form the RNP core. Also associated with the RNP core are two minor proteins, L (for large) and P (for phosphoprotein, originally designated NS); collectively the L and P proteins, in association with the core N protein, serve as the viral transcriptase. The L-protein gene represents 60% of the coding potential of the VSV genome, whereas the P gene codes for a protein of 222 amino acids. The P protein is phosphorylated to varying degrees, the most highly phosphorylated forms apparently having the greatest potential for supporting transcription. Using dark-field scanning transmission electron microscopy, Thoma et al. calculated the length of the VSV-Indiana nucleocapsid to be 3.3 to 3.7 mm, containing 3.7 megadaltons of RNA. When it contains full-length genomic RNA and the full complement of the three RNP proteins, the VSV nucleocapsid alone is infectious at an efficiency of 10^{-5} to 10^{-6} that of the membrane-enclosed complete virion.

AIMS

I. To evaluate our hypothesis that a reduction in HO-1 mRNA expression may change the endogenous CO production in the ischemic/reperfused retina.

If this is the case, HO-1-regulated endogenous CO production and its vasodilatator or cytoprotective activity may play a role in the control of reperfusion-induced retinal damage. The overall objectives of the present investigations were to study:

1. the formation of endogenous CO levels by using gas chromatography,
2. the role of HO-1 protein expression and endogenous CO production on reperfusion,
3. the effects of a flavonoid-rich extract obtained from the seed of sour cherry on HO-1 protein expression, enzyme activity, endogenous tissue CO formation and tissue Na^+, K^+ and Ca^{2+} contents in the ischemic/reperfused retina.

II. To investigate the susceptibility of the immortalized WK conjunctival cell line to vesicular stomatitis virus and to analyze the role of apoptosis in the VSV-mediated induction of cell death.

The potential oncolytic activity of VSV has previously not been evaluated in epithelial tumors of the conjunctiva.

The overall objectives of the present investigations were to study:

1. the replication of VSV in the WK conjunctival cell line,
2. the mechanism of its cytopathic effect,
3. the underlying molecular events implicated in the apoptotic properties of this virus.

MATERIALS AND METHODS

I. In the investigation of heme-oxygenase-1 related carbon monoxide and flavonoids in ischemic/reperfused rat retina

Experimental animals Rats were handled and received humane care in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Guidelines. The institutional review board of the University of Debrecen, Hungary, approved the research.

Measurement of tissue CO content Retinal CO content was measured using a gas chromatograph. The headspace gas from each vial was injected into the gas chromatograph using a gastight syringe in argon gas flow with a speed of 20 ml/min. Analysis took place during the next 90 seconds on a 240 cm stainless-steel column with a 0.3 cm inner diameter.

Values were expressed in mV, than peak's areas were integrated and expressed in arbitrary units. The column was packed with Chromosorb 80/100 mesh and maintained at 120 °C. The temperature of the injector was controlled and kept at 150 °C.

Western blot analysis Detached retinas were homogenized in Tris-HCl , glycerol , SDS , and β-mercaptoethanol. The same amount of soluble protein was fractionated by Tris-glycine-SDS-polyacrylamide gel electrophoresis, and Western blot was carried out with the use of an antibody to recombinant rat HO-1 protein . The relative HO-1 protein expression was analyzed by densitometry.

Assay of HO activity At the end of reperfusion, retinas were homogenized in phosphate buffer and centrifuged. The supernatant was removed and recentrifuged and the precipitated fraction was suspended in potassium phosphate buffer. Reaction mixtures consisted of (final volume 2 ml) potassium phosphate (pH 7.4), hemin, bovine serum albumin, biliverdin reductase, and microsomal fraction of retina. The reaction was allowed to proceed in dark in a shaking water bath and was stopped by placing the test on ice. Incubation mixtures were then scanned using a scanning spectrophotometer, and the amount of bilirubin was calculated as the difference between absorbance at 464 nm and 530 nm. Protein content was determined in the microsomal fraction.

Measurement of retinal Na^+, K^+, and Ca^{2+} contents Immediately, after the washing out period, the eye was enucleated and rapidly opened, and the retina was progressively detached from the retinal epithelium and finally dissociated by cutting from the optic nerve. Retinal tissue was dried for 48 hours at 100 °C and then placed at 550 °C for 24 hours to make ash. The ash was dissolved in nitric acid and diluted . Tissue Na^+ was measured at a wavelength of 330.3 nm, K^+ at 404.4 nm, and Ca^{2+} at 422.7 nm in an air acetylene flame by Perkin-Elmer atomic absorption spectrophotometer. Since a small amount of extracellular ions can contaminate the samples after washing out the extacellular space, the values obtained in our studies are termed retinal rather than intracellular ion contents.

Histology One hundred milliliters of the fixative solution (Bouin's solutions) followed immediately without any interruption of the NaCl perfusion. Then eyes were enucleated and rapidly cut open and divided into two halves by coronal section through the ora serrata. The vitreous was removed and the eye was immersed into fixative solution (Bouin's fluid). After postfixation, the tissue was dehydrated in graded series of ethanol and embedded in paraffin. Sagittal sections of 7 μm were cut and stained with hematoxyliun-eosin. Ischemia/reperfusion-induced cell swelling is well recognized and documented in the inner

plexiform layer of the retina. The average of the retina's thickness for each eye was measured in the sagittal section at near the optic nerve, and expressed in μm using a video-plan computer analyzer as we previously described. Migration of neutrophils was observed after 90 min ischemia followed by 24 hours of reperfusion.

II.In the investigation of apoptosis induced by vesicular stomatitis virus in the Wong-Kilbourne derivative of the Chang conjunctival cell line

Cell culture The Wong–Kilbourne derivative of the Chang conjunctival cell line (WK), obtained from the American Type Culture Collection (ATCC), was grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 7.5% fetal calf serum.

Virus The Indiana strain of VSV was propagated at a multiplicity of infection (MOI) of 0.001 plaque forming unit (PFU) per cell in L929 cell cultures for 3 days at 37 °C. Virus plaque assay was performed on confluent monolayers of Vero cells.

Methods used to detect the hallmarks of apoptosis

Quantitation of apoptosis by ELISA Cells were washed in PBS and the cell pellet was processed in a cell death-detection ELISA kit based on the detection of histones complexed with mono- and oligonucleosome fragments formed during cell death. This system allows the specific detection of DNA and histone proteins in the cytoplasmic fraction. Cells were incubated in lysis buffer for 30 min at room temperature and centrifuged at 12,000 rpm for 10 min, and the supernatants were tested by ELISA. The supernatants were transferred into a streptavidin-coated microplate and incubated with biotin-conjugated anti-histone and peroxidase-conjugated anti-DNA monoclonal antibodies for 2 h at RT. After washing, substrate solution (ABTS) was added to each well for 15 min. Absorbance was measured at 405 and 490 nm. The specific enrichment of mono- and oligonucleosomes was calculated as absorbance of VSV-infected cells/absorbance of corresponding non-infected control cells=enrichment factor (EF).

Terminal deoxynucleotidyltransferase (TdT)-mediated digoxigenin-dUTP end labelling (TUNEL) assay Cytospin cell preparations were fixed in 4% paraformaldehyde for 30 min and treated with proteinase K for 15 min at room temperature. TdT enzyme was applied to incorporate FITC -dUTP at sites of DNA breaks for 1 h at 37 °C. Nuclear fluorescence of stained cells was visualized by confocal microscopy. Apoptotic cells labeled with FITC display green fluorescence.

Methods used to identify proteins

Indirect immunfluorescence assay Cytospin cell preparations were incubated with a VSV G protein-specific MAb. After washing, samples were reacted with FITC-conjugated anti-mouse antibody. The slides were visualized by confocal microscopy. The ratio of positive to negative cells was determined after counting 1,000 cells in random fields.

Western blot assay Cells were homogenized in ice-cold lysis buffer and the mixture was then centrifuged. Protein concentrations of cell lysates were determined by using the Bio-Rad protein assay. Supernatants were mixed with Laemmli's sample buffer and boiled for 3 min. Aliquots of total protein were resolved by SDS-PAGE and electrotransferred onto nitrocellulose filters. Preblocked blots were reacted with specific antibodies to Bcl-2 and Bax. Blots were then incubated with species-specific secondary antibodies coupled to peroxidase. Filters were were developed by using a chemiluminescence detection system. The autoradiographs were scanned, and the relative band intensities were quantified by use of the ImageQuant software.

RESULTS

I. Investigation of heme-oxygenase-1 related carbon monoxide and flavonoids in ischemic/reperfused rat retina

Figure I/1 shows the sagittal section of rat retina in nonischemic control eye (Fig. I/1A), in retina subjected to 90 minutes ischemia followed by 24 reperfusion without treatment (Fig. I/1B), in ischemic/reperfused (90 minutes/24 hours) retina treated with 10 (Fig. I/1C) and 30 (Fig. I/1D) mg/kg sour cherry seed extract for 14 days, respectively.

In the drug-free ischemic/reperfused retina (Fig. I/1B), we observed many changes by using hematoxylin–eosin staining in the inner nuclear layer. There were more pyknotic, vacuolated spaces and degenerative changes in ganglion cells. These changes were markedly reduced in retinas subjected to ischemia/reperfusion and treated with 10 (Fig. I/1C) and 30 (Fig. I/1D) mg/kg sour cherry flavonoid–rich seed extract.

Figure I/2 shows the thickness (in micrometers), as an indicator of edema formation, of the retinal plexiform layer in eyes subjected to 90 minutes of ischemia followed by 24 hours of reperfusion in drug-free and drug-treated groups. Thus, a significant reduction in the thickness of the inner plexiform layer was observed in the ischemic/reperfused retina obtained from rats treated with 10 and 30 mg/kg of sour cherry flavonoid–rich extract, respectively

(Fig. I/2). The migration of neutrophils was reduced by 75% in the groups treated with 30 mg/kg sour cherry flavonoid–rich extract.

Figure I/3 shows representative chromatograms of CO peaks recorded (Fig. I/3 , chromatogram A) in the nonischemic retina; and after 90 minutes of ischemia followed by 24 hours of reperfusion (Fig. I/3, chromatogram E) in the drug-free retina; and in retina from rats treated with (Fig. I/3, chromatogram B) 30 mg/kg, (Fig. I/3, chromatogram C) 10 mg/kg, or (Fig. I/3, chromatogram D) 5 mg/kg flavonoid-rich sour cherry seed extract. Thus, the results clearly show that detectable endogenous CO production by GC can be observed in aerobically perfused rat retina (Fig. I/3, chromatogram A) and this CO peak was substantially reduced in ischemic/reperfused drug-free retina (Fig. I/3, chromatogram E). However, in the ischemic/reperfused retina, a moderate increase in endogenous CO production was measured in rats treated with 30, 10, or 5 /kg sour cherry flavonoid–rich extract, respectively, returning toward the concentration observed in the Figure I/3, chromatorgam A.

Insets in the Figure I/3 show the corresponding integrated and calculated peak areas (n = 5 in each group) of CO curves expressed in arbitrary units in nonischemic control (Fig. I/3A) retinas, in retinas obtained from rats treated with 30 mg/kg (Fig. I/3B; *P<0.05), 10 mg/kg (Fig. I/3C; *P<0.05), or 5 mg/kg sour cherry flavonoid–rich extract, respectively, and subjected to 90 minutes of ischemia followed by 24 hours of reperfusion.

Insets in the Figure I/3 show the expression of HO-1 protein in nonischemic aerobic control retina (Fig. I/3 , lane A), in ischemic/reperfused drug-free control retina (Fig. I/3, lane E), in ischemic/reperfused retina obtained from rats treated with 30 (Fig. I/3, lane B), 10 (Fig. I/3, lane C), or 5 (Fig. I/3, lane D) mg/kg flavonoid-rich extract. Thus, the downregulation in HO-1 protein expression (approximately 10-fold) was observed in ischemic/reperfused retina (Fig. I/3, lane E) in comparison with the aerobically perfused nonischemic control retina (Fig. I/3, lane A). In rats treated with 5, 10, or 30 mg/kg sour cherry seed flavonoid–rich extract and retinas were subjected to 90 minutes ischemia followed by 24 hours of reperfusion, the expression of HO-1 mRNA was increased approximately 1.5-, 5-fold, and 9-fold, respectively, in comparison with the ischemic/reperfused drug-free value.

HO enzyme activity (Fig. I/4) was significantly reduced after 24 hours of reperfusion in drug-free ischemic/reperfused retinas in comparison with the nonischemic group (Fig I/4, group 1). However, a significant increase in HO enzyme activity was observed in retinas (Fig. I/4, group 3 and group 4) treated with 10 or 30 mg/kg of flavonoid-rich sour cherry seed extract, respectively, compared with the ischemic/reperfused drug-free control group.

The effect of sour cherry flavonoid–rich seed extract was tested in rat retina for its protective action against the changes of retinal cation contents induced by 90 minutes ischemia followed by 24 hours of reperfusion. Table I/1 shows the basic levels of retina Na^+, K^+, and Ca^{2+} contents (first column)in nonischemic retinas. After 90 minutes of ischemia followed by 24 hours of reperfusion, there was a significant increase in retinal Na^+ and Ca^{2+} contents from nonischemic control levels of 51.7 ± 2.8 and 2.1 ± 0.1 μg/g dry weight to 94.2 ± 3.2 and 4.8 ± 0.2 μg/g dry weight, respectively. Tissue K^+ was reduced from the nonischemic control value of 303.4 ± 5.1 to 229.6 ± 4.2 μg/g dry weight (*P<0.05). These changes in ischemia/reperfusion-induced retinal Na^+ and Ca^{2+} gains, and K^+ loss were significantly reduced in rats treated with 10 and 30 mg/kg sour cherry flavonoid–rich extract (Table I/1).

Taken together, these data demonstrated that retinal ischemia/reperfusion resulted in a significant reduction (to 10 %) in HO-1 protein expression, enzyme activity, and HO-1-related endogenous CO production in the retina. These changes were accompanied by increases in retinal Na^+ and Ca^{2+} gains and loss of K^+. In rats treated with 10 and 30 mg/kg of sour cherry flavonoid-rich extract, after 24 hours of reperfusion, tissue Na^+ and Ca^{2+} accumulation and K^+ loss were pervented in comparison with the drug-free control.

II. Evaluation of VSV as a potential virotherapy vector for the treatment of malignant conjunctival tumors

To determine the susceptibility of the conjunctival WK cell line for VSV, inoculated cultures were examined using an inverted microscope for cytopathic effects (CPEs) for a period of 48 h. Mock-infected WK cells were small, polygonal, slightly elongated and formed a compact monolayer throughout the 48 h of culturing (Figs. II/1a and III/2). Cells infected at an MOI of 1 for 10 h displayed CPEs characterized by cell rounding and detachment, but cultures infected at lower MOIs showed no visible cytomorphological alterations (data not shown). The cultures infected at MOIs of 0.1 and 1 displayed strong CPEs within 24 h (Fig. II/1d and not shown, respectively), at which time point considerable proportions of cells (approximately 10–25%) seemed to be intact at MOIs of 0.01 and 0.001 (Figs. II/1c and II/1b, respectively). All of the cultures, infected even at MOIs as low as 0.001 and 0.01, were completely destroyed by VSV at 48 h after virus inoculation (Fig. III/2). These findings indicate that although the development of CPE in response to VSV infection follows a dose-dependent course, VSV leads to the demise of cells within 48 h. Thus, the WK conjunctival cell line is highly susceptible for the CPEs of VSV.

To evaluate VSV replication in the WK cell line, different methods were used. Indirect immunofluorescence assay revealed positive staining for the G protein at 24 h after VSV inoculation (Fig. II/1b-d, lower panels) in 55%, ≥98% and ≥99% of cells infected at MOIs of 0.001, 0.01 and 0.1, respectively. The experiments have also shown that ≥99% of cells infected at MOI of 1 were positive for expression of the G protein at 48 h after VSV inoculation (Fig. III/2). Western blot analysis revealed the presence of G protein in cultures infected at an MOI of 1 at 4 h and 6 h after virus inoculation (Fig. II/2). G protein was detectable in cells infected at MOIs of 0.1 and 1 at 12 h postinfection (Fig. II/2). G protein accumulated in every culture infected with VSV by 24 h. High-level expression of G protein was also detected at 48 h postinfection (Figs. II/2 and III/1). The production of progeny virus was determined by plaque titration of the culture supernatants taken from WK cells at 12, 24 and 48 h postinfection. Virus production, depending on the infectious dose, varied between 2.7×10^3 and 2.7×10^7 PFU/ml at 12 h postinfection (Table II/1). Virus titers increased thereafter and ranged from 3.5×10^5 to 1.2×10^8 PFU/ml at 24 h after virus inoculation (Table II/1) .Virus production of cells infected with various MOIs grew to titers of about 1×10^8 PFU/ml at 48 h of culturing (Table II/1). The maximum yield thus corresponded to 100, 110, 93, 97 and 110 PFU/cell at 0.0001, 0.001, 0.01, 0.1 and 1 MOI, respectively. These data demonstrate that the WK conjunctival cell line is highly permissive for VSV replication.

To detect the hallmarks of apoptosis, VSV-infected WK cells were analyzed by different methods and compared to mock-infected cultures. The extent of DNA fragmentation was measured by ELISA. At 24 h after virus inoculation, the EFs measured in cells infected at MOIs of 0.001, 0.01, 0.1 and 1 MOI were 19, 39.7, 45.6 and 41.7, respectively (Fig. II/3). TUNEL assay revealed very few positive cells in the mock-infected cultures, whereas 90-95% of cells in VSV-infected cultures displayed positive TUNEL staining at 48 h after virus inoculation (Fig. III/2). These data demonstrate that VSV induces apoptosis in the WK conjunctival cell line.

To determine whether VSV infection can alter the expressions of Bcl-2 family members, the steady-state levels of Bcl-2 and Bax proteins were determined by Western blot analysis. The analysis revealed endogenous expression of Bcl-2 and p21 Bax in mock-infected WK cells (Figs. II/4, II/5, III/3 and III/4). Endogenous expression of Bcl-2 in mock-infected cells remained constant during the 48 h of culturing (Figs. II/4 and III/4). No quantitative differences were revealed in the expression level of Bcl-2 protein between VSV-infected and control cultures at the 24-h time point (Fig. II/4). The VSV-infected cells showed decreased levels of Bcl-2 48 h after virus inoculation; 8%, 37%, 37% and 48% decreases were detected

in the level of this protein in cells infected at MOIs of 0.001, 0.01, 0.1 and 1, respectively (Figs. II/4 and III/4). These data demonstrate that VSV infection alters the levels of Bcl-2 protein. Endogenous expression of p21 Bax in mock-infected cells remained constant during the 48 h of culturing (Figs. II/5 and III/3). No quantitative differences were revealed in the expression level of p21 Bax protein between the VSV-infected and control cultures at the 24-h time point (Fig. II/5), but cells infected at MOIs of 0.1 and 1 displayed high levels of p18 Bax (Fig. II/5). Expressions of p21 Bax were downregulated in VSV-infected cells at 48 h after virus inoculation (Figs. II/5 and III/3). In contrast, VSV-infected cells showed increased levels of p18 Bax 48 h after virus inoculation; 54%, 58%, 81% and 91% increases were detected in the level of this protein in cells infected at MOIs of 0.001, 0.01, 0.1 and 1, respectively (Figs. II/5 and III/3). These results demonstrate that in parallel with a slight decrease in the level of p21 Bax, p18 Bax protein accumulated in VSV-infected WK cells. Thus, the expressions of Bcl-2, p21 Bax and p18 Bax proteins are differentially modulated by this virus.

Taken together, these data demonstrated that the WK cell line was highly permissive to VSV replication and was highly susceptible for the CPE of the virus. VSV infection elicited the apoptotic death of WK cells. Mock-infected cells exhibited endogenous expression of Bcl-2 and p21 Bax proteins. VSV infection caused a pro-apoptotic shift in the expression pattern of some Bcl-2 family member proteins.

DISCUSSION

I. Investigation of heme-oxygenase-1 related carbon monoxide and flavonoids in ischemic/reperfused rat retina

Occlusion of the central retinal artery is a true ophthalmic emergency, when each minute elapsed increases the chance of death of retina leading to vision loss. Although reperfusion is a prerequisite for the survival and recovery of ischemic tissue, it is emphasized that it is not without hazard, and reperfusion-induced injury could occur. Mammalian cells and tissues respond to injury by up- or downregulation of several stress-related genes, the protein products of which, may participate in the protection against cellular injury. Although the importance of HO-mediated endogenous CO production has been identified recently as a protective mechanism in the regulation of ischemia/reperfusion-induced damage in various organs (43, 44, 45, 46), the direct measurement of endogenous CO from retinal tissue, to our knowledge, has not yet been performed. Although the induction of HO-1 has recently been

extensively studied at the level of gene transcription (47, 48), the physiological function of this process is poorly understood in the context of its role in the mechanism and regulation of reperfusion-induced cellular injury. Therefore, in our study, we have endeavored to obtain additional circumstantial evidence for the involvement of HO-1–related endogenous CO production and its direct measurement in ischemic/reperfused rat retina.

Like nitric monoxide (NO), CO has been identified as an endogenous cellular messenger, and studies suggest an important role of CO in hemodynamic regulation (26). It has been shown that endogenously produced CO acts as a signal molecule (22) and an activator of guanylate cyclase responsible for the generation of cGMP in the vascular tissue (49). These findings suggest that vessel wall-derived CO could serve as an endogenous regulator of vascular tone and platelet activity. Thus, it has been suggested that CO generated by heme catabolism may modulate the activity of the metabotropic receptor suspected to be neuroprotective during ischemic neuronal damage (50). CO may also have the capacity to affect ischemic brain hemodynamics positively, through its inhibitory role on platelet aggregation, which may contribute to neuroprotection (51). In addition, the capacity of CO to induce cGMP, with upregulation of the anti-apoptotic Bcl-2 protein, together with inhibition of nuclear translocation of p53 transcription factor, indicates an important role for the HO system in modulating cell death/survival signals (50). It is also of interest to note that CO binds and inactivates neuronal nitric oxide synthetase leading to a reduced production of nitric oxide by calcium influx into hypoxic neurons (52) .

In our study involving measurement of retinal CO production, we now provide direct evidence consistent with the hypothesis that retinal CO production may play a key role in the development of reperfusion-induced damage through HO-1 protein induction in the retina. Using a flavonoid-rich extract of sour cherry seed to stimulate HO-1 protein expression and endogenous CO production, we demonstrate that increased endogenous CO production is associated with the prevention of reperfusion-induced retinal damage. Mechanisms by which elevated HO-1 expression leads to the increase of endogenous CO production may involve the following:

First, flavonoids, which are naturally occurring compounds, have been shown to modulate P-450–dependent metabolic activities *in vitro* and *in vivo* (53, 54) and to scavenge O_2^-- and OH-free radicals. Flavonoids also act as antioxidants (55, 56) and have anti-inflammatory activity (57). The specific action of flavonoids may have been to increase the binding affinity of a substrate (58) or to improve the electron transfer efficacy between NADPH-cytochrome P-450 reductase and the P-450 enzyme. The P-450 reductase, which

transfers electrons from NADPH to P-450 during P-450–dependent catalysis, is capable of reducing oxygen to yield superoxide anion, and the oxygenated intermediates of P-450 itself can decompose in a side reaction to release superoxide anion (59).

The second possible explanation for increased induction of HO-1 and CO formation may involve the elimination of reactive oxygen species and reducing their direct interaction with cellular components including gene transcription machinery. Therefore, with trapping of powerful oxidizing radical species, the rate of decay in HO-1 protein expression may decrease.

Third, today, when so many advances are being made in molecular biology and cell physiology, we tend to lose sight of the potential importance of basic ions (e.g., Na^+, K^+, and Ca^{2+}) in both experimental and clinical medicine. Our study emphasizes the importance of Na^+, K^+, and Ca^{2+} in the maintenance of ionic balance across cell membranes, because various clinical conditions are frequently complicated by vision loss that originates from the ionic imbalance through edema formation and apoptotic or necrotic cell death. Our findings provide a basis for inquiry but do not dissociate the different routes and pathways involved in the postischemic ion accumulation or loss in ischemic/reperfused retina. Thus, the connection between HO-1 gene and protein expression-related CO formation could modify cellular membrane ion transports, and an increase in HO-1 gene and encoded protein expression could protect against changes in cation concentrations caused by ischemia/reperfusion. Our results show that Na^+ and Ca^{2+} accumulate in retina. An increase in CO production by flavonoid-rich extract of sour cherry through the HO-1 protein expression resulted in a significant decrease in cellular Na^+ and Ca^{2+} contents leading to a significant reduction of edema formation (31) and mitochondrial calcium-overload–induced cell death, which is a key mediator or signal of necrosis and/or apoptosis (60,61).

II. Evaluation of VSV as a potential virotherapy vector for the treatment of malignant conjunctival tumors

Our study, aiming to evaluate the potential oncolytic activity of VSV on epithelial conjunctival tumors, revealed that VSV was capable of establishing an infection in the WK conjunctival cell line that followed a rapid time course and affected virtually all of the cells. VSV replication in the WK cell line yielded high titers of progeny virus. Our data have also demonstrated that VSV infection elicited strong CPE characterized by cell rounding and detachment leading to the demise of cultures within 48 h even at an MOI as low as 0.001. Our further results have shown that the cytopathogenicity evoked by VSV in WK cells was linked

to apoptotic mechanisms. Thorough previous investigations demonstrated that several cell lines derived from lung, renal, colorectal, ovarian, breast, endometrial, prostate, central nervous system, and hematological tumors and melanoma were permissive to VSV and highly susceptible for the deadly infection it caused (63, 64, 70, 73, 83, 85, 93, 96). Thus, in accord with the previous notion suggesting that VSV has inherent oncolytic activity, our data extend the known spectrum of malignancies susceptible for this virus to immortalized conjunctival epithelial cells.

A large body of experimental evidence indicates that the VSV-mediated induction of apoptosis is an important factor in the cytopathogenicity of this virus (62, 63, 76, 77). It has also been revealed that over-expression of Bcl-2 confers significant protection against the apoptotic effect triggered by VSV (78). Our experiments have shown that the anti-apoptotic Bcl-2 and the pro-apoptotic p21 Bax were simultaneously present in the mock-infected WK cells. VSV infection decreased the level of Bcl-2. Moreover, in parallel with a slight decrease in the level of p21 Bax, p18 Bax protein accumulated in virus-infected WK cells. Apoptosis is critically dependent upon the Bcl-2 family member proteins comprising pro-apoptotic, anti-apoptotic and Bcl-2 homology domain 3 (BH3)-only groups (80). These proteins are implicated in the regulation of mitochondrial membrane permeability and cytochrome *c* release. The structure of these proteins can be characterized by variable amounts of Bcl-2 homology (BH) domains and by a transmembrane region restricting their subcellular localization to the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane. The anti-apoptotic subgroup includes proteins possessing all four BH domains, such as Bcl-2, which promote cellular survival by inhibiting apoptosis. The multi-domain pro-apoptotic subgroup contains proteins having the BH1, BH2 and BH3 domains, such as Bax, which promote apoptotic demise of cells. The death promoting members belonging in the BH3-only subgroup possess BH3, but not BH1, BH2 or BH4 domains. BH3-onlies modulate the function of multi-domain family members through direct interaction with them. In living cells, the anti-apoptotic Bcl-2 proteins, by sequestering pro-apoptotic members inhibit the activation of the apoptotic cascade. Previous studies have also demonstrated that Bcl-2 is often over-expressed in immortalized cells to maintain survival by suppressing apoptosis (66, 88). It is also well documented that the expression of p21 Bax is widespread in normal tissues (79, 86). Certain malignant cancers display high levels of various Bax isoforms, while in other tumors the expression of Bax is lost due to mutational inactivation of the gene (74). The fate of cells depends on the stoichiometric ratio, dimerization pattern and posttranslational modifications of the pro-apoptotic and anti-apoptotic Bcl-2 family members (80). In viable

cells p21 Bax exists in an inactive form located in the cytosol (80). Death signals trigger conformational change in the structure of p21 Bax molecule, thereby leading to its activation (80). Activated p21 Bax translocates to the mitochondrion, resulting in the release of cytochrome c and caspase-9, which in turn leads to the inevitable execution of the apoptotic process. Interesting recent data have shown that the activity of p21 Bax is subject to regulation by calpain-mediated proteolytic cleavage in cells exposed to stress, such as irradiation, etoposide or cisplatin treatment (66). Cleavage of p21 Bax was shown to yield a p18 Bax product, which behaves like a sensitizer type of BH3-only proteins (67). The p18 truncated form of Bax is more potent in disrupting mitochondrial integrity and inducing apoptosis (66, 67). Thus, the endogenous expression of Bcl-2 and p21 Bax observed in the mock-infected WK cell line is consistent with previously published observations. Our data demonstrating simultaneous down-regulation of Bcl-2 and up-regulation of p18 Bax in WK cells inoculated with VSV indicate a pro-apoptotic shift, which in turn may play an important role in the apoptotic responses of the infected cells and may also sensitize to other apoptotic stimuli. The significant decrease in the expression level of Bcl-2 may abrogate the survival function of this anti-apoptotic protein in VSV-infected cultures. The dramatic increase detected in the level of p18 Bax following infection may represent an important event in the amplification of the apoptotic process and contribute to the powerful CPE of this virus.

Extensive investigational efforts focusing on the oncolytic activity of VSV have provided strong experimental evidence of the effectiveness of this virus in several histological types of tumors, irrespective of the molecular mechanisms implicated in the events leading to malignant transformation (63, 64, 70, 71, 73, 83, 85, 93, 96). VSV has been shown to be capable of repressing the in vitro and in vivo growth of tumors that overexpress Myc and Ras oncogenes or carry mutated p53 (63). It is well documented that cancer cells accumulate various genetic alterations and display impaired responsiveness to IFNs and defective control of mRNA translation initiation (63, 64). These abnormalities render cancer cells more permissive to VSV replication and thereby more susceptible to its CPE (63, 64). Normal cells are endowed with high-level natural resistance against VSV, due at least in part to the activity of their functional IFN system (62-64). Following intratumoral or intravenous administration of VSV to tumor-bearing mice, the virus could be detected only in the tumor tissue, and not in other organs. VSV-infected athymic nude (nu/nu) mice, which are almost completely deficient in T lymphocytes, exhibited hind-limb paralysis, a characteristic manifestation of VSV disease, while no sickness or overt symptoms of VSV disease were evident in immunocompetent mice (63). The major drawback of the systemic inoculation of the

therapeutic virus preparation is that the activated adaptive immune responses may inhibit virus replication and thereby abrogate its oncolytic activity. It is important to note that the treatment of ocular surface tumors would not require the systemic administration of VSV. Conjunctival tumors are unique in consequence of their easy accessibility for topical treatment, and thus may be perfect targets for virotherapy without the risk of systemic side effects. VSV installed directly on the surface of the tumor may trigger an inflammatory reaction in the conjunctiva and the cornea, but possibly elicits a much less extensive activation of the adaptive immune system, and hence can be used repeatedly without the loss of efficiency.

SUMMARY

I. Our results show that a downregulation in HO-1 protein, HO-1-related CO production and HO-1 enzyme activity may play a crucial role in the development of reperfusion-induced retinal damage. Further data demonstrate that a sour cherry seed flavonoid-rich extract reduces the changes in concentrations of retinal ions through HO-1-related endogenous CO production in the ischemic/reperfused retina and exerts a protective effect against reperfusion-induced injury. Thus, it is reasonable to suggest that interventions which are able to increase endogenous CO production through the HO-1 system could prevent the development of ischemia/reperfusion-induced injury, including changes in cation concentration and edema formation in the retina. However, additional studies are needed to resolve the links in the apparent cascade of the up- and downregulation of ischemia/reperfusion-induced HO-1 protein expression, HO-1-related CO generation and enzyme activity in the ischemic/reperfused retina.

II. Our data demonstrating that VSV replicates efficiently, induces strong CPE and triggers apoptosis in the WK conjunctival cell line raise the possibility that VSV may be developed into an effective modality for the treatment of malignant conjunctival tumors. However, an assessment of the clinical applicability of VSV in the treatment of epithelial conjunctival tumors and determination of the effects of VSV on the resident ocular surface cells would necessitate further *in vitro* studies and *in vivo* approaches.

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ANNEX