The Moran Foundation
DEPARTMENT OF PATHOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS 77030

PROGRESS REPORT

HISTOPATHOLOGIC EVALUATION OF THE GENE THERAPY FOR RETINOBLASTOMA:COMPARISON OF AdV-TK AND AdV-RB

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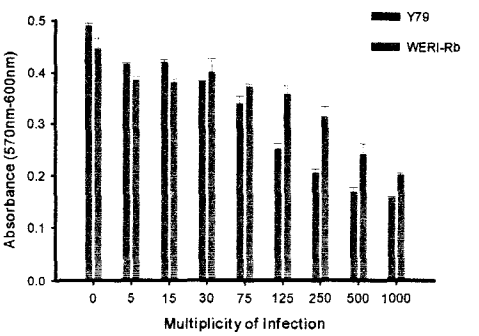
Preclinical studies of adenoviral-mediated gene therapy using replication-deficient adenovirus (AdV) containing the gene for Herpes simplex thymidine kinase (TK) followed by treatment with the prodrug ganciclovir (GCV) have suggested significant efficacy for retinoblastoma (Hurwitz et al., 1998). Since the defective gene associated with retinoblastoma is known (Francke and Kung, 1976; Knudson et al., 1976; Dryja et al., 1984; Lee et al., 1987), a comparison of gene-replacement therapy using AdV containing the normal Rb gene to the AdV-TK/ganciclovir "suicide" gene therapy would be significant. Comparison of these treatments using histopathology and immunohistochemistry as the efficacy criteria is the goal of this proposal.

In the first part of this project we have compared the efficacy in vitro and in vivo of two models of retinoblastoma using either Y79 or Weri-induced retinoblastoma tumors. Then these models were treated with replication-deficient adenovirus containing the normal human retinoblastoma gene (AdV-Rb) and compared to mice treated with the AdV-TK/ganciclovir treatment described above. Also control mice were studied using injections of PBS and of AdV-TK alone.

VIRAL VECTORS: Replication-deficient Ela and E3-deleted adenoviral vector containing the the Herpes thymidine kinase gene driven by the RSV promoter (AdV-TK) is produced at the Baylor College of Medicine Gene Vector Laboratory as described previously (Nyberg-Hoffman et al., 1997). AdV-Rb will be obtained from Canji Corp. (San Diego, CA). Viral vectors are stored at -80° C. They are diluted in virus dilution buffer (4% sucrose, 10 mM Trizma base pH 8, 2 mM $MgCl_2$) for *in vivo* experiments.

IN VITRO RESPONSE: AdV-Rb Transduction of Y79 Cells: Rb Protein Expression
Objective: To confirm that AdV-Rb transduction results in Rb protein expression
Methods: Y79 cells were cultured for 24 hours after transduction with various amounts of AdV-Rb. Protein expression was verified using Western blot analysis.
Results: Rb protein was expressed by the transducted cells as seen in Figure 1 and 2.

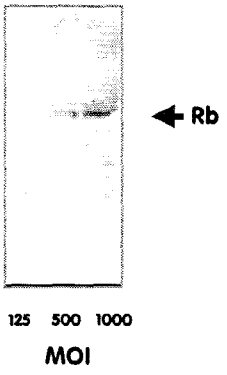
Figure 1. AdV-Rb Transduction of Y79 and WERI-Rb Cells



Incubation of Weri and Y79 retinoblastoma cells with AdV-Rb resulted in half maximal killing at MOIs of 60 and 500 respectively. These data correlated with the amount of

virus necessary to produce Rb protein in WERI Rb cells as monitored by western blot (Figure 2). The MOI of AdV-Rb necessary to affect growth of both Rb cell lines was similar to the MOI of AdV-□gal necessary to transduce a high percentage of both cell lines. Half-maximal killing of Weri Rb cells incubated with AdV-TK and ganciclovir was observed at an MOI of 2 and with Y79 Rb cells at and MOI of 30. The concentrations of AdV-TK necessary to affect growth of both Rb cell lines were less than AdV-Rb.

Figure -Rb Transduction of Y79 Rb Protein



DEVELOPMENT OF INTRAOCULAR TUMORS: Y79Rb or Weri cells were injected into the vitreal cavities of adult transgenic Rag-2 "knockout" immunodeficient mice (Shinkai et al., 1992). Animals were handled at all times following the Association for Research in Vision and Ophthalmology Statement of the Use of Animals in Ophthalmology and Vision Research. Each animal was first anesthetized with an intraperitoneal injection of 20-30 μl of sodium pentobarbital (50 mg/ml). The pupil were then dilated with 2-3 drops of 2.5% phenylephrine hydrochloride solution and a drop of the topical anesthetic proparacaine hydrochloride (0.5%) was applied. Cellulose eye drops (2.5%) and a glass contact lens were used to aid in visualization during the surgical procedures. The conjunctiva of the temporal area of the eye were dissected and a scleral sulcus were made with a #11 disposable scalpel. While observing the retina through a TopCon OMS 75 operating microscope, $2x10^4$ Y79Rb cells in 2 μl PBS were injected through the incision and into the vitreal space using a 10 μl Hamilton syringe with a 33g needle. Injections were made with the tip of the needle placed just above the retina and in the center of the field of vision. This placement allows easy visualization of the resulting tumor. The cells were delivered slowly over 30 seconds, and the needle was left in the eye an additional 30 seconds to allow for diffusion of the cells. Special care was taken to prevent lens damage or posterior retinal punctures.

IN VIVO TRANSDUCTION: Ten days after injecting the retinoblastoma cell line, eyes were injected with a viral vector in a volume of 2 μl. Untreated control animals received sham injections containing an equal volume of viral dilution buffer. Animals were anesthetized and prepared for injection as described above. Care was taken to place the needle within the tumor before delivery of the viral vector. Animals received either AdV-TK or AdV-Rb. Animals receiving AdV-TK (or the sham-treated control animals) received ganciclovir (33 μg in 2 μl of PBS) or PBS directly into the vitreous 1 and 4 days after vector delivery. Mice were sacrificed one week after vector administration and their eyes were examined histologically. Differences in the response of the groups of animals were analyzed in a blinded fashion and the statistical analysis using the unpaired Student's *t* test is in process. Mice in progression-free survival studies will be sacrificed after visible tumor recurrence and the presence of tumor verified histologically.

The amount of Rb protein expresed by the tumors in each group will also be analyzed using immunohistochemical studies. Data will be analyzed using a Kaplan-Meier plot. The significance of the differences between treatment groups will be determined by the log-rank test.

HISTOPATHOLOGY: After dissection, the eyes were immediately fixed in 10% formalin. The tissues were processed and embedded in paraffin using conventional automated systems. The process of embedding required especial attention to orientation of the eye to obtain a pupillary-optic nerve section. The blocks were sectioned to obtain levels and serial sections 4-5 microns thick. Two alternating sections were stained with conventional hematoxylin-eosin (H&E). The remaining sections were mounted in glass slides specifically used for immunohistochemistry. The H&E slides were examined and scored in a blinded fashion. The results of the group using Y79 tumors are summarized in Table 1.

Table 1. Comparison of response of Y79 retinoblastoma tumors treated with AdV-Rb versus AdV-TK

Treatment	CR/ PR	%	NR	%
Control – PBS	(1/2)	30	(7)	70
Rb	(4/4)	80	(2)	20
TK	(0/3)	38	(5)	62
TK + GC	(3/6)	90	(1)	10
TK + Rb + GC	(1/2)	30	(7)	70

CR = Complete response
PR = Partial response
NR = No response
Rb = AdV + Rb vector
TK = AdV + TK vector
GC= Ganciclovir

The results of the group using Weri retinoblastoma tumors are summarized in Table 2 and Fig.2. These tumors as

Table 2. Comparison of response of Weri retinoblastoma tumors treated with AdV-Rb versus AdV-TK

Treatment	CR/ PR	%	NR	%
Control – PBS	(1/0)	12.5	(6)	87.5
Rb	(0/6)	60	(4)	40
TK	(1/3)	57.1	(3)	42.9
TK + GC	(3/5)	100	(0)	0
TK + Rb + GC	(2/4)	100	(0)	0

CR = Complete response
PR = Partial response
NR = No response
Rb = AdV + Rb vector
TK = AdV + TK vector
GC= Ganciclovir

The histopathologic changes of the in vivo models show similar features to those seen in the in vitro study. However, there is a striking difference between the response of the Y79 Rb and the Were Rb tumors when treated with the combination of AdV-TK-Rb + ganciclovir. Overall, both tumor models have a better response (CR/PR) of 90% for Y79 Rb tumors and of 100% for the Weri Rb tumors. In the Y79 Rb tumors treatment with AdV-Rb resulted in the second better response with a CR/PR of 80%. The Weri Rb tumor group has a CP/PR of 60% when treated with AdV-Rb. The main difference between both models is that the Y79 Rb group has a poor response to the combination of AdV-TK-Rb + ganciclovir of 30% (CR/PR) in contrast to the Weri Rb group which shows a response of 100% (CR/PR) with the same treatment. The remaining groups show similar responses with adequate controls.

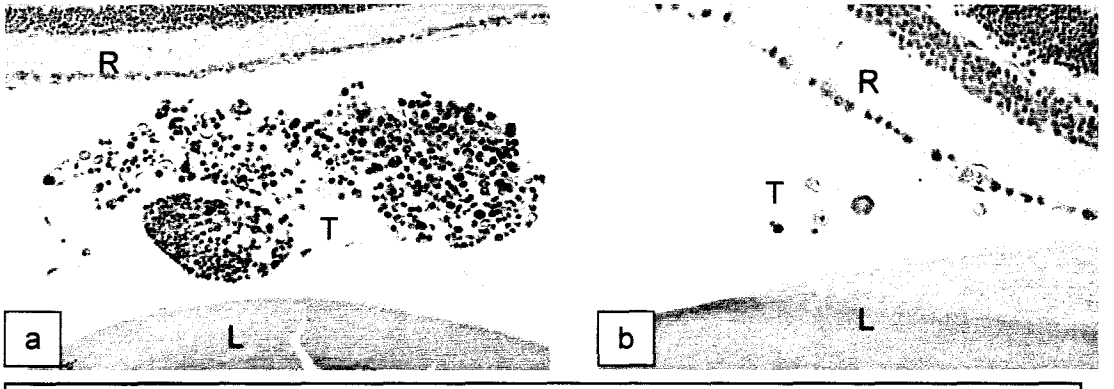


Figure 1. *In vivo* Weri-retinoblastoma tumor.
a) Example of no response (NR). Retinoblastoma tumor (T) treated with AdV-Rb only. The tumor partially fills the vitreal cavity. L= lens, R = retina. H&E, original magnification 25X.

b) Example of partial response (PR) in tumor treated with AdV-TK and ganciclovir. Rare mostly necrotic retinoblastoma tumor cells (T) are present in the vitreal cavity . H&E, original magnification 50X.

Immunohistochemistry. Rb protein antibody is being search for in the sections of the tumors after transduction of the vectors AdV-TK or AdV-Rb using conventional immunohistochemistry. The sections used for this study alternate with those employed for regular H&E staining for the evaluation of tumor response. Control animals are included (those with tumors and sham injections) to compare the Rb expression of the Y79 human retinoblastoma cells *in vivo* with the expression of the transduced tumors (AdV-TK or AdV-Rb).

CONCLUSIONS

AdV-TK followed by ganciclovir is superior to AdV-Rb at affecting the growth of the Rb cells lines *in vitro.*

In vivo experiments using Y79 retinoblastoma tumors show similar response to both constructs, the AdV-Rb (80%) and the AdV-TK (90%). However, Y79 Rb tumors show no response (30%), similar to the control group, when treated with AdV-Rb-TK followed by ganciclovir.

In vivo experiments using Weri retinoblastoma tumors show less response to treatment with the AdV-Rb (60%) than treatment with AdV-TK followed by ganciclovir (100%). However, Weri Rb tumors show similar response when treated with AdV-Rb-TK or AdV-TK follow by ganciclovir.

The overall results are consistent with the interpretation that the bystander effect plays a role with AdV-TK/ganciclovir therapy and not with AdV-Rb.

The striking difference of the response between Y79 Rb tumors and Weri Rb tumors to treatment with AdV-Rb confirms the difference of biological behavior between the two cell lines as described previously (Chevez-Barrios,1999). Most of the Weri Rb tumors without response were smaller than the similar group in the Y79 Rb tumors. As described previously Y79 Rb tumors are more aggressive in invading and more mitotically active. Weri Rb tumors are slower growers and do not behave as aggressive invasive tumors. This may explain why the Y79 might incorporate better the Rb gene and produce the Rb protein making the cells less vulnerable to the TK/ganciclovir treatment. However, Weri Rb tumors being less mitotically active may delay incorporation of the Rb gene and production of the protein allowing the cells to incorporate the TK gene and responding to the ganciclovir treatment. Immunohistochemistry in the sections of these eyes will prove if the Rb protein is expressed in both Y79 Rb tumors and Weri Rb tumors in a different fashion.

Outline of current research

Immunohistochemistry for localization of the Rb protein in the sections of the different tumor models is undergoing. Reading and analyzing the results is expected to be our immediate next step in the current project. Statistical analysis for both morphological changes and results of immunohistochemistry will be performed. A paper is in preparation and will be submitted shortly for publication when results are completed.

These results further support the roll of the intimate interactions taken between the tumor biology and the treatment (gene therapy). In further investigating these differences we have described found that the Y79 Rb model forms tumors that progressively invade the retina, choroid and optic nerve and metastasize to the brain. In contrast, Weri Rb tumors invade locally into the anterior portion of the eye, the ciliary body and the iris. To examine potential biological differences *in vitro,* the retinoblastoma cell lines were co-cultured with adherent choroid cells or adherent glioma cells which represent the targets of metastatic retinoblastoma *in vivo.* Consistent with the *in vivo* observations, Y79 cells but not Weri-Rb cells adhere specifically to both the choroidal and the glioma cell lines. The attachments were destroyed by using trypsin suggesting the presence of a protein on the surface of the cells (receptor). Furthermore, this simple in vitro binding assay may help predict which patients with retinoblastoma might be prone to develop metastases. (See attached document submitted to the American Journal of Pathology for publication).

Metastasis is a complex process that requires sequential interactions between the invasive cell and the extracellular matrix. These interactions are characterized by cell adhesion and migration. Cell adhesion involves specific receptors. Migration requires the induction and secretion of proteolytic enzymes belonging to the matrix metalloproteinases (MMP) family. In most cancers, stromal cells secrete collagenases or gelatinases under the influence of cancer cells. The MMPs are secreted as inactive forms. In order to cross basement membrane and then to reach the extracellular matrix, the MMPs undergo an activation step which involves plasmin, growth factors or membrane-type matrix metalloproteinases (MT-MMPs). MMPs are associated with tissue inhibitors of metalloproteinases (TIMPs) with which they form high affinity non covalent 1:1 complexes. Upregulation of MMPs or down regulation of TIMPs lead to an imbalance of this ratio which favors an invasive process.

Our preliminary studies using the *in vitro* tumors Y79 and Weri cells lines co-cultivated with glioma cells and choroidal cells show than only Y79 cells will induced the production of metalloproteinases type B when attached to glioma or choroidal cells. These promising results are encouraging for pursuing this line of research as a potential inhibitor of these MMPs could be useful in a clinical setting to prevent metastasis.

Manuscripts and presentations

The generous support from the Moran Foundation has made possible a large part of this research and has produced two manuscripts, three presentations and three abstracts listed bellow (please also see attached material):

Manuscripts:

1. Chévez-Barrios P, Hurwitz MY, Louie K, Marcus TK, Holcombe VN, Schafer P, Aguilar-Cordova CE, and Hurwitz RL: Retinoblastoma: Animal Models of Tumor Progression. *Submitted to American Journal of Pathology, September 1999.*

2. Chévez-Barrios P, Marcus TK, Holcombe VN, Hurwitz MY, Hurwitz RL: Gene therapy for retinoblastoma: Comparison of gene replacement with the

retinoblastoma gene to suicide gene therapy with herpes simplex thymidine kinase and ganciclovir. *In preparation.*

Abstracts and presentations:

1. Chévez-Barrios P, Marcus TK, Holcombe VN, Hurwitz MY, Hurwitz RL: Gene therapy for retinoblastoma: Comparison of gene replacement with the retinoblastoma gene to suicide gene therapy with herpes simplex thymidine kinase and ganciclovir. *The Methodist Hospital Oncology meeting, 1999. Presented as Poster.*

2. Hurwitz MY, Schafer P, Holcombe VN, Chévez-Barrios P, Hurwitz RL: Determination of metastatic properties of retinoblastomas: Development of an in vitro assay. Investigative Ophthalomology and Visual Science, 1999, 40(4) suppl:647. *Presented as poster in the ARVO Annual Meeting, Fort Lauderdale, Florida, May 9-14, 1999.*

3. Hurwitz RL, Rivera AL, Holcombe VN, Chévez-Barrios P, Hurwitz MY: Gene therapy for retinoblastoma: Comparison of gene replacement with the retinoblastoma gene to suicide gene therapy with herpes simplex thymidine kinase and ganciclovir.. Investigative Ophthalomology and Visual Science, 1999, 40(4) suppl:761. *Presented as poster in the ARVO Annual Meeting, Fort Lauderdale, Florida, May 9-14, 1999.*

BUDGET EXPENDINGS:

HISTOLOGY		$1895.00
Process, embedding, cutting and H&E staining		
$6.00 X each block (80)		
$7,00 X each block for immunohistochemistry (70)		
IMMUNOHISTOCHEMISTRY		
Immunostaining		
(Rb protein) antibody (each100□l =$199.00) (10)	(pending)	$1990.00
Titration fee	(pending)	$25.00
IMAGE RECORDING		
COOLSCAN 2000 FILM SCANNER (Nikon)		$1670.00
Auto slide feeder for Coolscan		$450.00
REWRITABLE W/CD-RW CD SPEED RACER		$399.00
TOTAL EXPENDED		**$ 4414.0**
TOTAL REQUESTED		**$ 5595.00**

The process and staining of the eyes were performed in the Baylor College of Medicine Pathology laboratory and the above estimate prices are obtained from their list of research prices.

We are confident that with a continuous support from the Moran Foundation this promising line of research could produced useful results for a possible prevention treatment for metastasis in retinoblastoma.

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4020—9:45

IN VIVO DETERMINATION OF CONE PROMOTER REQUIREMENTS USING A HUMAN RED OPSIN PROMOTER IN A RECOMBINANT AAV Q. Li, A. M. Timmers, W. Yan, V. A. Chiodo, W. W. Hauswirth. University of Florida.

Purpose: To determine the character and function of promoter elements needed for cone specific gene expression in vivo using recombinant Adeno-Associated Virus (rAAV) as the vector. Methods: Recombinant AAV vectors were constructed containing various elements upstream of the human red opsin gene to direct green fluorescent protein (GFP) expression. Each recombinant virus was injected subretinally into rat eyes. GFP expression was analyzed 4-6 weeks after injection by confocal fluorescence microscopy and immunocytochemistry. Results: A 2.1kb human red opsin gene upstream sequence containing a 1.5 kb deletion between the Locus Control Region, LCR, and the proximal promoter supported GFP expression specifically in rat cones because GFP and cone-opsin antibody co-localized to the same photoreceptor cells. Further deletions of this promoter, both upstream and/or downstream of the LCR also lead to cone specific expression as long as the proximal 445bp promoter was present. However the relative strength of these multiply deleted promoters, as judged by the number of positive cones, was reduced significantly. Even in the absence of the LCR, the 445bp proximal promoter alone supported specific but inefficient cone expression. When a small region containing the LCR was tandemly repeated just upstream of the proximal promoter, cone expression returned to levels seen with the parental 2.1kb promoter. Conclusions: In vivo cone targeted gene expression is obtainable in rats with a simple human red opsin gene upstream region. Therefore, the DNA elements required for cone photoreceptor selectivity must be well conserved among mammals. Even a small human red cone opsin promoter (-445 to -1) lacking an LCR is sufficient to direct cone-specific gene expression in vivo. The LCR is therefore not a determinant of cone expression specificity, but appears to be an enhancer of basal cone transcription. Supported by NIH grants EY07864, EY11123, Research to Prevent Blindness Inc. and The Foundation Fighting Blindness.
CR: None

Room 315
Thursday 8:30 — 10:15 AM

Immunology & Microbiology, Cornea

Herpes Virus: Pathogenesis, Diagnosis, and Treatment

MODERATORS: Robert L Hendricks
Jerry L Taylor

PGM#	TIME	AUTHORS
4022	8:30	Hendricks, Kodukula, Liu, Jager, van Rooijen
4023	8:45	Stumpf, Shimeld, Hill, Easty
4024	9:00	Taylor, Tsao, Hill, Marquart, Loutsch, Zheng
4025	9:15	Zheng, Marquart, Loustch, O'Callaghan, Kaufman, Hill
4026	9:30	Kriesel, Hwang, Jones
4027	9:45	Maertzdorf, van der Lelij, Remeijer, Niesters, Osterhaus, Verjans
4028	10:00	Remeijer, Dings, Osterhaus, Verjans

NOTE: Potential conflicts of interest of presenters and authors are noted at the end of the Abstract either by "None" or with codes (see code definitions under the section "Abstract Content/Commercial Relationships/ Disclosure Codes" in the front matter).

4021—10:00

GENE THERAPY FOR RETINOBLASTOMA: COMPARISON OF GENE REPLACEMENT WITH THE RETINOBLASTOMA GENE TO SUICIDE GENE THERAPY WITH HERPES SIMPLEX THYMIDINE KINASE AND GANCICLOVIR R. L. Hurwitz, A. L. Rivera, V. N. Holcombe, P. Chevez-Barrios, M. Y. Hurwitz. Baylor College of Medicine.

Purpose: To compare the efficacy of gene replacement therapy using an adenoviral vector delivering the retinoblastoma gene (AdVRb) with an adenoviral vector delivering the suicide gene Herpes simplex thymidine kinase (AdVHSV-TK) followed by ganciclovir for the treatment of retinoblastoma. Methods: AdVRb (Canji Corp.), AdVHSV-TK or AdVBgal (Baylor College of Medicine Gene Vector Laboratory) were incubated with the retinoblastoma cell lines WERI Rb or Y79 Rb at multiplicity of infection (MOI) of 1 to 10,000 in a 96-well microtiter plate (2 x 103 cells/well). After 6 days of incubation, the viability of cultures containing AdVRb or AdVHSV-TK was monitored with Alomar blue. The B-galactosidase substrate X-gal was added after 18 hours to cultures containing AdVBgal and the percentage of cells that were blue were counted. The expression of Rb protein was monitored by western blot. Results: Incubation of WERI Rb and Y79 Rb cells with AdVRb resulted in half maximal killing at MOIs of 60 and 500 respectively. These data correlated with the amount of virus necessary to produce Rb protein in WERI Rb cells as monitored by western blot. The MOI of AdVRb necessary to affect growth of both Rb cell lines was similar to the MOI of AdVBgal necessary to transduce a high percentage of both cell lines. Half-maximal killing of WERI Rb cells incubated with AdVHSV-TK and ganciclovir was observed at an MOI of 2 and with Y79 Rb cells at an MOI of 30. The concentrations of AdVHSV-TK necessary to affect growth of both Rb cell lines were less than AdVRb. Conclusions: AdVHSV-TK followed by ganciclovir is superior to AdVRb at affecting the growth of Rb cell lines. The results are consistent with the interpretation that a bystander effect plays a role with AdVHSV-TK/ganciclovir therapy and not with AdVRB.
CR: None Support: The Foundation for Research, Retina Research Foundation, and The Moran Foundation

4022—8:30

Macrophage Control of Herpes Simplex Virus Type 1 Replication in the Peripheral Nervous System R. L. Hendricks[1], P. Kodukula[2], T. Liu[1], M. J. Jager[3], N. Van Rooijen[4]. University of Pittsburgh[1], University of Illinios at Chicago[2], Leiden University Medical Center[3], Free University, Amsterdam[4].

Purpose: Latently infected sensory neurons of the trigeminal ganglion are the source of HSV-1 during recurrent herpetic corneal disease. The goal of these studies was to define mechanisms by which the immune system contributes to the control of HSV-1 replication in sensory neurons. Methods: Cellular infiltration and cytokine production in the HSV-1 infected TG was evaluated at the mRNA level with a multi-probe RNase protection assay (RPA) or PCR, and at the protein level by immunohistochemistry. Then in vivo depletion and neutralization studies were used to establish the role of the identified cells and cytokines in preventing HSV-1 replication in the ganglion. Results: During the period of HSV-1 replication, macrophages and γδT cells infiltrate the TG, and TNF-α, IFN-γ, and the inducible nitric oxide synthase (iNOS) enzyme, and IL-12 are expressed. TNF-α, IFN-γ and the iNOS product nitric oxide all inhibit HSV-1 replication in vitro. Macrophage and γδT cell depletion studies demonstrated that macrophages are the main source of TNF-α and iNOS, while γδT cells produce IFN-γ. Macrophage depletion, aminoguanidine inhibition of iNOS, and neutralization of TNF-α or IFN-γ all individually and synergistically increased HSV-1 titers in the TG after HSV-1 corneal infection. Moreover, individually depleting macrophages or neutralizing TNF-α or IFN-γ markedly reduced the accumulation of both macrophages and γδT cells in the TG.Conclusions: Our findings establish that following primary HSV-1 infection the bulk of virus replication in the sensory ganglia is controlled by macrophages and γδT lymphocytes through their production of antiviral molecules TNF-α, NO, and IFN-γ. Our findings also strongly suggest cross-regulation between these two cell types is necessary for their accumulation and function in the infected TG.
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4023—8:45

Cytokine production in a murine model of recurrent herpetic stromal keratitis . T. Stumpf[1], C. Shimeld[1], T. J. Hill[2], D. L. Easty[1]. Department of Ophthalmology, University of Bristol, UK[1], Department of Path & Micro, University of Bristol, UK[2].

Purpose: Herpetic stromal keratitis is an immunopathological disease caused by reactivation of latent herpes simplex virus type 1 (HSV-1) and recurrent infection of the cornea. Using our murine model of recurrent HSV-1 infection, we have investigated the pattern of cytokine production in the cornea and its relationship with viral antigens. Methods: 8 week old female NIH mice were passively immunised with anti HSV serum 24 hours prior to corneal inoculation with HSV-1 (McKrae). Control mice were mock inoculated. After 6 weeks, latently infected mice with normal corneas and control mice were irradiated with ultraviolet light (UV). Mice were examined daily for signs of disease and viral reactivation. The eyes of 5 mice with recurrent corneal disease and 2 control mice were taken, fixed and paraffin wax embedded on days 4, 7, 10 and 14. Serial sections were double stained using immunohistochemistry for viral antigens and the following cytokines: IL-2, IL-4, IL-6, IL-10 and IFN-γ. Cytokine positive cells were counted in two areas 450 μm apart.Results: 40 % of mice shed virus in their tears and the severity of recurrent disease peaked on day 7 post UV irradiation. There was a significant cellular infiltrate in the stroma of all the corneas with recurrent disease and the predominant cytokines were IL-6, IL-10 and IFN-γ, all present on day 4 and peaking on day 10 (50, 65, 86 cells/0.04 mm^2 respectively on day 10). There were very few cells producing IL-2 and IL-4 (1, 2 cells/0.04 mm^2 respectively on day 10). Control eyes had no significant cytokine producing cells. Conclusions: The large number of IFN-γ producing cells and the relative absence of $IL\text{-}4^+$ cells, suggest a Th1 response. The very low number of IL-2 producing cells indicates that T cell proliferation is likely to be extraocular. The large number of IL-10 producing cells is interesting and may suggest a local inhibitory effect on lymphocytes but the overall balance of cytokine production appears to be proinflammatory, with large numbers of infiltrating neutrophils.
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3406—B264

Discordant Retinoblastoma (Rb) in Genetically Proven Monozygotic Twins ((GI Papastergiou[1], SE Brooks[1], A Pandia[2], M Patel[1], DM Marcus[1])) Department of Ophthalmology, Medical College of Georgia, Augusta, GA[1], Department of Human Genetics, Medical College of Virginia, Richmond, VA[2].

Purpose: To report a case of discordant Rb in twins shown to be monozygotic by means of genetic analysis. **Methods:** Repeated clinical examinations of both twins were performed from ten months to four years of age. To determine zygosity we employed the method of DNA fingerprinting with the use of polymorphic microsatellites. **Results:** Twin A exhibited bilateral multifocal Rb. The right eye of twin A was enucleated. The left eye of Twin A was treated with external beam irradiation followed by chemoreduction with local laser and cryotherapy for recurrence. A total retinal detachment led to enucleation of this eye as well. Histologic examination confirmed the diagnosis of Rb. Twin B is four years old and remains free of Rb. DNA fingerprinting showed that the possibility that these same sexed twins are monozygotic is greater than 98%. Single stranded conformation polymorphism and Southern blot analysis confirmed monozygosity but failed to identify a Rb gene mutation. **Conclusions:** To our knowledge this is the first report of discordant Rb in monozygotic twins confirmed by molecular genetic analysis. Possible causes for discordance include early post-zygotic Rb gene mutation, post-zygotic events leading to mosaicism, influence of modifying genes, stochastic and environmental factors. Given that low-penetrance Rb usually leads to a less aggressive form of Rb in affected individuals (not observed in Twin A) early post-zygotic events are more likely possibilities.

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3407—B265

THERMOTHERAPY FOR RETINOBLASTOMA : TUMOR CONTROL AND COMPLICATIONS IN 191 CASES
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Purpose. To evaluate tumor control and complications for thermotherapy. **Methods.** Retrospective review. **Results.** Of 190 retinoblastomas managed with thermotherapy, the mean tumor base was 3 mm and thickness 2 mm. Coupled chemotherapy was used in 58%. Delivery was via indirect ophthalmoscopy in 110 cases, operating microscope in 70, and transcleral probe in 3. After a mean total treatment time of 27 minutes per tumor, the tumor was controlled in 163 cases (86%). The main complications were iris atrophy (36%), often with paraxial retroiridic focal lens opacity (24%). **Conclusions.** Thermotherapy offers promising tumor control for small intraocular retinoblastoma.

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3408—B266

OBSERVATIONS ON 22 PATIENTS WITH RETINOCYTOMA
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Oncology Service, Wills Eye Hospital, Thomas Jefferson University, Philadelphia, PA.

Purpose. To study the clinical profile, tumor characteristics and topography of retinocytoma. **Methods.** Data on 22 patients with diagnosis of retinocytoma was reviewed for clinical profile, fundus appearance, natural history, and association of retinocytoma to retinoblastoma (RB) and second malignant neoplasms (SMN). **Results.** Of the 920 patients with RB, we identified 22 patients (2.4%) with 35 retinocytomas at a median age of 15 years (range 1.4-45 years). 4 cases transformed into active RB. None of the cases developed SMN. Familial cases had normal penetrance RB. 77% tumor foci were extra macular and lacked excess distribution along the horizontal meridian. **Conclusions.** Retinocytoma may be associated with normal or low penetrance RB. Topographic distribution suggests origin from rod photoreceptor precursors. Patients with retinocytoma should be carefully observed for tumor activation.

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3409—B267

CHEMOPROPHYLAXIS FOR HIGH RISK FACTORS ON HISTOPA EXAMINATION OF RETINOBLASTOMA EYES J. M. O'Brien[1], M. Uusitalo[1], K. I. Scott[2], T. Murray[2]. Department of Ophthalmology, University of California San Francisco[1], Department of Ophthalmology, Bascom Palmer Eye Institute, Miami, Florida[2].

Purpose: This study evaluated the need for chemoprophylaxis in patients with retinoblastoma who underwent enucleation and were found to have high risk features on histopathologic examination. Patients with unilateral retinoblastoma were selected because they typically present with more advanced disease and were not treated with chemoreduction for visual salvage. **Methods:** The medical records of all unilateral retinoblastoma patients since the routine institution of post-enucleation prophylactic chemotherapy at Bascom Palmer and UCSF were reviewed (UCSF 1977-1998, BPEI 1991-1998). All histopathologic specimens were re-examined by light microscopy. **Results:** Ninety-two of 153 patients demonstrated notable histopathologic findings on review of specimens. Optic nerve involvement beyond the lamina cribrosa was present in 14 patients, and 4 of these patients had tumor extension up to the cut end. These fourteen patients met institutional criteria for chemoprophylactic therapy, and nine of these patients received chemoprophylaxis. No patient developed metastatic disease. **Conclusions:** Chemoprophylaxis appears to be of benefit in preventing metastasis in patients with optic nerve involvement to the cut end or beyond the lamina cribrosa.
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3410—B268

DETERMINATION OF METASTATIC PROPERTIES OF RETINOBLASTOMAS: DEVELOPMENT OF AN IN VITRO ASSAY M. Y. Hurwitz, P. Schafer, V. N. Holcombe, P. Chévez-Barrios, R. L. Hurwitz. Baylor College Of Medicine.

Purpose: An in vitro assay was developed to analyze the metastatic properties of retinoblastomas. **Methods:** Two human retinoblastoma cell lines, WERI Rb and Y79 Rb, have previously been used to develop murine models of retinoblastoma. WERI Rb cells formed non-metastatic tumors when the cells were injected into the vitreal cavities of immuno-incompetent mice. Y79 Rb cells, however, formed retinoblastomas with metastatic characteristics that mimic those observed in children with advanced disease. Y79 Rb cell-induced tumors fill the vitreal cavity, invade the choroid, migrate along the optic nerve, and progress through the optic chiasm to the contralateral optic nerve. To develop an in vitro assay that will allow analysis of candidate genes responsible for these specific metastatic migration characteristics of retinoblastomas, three adherent cell lines, C6 rat glioma cells, RF/6A rhesus monkey choroid retina cells, and HEK 293 cells (control), were examined. Suspension cultures of WERI Rb or Y79 Rb cells were seeded over the adherent cell monolayers. After incubation at 37°C, the nonadherent cells were removed and the remaining cells were washed prior to analysis and photography. **Results:** Significantly more Y79 Rb cells than WERI Rb cells remained in tight contact with either C6 glioma or RF/6A choroid retina cells. Neither cell line adhered significantly to the HEK 293 control monolayers. **Conclusions:** A simple in vitro assay to identify retinoblastoma cells with the potential to specifically adhere to cell types normally found along the metastatic migration path has been developed. This assay will be useful for the identification of candidate genes that may be involved in metastatic properties of retinoblastoma.
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3411—B269

Efficacy of the differentiating agent sodium butyrate in a murine transgenic model of retinoblastoma M. S. Ibarra[1], M. Madigan[2], M. Uusitalo[1], J. Smith[1], J. O'Brien[1]. Department of Ophthalmology, University of California San Francisco[1], Department of Clinical Ophthalmology, Sydney Hospital, Australia[2].

Purpose: In vitro studies have demonstrated that sodium butyrate (SB) can induce apoptosis in human retinoblastoma (Rb) cell lines. The purpose of this study was to investigate the effects of subconjunctival administered SB on tumor growth in a murine transgenic model of Rb. **Methods:** Twenty four SV40 Tag positive mice were identified using polymerase chain reaction. Eighteen 5 week old transgene bearing mice were injected subconjunctivally in the right eye with either 1.0 mM, 4.0 mM, or 8.0 mM of SB diluted in phosphate buffered saline (PBS, pH 7.4). 25 ul injection volume was delivered every 48 hours for a period of 2, 4, or 6 weeks. The untreated left eye served as an internal control. Six 5 week old transgene bearing mice were treated identically with PBS only. Mice were sacrificed at 7, 9, or 11 weeks of age; eyes were enucleated and processed for light microscopy. Frozen sections were immunolabeled with antibodies to GFAP (macroglia) and PCNA (proliferating cells), or TUNEL-labeled to assess cell death in retinae. **Results:** After 2 weeks of SB treatment (all doses), both control and treated retina appeared similar. Occasional TUNEL+ cells were seen in the ganglion cell layer, and GFAP immunoreactive (-IR) astrocytes and radial Muller cells were visible. Foci of PCNA-IR tumor cells were more apparent across the retina with increasing age, reflecting tumor development. Four weeks after 4 mM SB treatment, some evidence of retinal thinning and reduced PCNA-IR foci were visible in treated eyes compared with controls. Astrocyte disorganization was also seen both in control and SB-treated eyes. **Conclusions:** Subconjunctival administration of SB did not appear to be associated with systemic or retinal toxicity. Combined with previous in vitro observations, these preliminary results suggest SB may be able to affect Rb growth in vivo, although longer time-courses need to be examined.
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