rd1 Mouse Retina Shows an Imbalance in the Activity of Cysteine Protease Cathepsins and Their Endogenous Inhibitor Cystatin C

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PURPOSE. To compare in vivo levels, spatial localization, and in vitro secretion of cysteine protease cathepsins and cystatin C (cysC) in the retinal degeneration 1 (*rd1*) mouse model of retinitis pigmentosa and control (*wt*) mouse retinas.

METHODS. The spatial localization, protein contents, cysC levels and cathepsin-B, -S, and -L activities in *wt* and *rd1* retinas at postnatal (PN) days 2, 7, 14, 21, and 28 were analyzed by immunostaining, spectrophotometry, ELISA, and fluorescence spectrophotometry. The in vitro secretion of cysC and cysteine proteases by PN7 retinal explants into the conditioned medium (RCM) was quantified.

RESULTS. The pigment epithelium, photoreceptors, and inner retinal and ganglion cell layers of both *wt* and *rd1* retinas showed cysC and cathepsin-B labeling. CysC immunostaining was extensive in the optic nerve head fibers. The *rd1* explants secreted higher amounts of cysteine protease into the RCM. The protein content in *wt* and *rd1* retinal extracts increased up to PN14, then decreased in *rd1* but not in *wt*. In *rd1* extracts at PN14 to -28, cathepsin activity was higher and increased with age, but the cysC level was higher and constant. The ratios of cathepsin activity to cysC (cathepsin-L at PN2 and total, -B, and -L at PN14 to -28) were higher in *rd1* extracts.

CONCLUSIONS. Similar localization of both cathepsin-B and cysC in *wt* and *rd1* retinas along with lower proteins and higher cathepsin activity in *rd1* retinal extracts and RCM are consistent with their localization in extracellular matrix and a role in physiopathologic remodeling in *wt* and *rd1* retinas. (*Invest Ophthalmol Vis Sci.* 2008;49:1089–1096) DOI:10.1167/iovs.07-0549

The retinitis pigmentosa (RP) group of diseases is a common cause of retinal degeneration in patients. Similar to a form of RP in humans, the retinal degeneration 1 (*rd1*) mouse has a mutation in the gene for the β-subunit of cGMP phosphodiesterase-6 (PDE-6). Consequently, *rd1* rod photoreceptors have elevated cGMP and Ca^{2+} ion concentrations and degenerate between postnatal days (PN)10 and -21.[1] Elevated cGMP modifies the expression of more than 60 genes involved in transcription in neural cells, proliferation, apoptosis, extracellular matrix (ECM) turnover and angiogenesis in different tissues.[2] Such changes can be expected to modify multiple biochemical pathways of retinal degeneration, which are at present poorly delineated. Oxidative stress, proteases, and the ubiquitin-proteasome system are some of the known factors involved in neuronal cell death and the pathogenesis of retinal degeneration. Their effects are evident from the in vivo and in vitro rescue of *rd1* mouse rods by antioxidants and other neuronal cells by proteasome inhibition.[3–6]

The serine proteases, matrix metalloproteases (MMPs), aspartyl and cysteine cathepsins, and calpains modulate many processes, such as protease activation, ECM degradation, angiogenesis, immune response, genetic stability, embryonic development, cell signaling, growth, invasion, and death. Serpins, tissue inhibitors of MMPs (TIMPs), cystatin C (cysC), and calpastatin inhibit these proteases, may act as growth factors, and are susceptible to degradation by cathepsins and serine proteases.[3–5,7–9] A transient imbalance between activated proteases and their endogenous inhibitors is physiological, but a persistent imbalance induces cell death and ECM degradation.[10, 11]

Calpain and lysosomal cathepsins (types B, L, and S) are cysteine proteases. The ubiquitous endo- and exopeptidase cathepsin-B (E.C.3.4.22.1) and the endopeptidase cathepsin-L (E.C.3.4.22.15) are normally active at acidic pH. However, cathepsins can be protected by sulfated glucosaminoglycans (GAGs) to remain active at neutral pH.[3,12] In contrast, endopeptidase cathepsin-S (E.C.3.4.22.27), with restricted tissue distribution and specific functions, is active both at acidic and neutral pH.[13] Protease activity is regulated at the transcription level by growth factors, hormones, and cytokines and at the protein level by metal ions, inhibitory peptide cleavage, and endogenous inhibitors.[3–5] Tumor necrosis factor-α, platelet-derived growth factor, basic fibroblast growth factor, and interferon-γ increase cathepsin-B and/or -L induction and secretion by synovial fibroblasts.[14] Cathepsins are released or secreted intracellularly and extracellularly and are also localized in the ECM, where they can participate in cell death and tumor metastasis.[15,16]

Between PN0 and -10, mouse retinal ECM must be remodeled by proteases for neuronal and vascular network restructuring, which originates at the optic nerve head.[17] The MMPs and elastases degrade collagens, forming antiangiogenic peptides like endostatins which are further cleaved by cysteine cathepsins but not by MMPs.[7,12,18] An imbalance in the MMP/TIMP axis activates microglial cells to degrade ECM in *rd1* mouse retinas,[3] but this process may require other proteases such as lysosomal cathepsins and serine proteases. Both MMPs and cysteine cathepsins process cytosolic and ECM-membrane–associated molecules for physiopathologic angiogenesis

and retinal cell death.[19,20] Thus, remodeling and degeneration of the *rd1* mouse retina can be expected to be influenced by the imbalance between active proteases and their endogenous inhibitors.[21,22] However, such relationships have not been investigated in the retina.

Increased reactive oxygen species oxidize lipids, proteins, and nucleic acids and permeabilize membranes of cells and cell organelles, which results in the release of hydrolases. Aberrant and oxidized proteins are degraded by the proteasome system and by active lysosomal cathepsins.[3–5,22,23] The intracellular and extracellular leakage of cathepsins and their participation in cell death have also been reported in some well-defined models of apoptosis.[15] Cathepsins and cysC are expressed in all cell types, including those of the retina.[24,25] Retinal activities of calpains, cathepsin-S, and other unidentified lysozymes are increased in several animal models of retinal degeneration (e.g., *rd1*, *rds*, and BALB/c mice under oxidative stress) but their specific roles in cell death are not fully explained.[15,26–29]

The interrelationship between the activities of different types of proteases through auto- and heteroprotease activation, inhibition by their respective endogenous inhibitors, and cleavage of the latter has been poorly delineated in retinal physiopathology. Therefore, in continuation of our previous work on an imbalance in the MMP/TIMP axis, we evaluated the status of cathepsins and cysC in the developing and degenerating retina.

METHODS

Animals and Collection of Retinal Tissue

Congenic *wt* and homozygous *rd1* C3H mice from the animal colonies at Lund University were used. PN2 and -7 pups were decapitated and PN14, -21, and -28 mice were asphyxiated on dry ice, and the eyes were enucleated. Retinas with attached pigment epithelium (RPE) freed from the choroid were dissected and frozen at −80°C until analyzed. For immunohistochemical studies, dissected eyes were immediately fixed, cryoprotected, and sectioned, and the frozen sections were stored at −20°C for immunohistochemical evaluation. All experiments were conducted with the approval of the Swedish National Animal Care and Ethics Committee (M-213-03) and according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation and Analyses of Retinal Extracts

From each age group at the ages of PN2, -7, -14, -21, and -28, four to six *wt* and four to six *rd1* mice were taken, and the two retinas from each mouse were pooled separately, homogenized in HEPES buffer (pH 7.2, 20 mM; [4-2-hydroxyethyl-1-piperazineethane sulfonic acid], 4% CHAPS [3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate], and 0.5 mM EDTA) and analyzed in duplicate for proteins, cysteine cathepsins and cysC.

Proteins

Total proteins were estimated by using a micromethod adaptation of a kit (D_C kit; BioRad Laboratories, Hercules, CA) for protein assay. Absorbance was recorded with a multiwell plate reader (Multiskan Ascent; Thermo Labsystems, Helsinki, Finland).

Cysteine Cathepsin. The fluorogenic substrates carbobenzyloxy-L-phenyl-L-arginylamido-4-methyl coumarin and carbobenzyloxy-L-arginyl-L-arginylamido-4-methyl coumarin were used, respectively, for measuring total cysteine protease cathepsin and cathepsin-B in retinal extracts. Complete inhibition of substrate cleavage with E-64 (*N*-[*N*-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine), a general inhibitor of cathepsin type cysteine proteases, confirmed the cysteine protease activity. Total cathepsin and cathepsin-B activities were measured at room temperature for up to 3 hours after activation in 0.4 M phosphate buffer at pH 6.5 (containing DL-dithiothreitol to activate cysteine cathepsin and EDTA to inhibit metal ion requiring proteases). *N*-methyl coumarin (NMeC) served as a standard. The fluorescence was measured in a microplate fluorometer (FluoStar+ Optima; BMG Labtechnologies, Offenburg, Germany) at excitation/emission wavelengths of 390/460 nm. Cathepsin-S activity in retinal extracts was measured at pH 6.5, as described for cathepsin-B after inactivation of other cysteine cathepsins at pH 7.5 in phosphate buffer, 37°C for 60 minutes.[13] Cathepsin-L activity was obtained by subtracting the sum of cathepsin-B and -S from that of total cysteine cathepsin.

Cystatin C. CysC content in retinal extracts was measured by ELISA with a multiwell plate reader (Molecular Devices Corp., Sunnyvale, CA).[25,30] The ratios of cysteine cathepsin activities to cysC were determined, as they indicates the extent of inhibition of proteolysis.

Fluorescent Immunohistochemical Localization of CysC and Cathepsin-B

Eight-micrometer-thick sections of frozen eyes embedded in an albumin-glycerin medium were cut on a cryostat, assembled on glass slides and fixed for 10 minutes at 20°C in cold methanol-acetic acid (3:1, vol/vol). The sections were then rinsed three times (5 minutes each) in phosphate-buffered saline (PBS), and preincubated for 30 minutes in PBS containing 1% bovine serum albumin and 0.25% Triton X-100. Primary antibodies were used for spatial localization of cysC (rabbit polyclonal antibodies[25,30] against human cysC, dilution 1:500) and cathepsin-B (mouse monoclonal antibodies, dilution 1:200; Oncogene Research Products, San Diego, CA). Appropriate secondary antibodies conjugated to Alexa fluorophores were used to visualize the cellular binding sites of the primary antibodies. In negative control subjects, primary antibodies were omitted. Immunostained sections were photographed with a fluorescence microscope (Nikon, Tokyo, Japan), equipped with a digital camera (DP-70; Olympus, Tokyo, Japan. For preparation of the figures, all images were transferred to image-management software (Photoshop; Adobe, San Diego, CA), and identical adjustments in brightness were applied to all images.

In Vitro Culture of Retinal Explants and Analyses of Conditioned Medium

Retinal explants generated from PN2 and -7 *wt* and *rd1* mice were cultured in R-16 medium for 26 and 21 days, respectively. Their histologic characteristics are similar to those of the age-matched *wt* and *rd1* retinas developing in vivo.[31] The R-16 medium was replenished at 2- to 3-day intervals. The in vitro secretion of cysteine proteases and cysC by *wt* and *rd1* explants into retinal conditioned medium (RCM) would be commensurate with their secretion into retinal ECM in vivo.[15,16] The RCM collected and stored at −20°C was analyzed in duplicate for cysteine proteases and cysC. The RCM collected over 21 days or the last 11 days of culture of PN7 explants correlated, respectively, to secretions from PN9–10 to PN28 and PN17 to -28 retinas in vivo.

Cysteine Proteases and CysC in RCM

BODIPY-FL casein conjugate (Invitrogen-Molecular Probes, Leiden, The Netherlands) was used as a general substrate for estimation of cysteine protease activity.[32] Cysteine proteases in RCM (100 μL), positive control (papain, a cysteine protease, 100 μL, 0.25 mg/mL; 1:100 dilution) and negative control (R-16 medium or 0.01% Brij-35 detergent) were measured. The change in fluorescence (ΔF) was determined with a luminescence spectrometer (LS 50B; Perkin Elmer, Boston, MA), before and after incubation for 2 hours by excitation/emission at 485/530 nm. The cysteine protease activity secreted into the RCM was expressed as an increase in fluorescence (ΔF/100 μL RCM per 2 hours). Complete inhibition of substrate cleavage with E-64, a general inhibitor of cathepsin type cysteine proteases, confirmed the cysteine protease activity. CysC content in RCM was measured as described for the retinal extract.[25,30]
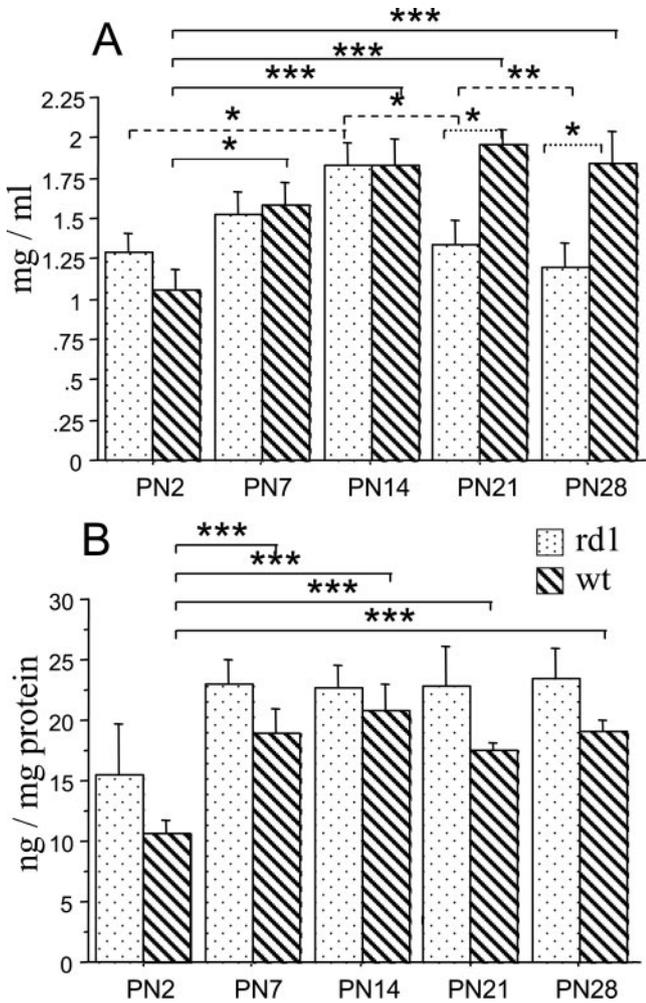
FIGURE 1.   (A) Total protein (two retinas were homogenized in 200 μL buffer) and (B) cysC contents in *wt* and *rd1* retinas at different stages of development: total proteins increased with age in *wt* retinas but decreased in *rd1* after a peak at PN14 (A). This result suggests a net protein biosynthesis in *wt* and *rd1* at the PN2–14 and degeneration in *rd1* retinas at the PN21–28 stages of development. At PN7–28, cysC was higher and unchanged in *rd1* compared with *wt* retinas (B), but the increase in cysC was insufficient to protect *rd1* retinas from degeneration. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *Solid brackets*: differences within *wt*; *dashed brackets*: differences within *rd1*; *dotted brackets*: differences between *wt* and *rd1*.

Statistical Analysis

Statistical analysis was by one-way analysis of variance and Fisher's protected least significant differences post hoc comparisons (StatView Software; SAS, Chicago, IL). $P < 0.05$, 0.01, and 0.001 represent the level of significance.

RESULTS

Protein Metabolism in *wt* and *rd1* Retinas

The overall protein content in *rd1* retinal extracts was significantly lower than that in *wt* extracts (Fig. 1A). The protein content in *wt* extracts increased significantly with age, whereas in *rd1* extracts, it did so up to PN14 and then decreased significantly at PN21 and -28. This indicated net protein biosynthesis in the former and degradation in the latter stages.

rd1 Retinal Imbalance in the Active Cathepsin/CysC Axis

At all ages, cysC content in *rd1* was higher than that in *wt* retinal extracts, and the levels in *wt* and *rd1* extracts at PN2 were lowest. In *wt* extracts, cysC content increased up to PN14 and then decreased, whereas in *rd1* it increased nonsignificantly up to PN7 and then remained unchanged and higher (overall) than that in *wt* extracts (Fig. 1B). CysC levels in *rd1* and *wt* extracts at PN7 to -28 were 1.5- and 1.8- to 2.0-fold higher, respectively, than those at PN2, suggesting that cysC may have effects other than inhibition of cathepsins (see corresponding cathepsin data in the following sections).

The higher cathepsin levels in *rd1* retinal extracts were independent of the variation in protein content (Fig. 2). Despite an increase in cysC levels, *rd1* extracts showed increased activity of cathepsins and suggested an imbalance in favor of cathepsins.

Total Cathepsins and CysC. Earlier, we reported (Fig. 2, Ref. 4) the total cysteine cathepsin activity in PN2, -7, -14, -21, and -28 *wt* and *rd1* retinas. The overall activity of total cysteine cathepsins in *rd1* retinal extracts was significantly higher than that in *wt* extracts.[4] Total activity of cysteine cathepsins in *wt* extracts at PN2 was highest and similar at later ages, but in *rd1*,
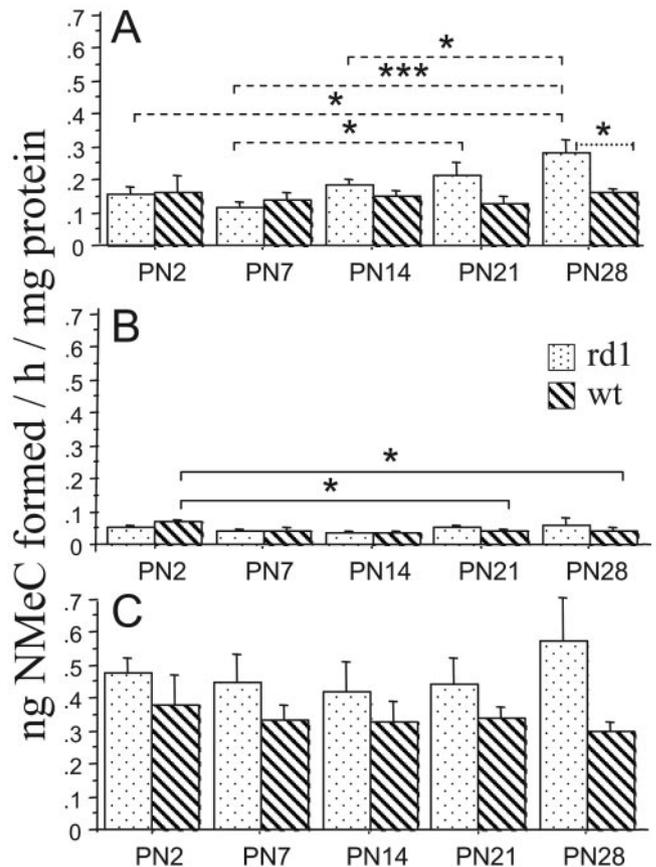

FIGURE 2.   Activities of cysteine cathepsins in *wt* and *rd1* retinas at different stages of development. (A) Cathepsin-B, (B) cathepsin-S, and (C) cathepsin-L. Cathepsin (total,[4] B, and S) activity increased with age in *rd1* but was unchanged (being highest at PN2) in *wt* retinas. Independent of the protein content, cathepsin activity was higher in *rd1* extracts. The proportion of cathepsin activity decreased in the following descending order: cathepsin-L < cathepsin-B < cathepsin-S. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *Solid brackets*: differences within *wt*; *dashed brackets*: differences within *rd1*; *dotted brackets*: differences between *wt* and *rd1*.
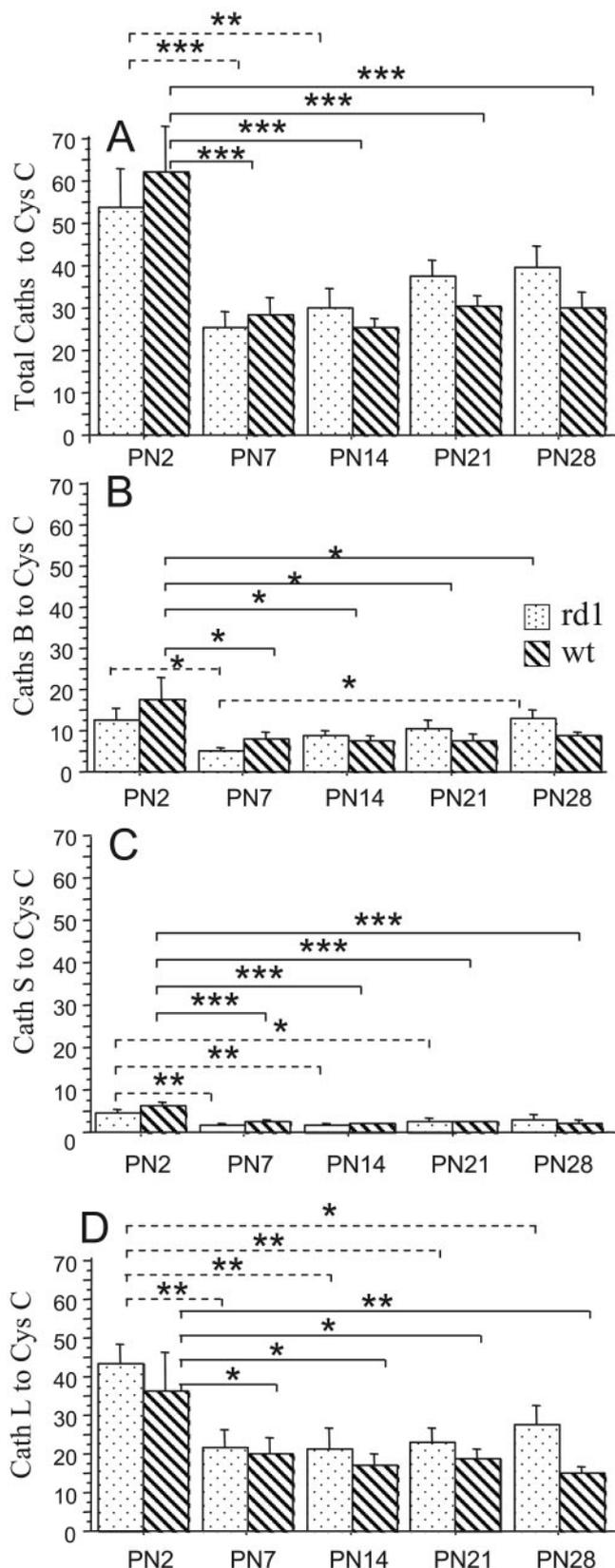
FIGURE 3. Ratio of cysteine cathepsins (Caths) to cysC in *wt* and *rd1* retinas at different stages of development. (**A**) Total cathepsin, (**B**) cathepsin-B, (**C**) cathepsin-S, and (**D**) cathepsin-L. The higher ratio of cysteine cathepsin activity (total, B, S, and L) to cysC at PN2, both in *rd1* and *wt* retinas, suggests lower inhibition of cathepsins to remodel ECM. Subsequently, in *rd1* but not in *wt* retinas, the ratio increased

it decreased at PN7 and then increased. Total cathepsin activity in *rd1* at PN21 and -28 was significantly higher than that in the corresponding *wt* extracts. The ratio of total cathepsin to cysC in *rd1* and *wt* extracts at PN2 was significantly higher than that at other ages (Fig. 3A). In *rd1* but not in *wt* extracts, this ratio increased from PN7 to -28.

Cathepsin-B and CysC. Overall, the cathepsin-B activity in *rd1* was significantly higher and increased with age compared with that in *wt* retinal extracts, which did not show age-dependent changes (Fig. 2A). The cathepsin-B-to-cysC ratio in *wt* extracts at PN2 was higher than at other ages, when it remained constant. In *rd1* extracts, the ratio decreased from PN2 to -7 and then increased with age (Fig. 3B).

Cathepsin-S and CysC. Cathepsin-S activity in *wt* retinal extracts at PN2 was significantly higher than at other ages, when it remained constant. In *rd1* extracts, it decreased until PN7 and then nonsignificantly increased with age (Fig. 2B). The cathepsin-S-to-cysC ratio in *wt* and *rd1* extracts at PN2 was significantly higher than at other ages. Thereafter, it remained constant in *wt* but increased in *rd1* extracts from PN7 to -28 (Fig. 3C).

Cathepsin-L and CysC. Overall, cathepsin-L activity in *rd1* was higher than that in *wt* retinal extracts and increased with age from PN14 to -28, when it was nonsignificantly higher. Cathepsin-L activity in *wt* extracts did not vary with age (Fig. 2C). Cathepsin-L to cysC ratios in *wt* and *rd1* extracts at PN2 were significantly higher than at other ages. After the age of PN2, the ratio was constant in *wt* but increased in PN 21 to -28 *rd1* extracts (Fig. 3D).

The rodless PN21 and -28 *rd1* retinas also showed significantly increased levels of cysC and activities of different types of cysteine cathepsins (Figs. 1, 2), suggesting that the inner retina, especially of *rd1* has higher activity of these proteases.

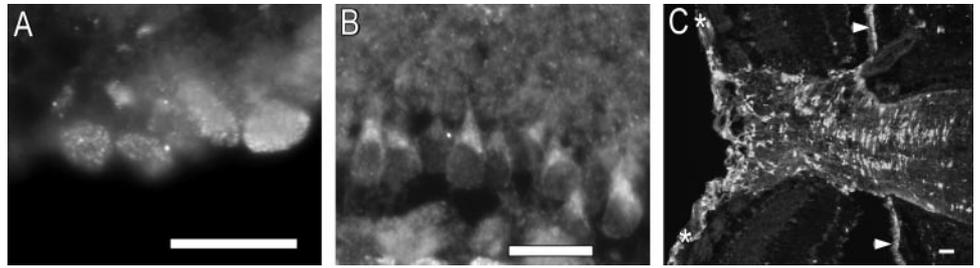## CysC and Cathepsin-B Localization in *wt* and *rd1* Retinas

The spatial localization of cysC in both *wt* and *rd1* retinas was similar, and the ame was the case for cathepsin-B. Retinal ganglion cells (RGCs) in *rd1* and *wt* retinas showed punctate cytosolic labeling for cathepsin-B (Fig. 4A) and polar cytosolic labeling for cysC (Fig. 4B). The cysC labeling decreased from the intraocular to intraorbital segment of the optic nerve. Extensive labeling for cysC in the optic nerve was probably localized to glial cells (Fig. 4C).

CysC Immunostaining in the Developing RPE and OLM in Retinas

***wt* Retinas.** At PN2 (Fig. 5A) the RPE, ganglion cell layer (GCL), subretinal space, and regions of the ILM showed cysC labeling. At PN7 (Fig. 5B) the outer limiting membrane (OLM), outer plexiform layer (OPL), and possibly the horizontal cells were also labeled. At PN14 to -28 (Figs. 5C–E) the RPE, OLM, inner retina (with some unidentified immunostained cells), RGCs, and ILM showed labeling for cysC. In the RGCs present on GCL, cysC labeling was focused at the proximal end at the point of entry of the processes into the inner plexiform layer (IPL). Immunostaining of RPE, OLM, and ILM increased with age, especially up to PN14.

with age but was significant only for cathepsin-B and suggested imbalance in the cathepsin/cysC axis, which is consistent with its role in retinal degeneration. The ratios of different cathepsin activity to cysC decreased in the following descending order: Total cathepsin < cathepsin L < cathepsin-B < cathepsin-S. *P < 0.05; **P < 0.01; ***P < 0.001. *Dotted brackets*: differences between *wt* and *rd1*; *solid brackets*: differences within *wt*; *dashed brackets*: differences within *rd1*.

FIGURE 4. Immunostaining for cathepsin-B and cysC. RGCs in *wt* and *rd1* retinas showed punctate cytosolic labeling for cathepsin-B (**A**) and polar cytosolic labeling for cysC (**B**, enlargement of Fig. 5H). Higher cathepsin-B may be involved in the developmental death of RGCs, both in *wt* and *rd1* retinas at PN 2 and -7; but, during degeneration in *rd1* retinas at PN 21 to -28, it may induce the RGCs to activate microglia cells. CysC-labeled structures situated perpendicular to the direction of the optic nerve (**C**) may represent astrocyte and oligodendrocyte glial cells surrounding the optic nerve axons. The extensive cysC label decreased in descending order from the optic nerve head in the intraocular through intraorbital segments of the optic nerve, suggesting higher synthesis of cysC in the optic nerve. (**A–C**) Immunolabeling in PN21, -14, -14 *rd1* retinas, respectively. ✶, *arrowhead*, respectively, indicate the GCL and RPE. Scale bar, 20 μm.

rd1 Retinas. At PN2 and -7 (Figs. 5F, 5G) the spatial localization of cysC was similar to that for age-matched *wt* retinas. At PN14 to -28 (Figs. 5H–J) the RPE, inner retina (with relatively more unidentified immunostained cells), and GCL showed increased label for cysC.

Cytosolic Cathepsin-B Labeling of RGCs

wt Retinas. At PN2 the RPE, subretinal space, IPL, GCL, and ILM showed cathepsin-B labeling (Fig. 6A). At PN7 the RPE, OLM, OPL, INL, IPL, GCL, and ILM showed cathepsin-B labeling (Fig. 6B). At PN14 to -28, the RPE, OLM, inner retina (with some unidentified immunostained cells), GCL, and ILM showed cathepsin-B labeling (Figs. 6C–E).

rd1 Retinas. At PN2 and -7 (Figs. 6F, 6G) the spatial localization of cathepsin-B in *rd1* was similar to that for age-matched *wt* retinas. At PN14 to -28 (Figs. 6H–J) the inner retina (with relatively more unidentified immunostained cells) and RGCs showed cathepsin-B labeling.

Secretion of Active Cysteine Proteases by Explants into RCM

PN2 explants had limited ability to secrete cysC and cysteine proteases, as their secretion decreased to negligible levels after 6 days in culture (data not shown). PN7 *rd1* explants secreted higher levels of cysC and active cysteine protease into the RCM collected both during all the 21 days and the last 11 days in culture (Table 1). Such results are consistent with the extracellular secretion of cysteine proteases and MMPs (Ahuja S, Ahuja P, Caffé AR, Abrahamson M, Ekström PAR, van Veen T, unpublished data, 2003) for their in vivo role in physiopathologic hydrolysis of ECM-associated proteins in different cell layers of retina.

Discussion

The *rd1* mouse retina shows degeneration of rod photoreceptors, swelling of mitochondria, increased sulfated GAGs, decreased proteins, imbalances in MMP/TIMP, and remodeling of
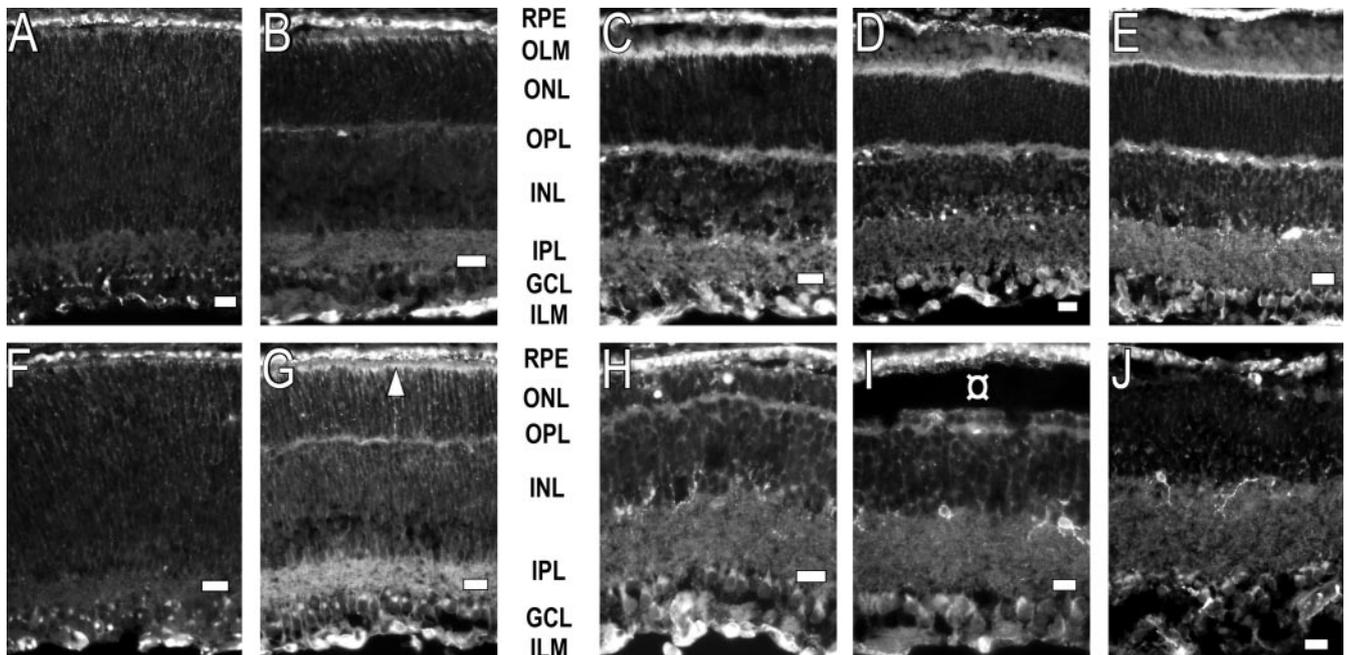

FIGURE 5. Cellular labeling for cysC in developing *wt* (**A–E**) and *rd1* (**F–J**) retinas: PN2 (**A, F**), PN7 (**B, G**), PN14 (**C, H**), PN21 (**D, I**), and PN28 (**E, J**). The *wt* retinas at PN2 showed cysC labeling in the RPE, GCL, and ILM (**A**). At PN7 cysC labeling was also observed in the OLM (*arrowhead*) and OPL (**B**); at PN14, -21, and -28, increasing cysC labeling was seen in the RPE, OLM, inner retina, GCL, and ILM (**C–E**). The *rd1* retinas at PN2–7 had cysC labeling similar to that in age-matched *wt* retinas (**F, G**); at PN14, -21, and -28, the RPE, INL, GCL, and ILM showed higher labeling for cysC (**H–J**). (¤) Gap in the section created during cryosectioning of the PN21 *rd1* retina (**I**). Scale bar, 20 μm.
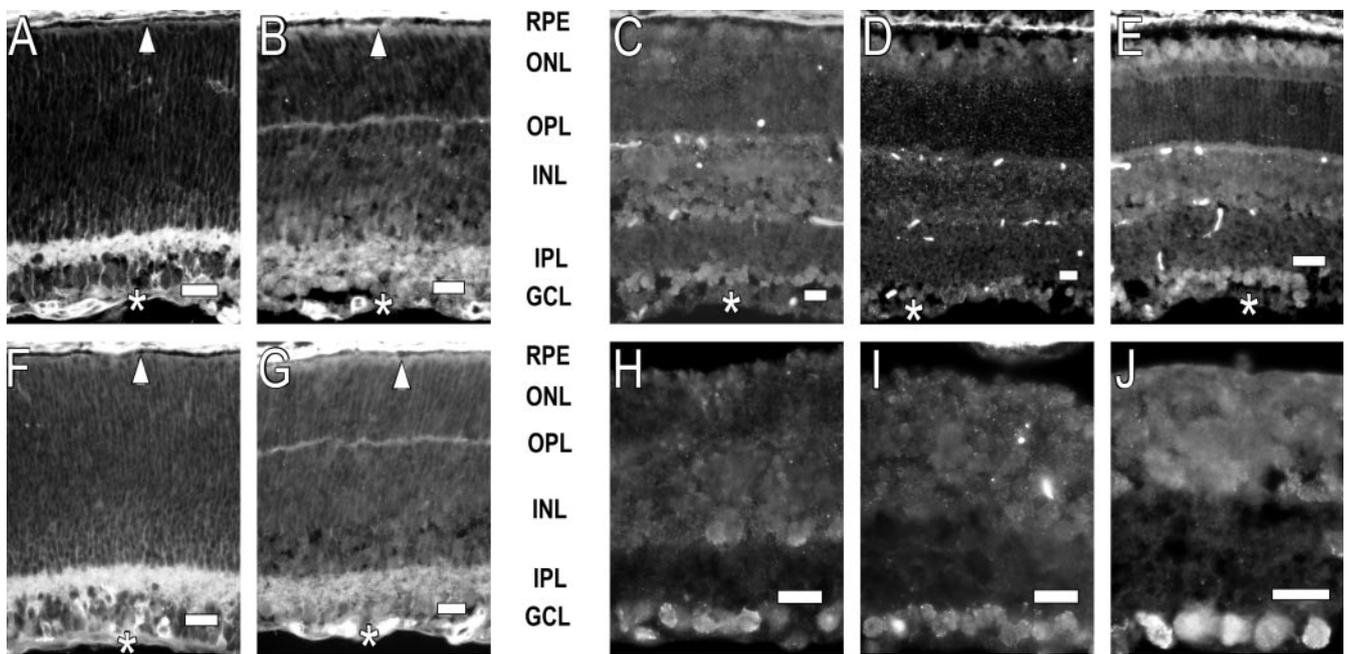
FIGURE 6. Cellular labeling for cathepsin-B in developing *wt* (**A–E**) and *rd1* (**F-J**) retinas: PN2 (**A, F**), PN7 (**B, G**), PN14 (**C, H**), PN21 (**D, I**), and PN28 (**E, J**). Both *wt* and *rd1* retinas at PN2 showed cathepsin-B labeling in RPE, subretinal space (*arrowhead*), IPL, ILM (✱) and GCL (**A, F**); at PN7 cathepsin-B labeling was also seen in the OPL and INL (**B, G**). Cathepsin-B immunostaining in the IPL and GCL at PN 2 and -7 suggests synaptogenesis and developmental death of RGCs in both genotypes. At PN14, -21, and -28, the *wt* (**C–E**) and *rd1* retinas (**H-J**) showed cathepsin-B labeling in the RPE (part of the section not included in **H, J**), INL, and GCL but not in the OPL and ILM. Scale bar, 20 μm.

neural and vascular networks. Some of these changes are observed in retinal cells, even during differentiation.[1,3,21,33] The present study also showed an imbalance in the cysteine cathepsin/cysC axis. The transient release or secretion of cathepsin-B, -L, and -S remodeled the ECM to accommodate both developing vasculature and differentiating and migrating cells. However, their persistence degenerates the ECM in retina and other tissues.[17,34,35] The increased Ca^{2+} ion concentrations reported in the *rd1* retina[1] may activate membrane bound μ-calpain, which in turn releases lysosomal cathepsin-B, as reported during the metastasis of tumors.[15,16,29] Furthermore, reactive oxygen species induce various degrees of lysosomal permeabilization during transient brain ischemia and *rd1* retinal degeneration.[1,23] Depending on the extent of lysosomal permeabilization in cultured mouse macrophages and apoptosis model cells, the released cathepsin-B activates caspases and proapoptotic proteins and determines the level of cell death by a calpain-cysteine cathepsin lysosomal pathway.[15,23,36]

TABLE 1. Secretion of Cysteine Proteases and Cystatin C into the RCM by PN7 *wt* and *rd1* Retinal Explants Cultured In Vitro for 21 Days

	wt (n = 9)	*rd1* (n = 9)
Cysteine proteases*‡	6.44 ± 1.60	12.00 ± 2.38
Cysteine proteases*§	3.83 ± 0.54	8.83 ± 1.89
CysC†	0.195 ± 0.03	0.257 ± 0.06

Data are expressed as the mean ± SE.
* ΔF/100 μL RCM.
† μg/ml RCM/day.
‡§: Analyses of RCM collected at 2–3 day intervals during ‡the total period of 21 days and §the last 11 days in culture. These data represent secretions from PN9–10 to -28 and PN17 to -28 retinas in vivo. After 21 days in culture PN7 *wt* explants mature and *rd1* explants showed degeneration of rods similar to that seen in vivo.[31]

Function of Active Cathepsins in PN2 and -7, and PN21 and -28 *rd1* Retinas

A transitory but inconsistent imbalance in proteases and their endogenous inhibitors is critical for remodeling ECM during retinal development. Different proteases would release growth factors and adhesion molecules into the ECM at PN2 and -7 which modify signaling processes and retinal development, as has been reported in the contexts of ischemia and cell death.[7,17,20,34,35] The secretion or release of cysteine proteases into the RCM demonstrated in the present study is consistent with their in vivo role in the hydrolysis of proteoglycans in the ECM. At PN2 and -7, the higher ratio of cathepsin (total, B and S, but not L) activity to cysC in *wt* compared with *rd1* retinas indicated a relatively higher activity of cathepsins in *wt* extract. Therefore, similar to the imbalance in the MMP/TIMP axis, the temporarily increased cathepsin (total, B, and S) activity and the cysC and protein contents in the *wt* retinal extracts at PN2 and -7 are consistent with the physiological role of cathepsins during development of the retina.[3] Although *rd1* retinas at PN2 and -7 also showed a trend similar to that in *wt*, a pathologic source of these changes cannot be ruled out.[1,3,21,33] In fact, abnormal remodeling of ECM has been shown when cysC inhibits cathepsin-S and -B and also during a combined but not an individual decrease in the activities of cathepsin-B and MMP-9.[37,38] Because cathepsin-S is required for growth of microvasculature, the higher activities of cathepsin-S and -B in PN2 *wt* and *rd1* retinas would be favorable for retinal remodeling at this stage.[39]

Despite the increase in cysC, cathepsin (total, B, S, and L)-to-cysC ratios in *rd1* retinal extracts at PN14 to -28 and the corresponding RCM were higher and indicated an imbalance between the two in favor of the cathepsins. Persistently higher cathepsin (total, B, S, and L) activity relative to cysC and decreased proteins in PN 21 to -28 *rd1* retinal extracts are consistent with a pathologic role in the degradation of retinal

ECM and the development of RP.[40] These findings are complementary to those in earlier studies showing an imbalance of the MMP/TIMP axis in the *rd1* mouse retina.[3] Independent of its inhibitory effect, an increased cysC level also suppresses the expression of cathepsins during oxidative stress and, by unknown mechanisms, modifies glial cell development, neuronal stem cell proliferation, and protect rat nigral dopaminergic neurons.[3,14,41–44] However, the increased levels of cysC/TIMP in *rd1* retinas at PN 21 to -28 may be insufficient to rescue the neurons by these mechanisms. The presence of cysC and cathepsin-B observed in RPE, INL, and GCL layers of *wt* and *rd1* retinas during this study have also been reported in other species.[15,24–27] This localization along with higher cathepsin (total, B, S, and L) activity in *rd1* retinal extracts and RCM are consistent with a role of cathepsins in the lysosomal pathway of *rd1* retinal degeneration.

In our study, *rd1* retinal degeneration involved oxidative stress, which correlated with an imbalance in the activities of different types of proteases relative to the levels of their respective endogenous inhibitors. Such a link between oxidative stress and proteases is also indicated in neuronal and PC12 cells protected from oxidative stress by proteasome inhibition and by increased cysC expression.[5,44] Therefore, antioxidant and protease-inhibitor–based therapies to rescue photoreceptors and inner retinal cells are worth investigation.

Cysteine Cathepsins and MMP Action

Cathepsin-L is 10- to 100-fold more active than cathepsin-B on most protein substrates, and cathepsin-S is active at neutral pH.[19,20] The increased potential of cathepsin-L, -S, and -B for proteolysis and decreased protein content in *rd1* retinas at PN21 to -28 represent degradation of ECM-associated proteins during retinal degeneration. These results are in agreement with the role of both cathepsins and MMPs in the remodeling of ECM.[34]

CysC Labeling in *wt* and *rd1* during Retinal Development

During photoreceptor differentiation in *wt* and *rd1* retinas at PN2 and -7, cysC labeling in the subretinal space increased with age. Similarly, cysC labeling in rat RPE increases after the embryonic stage.[23] CysC labeling was extensive in the RPE, OLM, and optic nerve head fibers at the intraocular and intraorbital segments; moderate in the GCL; and lowest in some cells of the INL of both genotypes. The cysC-labeled structures perpendicular to the direction of the optic nerve may be astrocyte and oligodendrocyte glial cells surrounding the optic nerve axons. Different glial cell types have been reported in the intraocular and intraorbital segments of the optic nerve.[45] Extensive cysC labeling in the optic nerve fibers and at the origin of the RGC dendrites suggests considerable cysC synthesis in these structures. CysC expressed and produced mainly by glial cells may be transported axonally from the optic nerve segments to the retina for sustenance, as has been reported for neurotrophins.[46,47] This finding was also supported by the transport of fluorescence-labeled cysC from the vitreous body to all retinal cell types normally showing cysC in rat retina.[48]

Higher cysteine cathepsin activity in *wt* and *rd1* retinas and punctate lysosomal localization of cathepsin-B in the GCL at PN2 and -7 indicated that cathepsins may participate in the developmental death of RGCs, which normally peaks between PN2 and -5.[49] However, higher cathepsin-B activity in *rd1* retina at PN 21 to -28 may induce RGCs to recruit and activate microglia cells, as reported in cultured rat retina.[50]

The present results showed similar localization of both cathepsin-B and cysC in the RPE, INL, and GCL regions of *wt* and *rd1* retinas; increased in vitro secretion of active cysteine proteases by PN7 *rd1* explants; and increased cysteine cathepsin (total, B, S, and L) activity in PN14 to -28 *rd1* and PN2 and -7 *wt* and *rd1* retinal extracts, with respectively decreased and increased protein content. Such results are consistent with the localization of imbalances in the active cathepsins/cysC axis in the ECM associated with the RPE, INL, and GCL of *wt* and *rd1* retina and with its role in physiopathologic remodeling of ECM at these sites.

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