Opticin Binds Retinal Growth Hormone in the Embryonic Vitreous

Esmond J. Sanders,[1] *Michael A. Walter,*[2] *Eve Parker,*[1] *Carlos Arámburo,*[3] *and Steve Harvey*[1]

Purpose. Opticin is a small leucine-rich repeat proteoglycan that is abundant in several ocular tissues, including the vitreous. Like other proteoglycans, opticin may have the ability to bind and regulate the release of growth factors. Previous work has shown that isoforms of growth hormone (GH) are present in the embryonic retina, where they may act as a growth factor. The current study was conducted to investigate the possibility that opticin binds retinal GH in the vitreous of the chick embryo.

Methods. The vitreous and retina of embryonic day-8 chicks were examined for the presence of opticin and GH, by Western immunoblot analysis, coimmunoprecipitation, and immunocytochemistry.

Results. Opticin associated with GH in the embryonic vitreous to produce a 60- to 62-kDa complex. Opticin and GH were also colocalized in the retina in retinal ganglion cells.

Conclusions. The binding of retinal GH by opticin in the vitreous suggests that GH, secreted by the retinal ganglion cells, may be sequestered and concentrated in the vitreous and could act there as a paracrine differentiation factor in ocular development. During development, opticin could therefore regulate growth factor–like actions of retinal GH, both in the vitreous and the retinal ganglion cells. The physiological roles of GH in this location remain to be determined, but may include the regulation of cell proliferation and cell death. (*Invest Ophthalmol Vis Sci.* 2003;44:5404–5409) DOI: 10.1167/iovs.03-0500

It is now well established that growth hormone (GH) is not solely a pituitary-derived endocrine hormone, but is also synthesized in extrapituitary sites, where it can act as a paracrine growth factor.[1] Further, GH is widely distributed in tissues of the early chick embryo, before the morphogenesis of the pituitary gland is complete[2] and probably acts as an important paracrine differentiation factor during early development.[3] We have shown that growth hormone–immunoreactive proteins are readily detectable in the embryonic neural retina of the chick,[4] and we have speculated that embryonic retinal GH has hitherto unsuspected roles in ocular development and differentiation.[5] This possibility is supported by studies that show that GH is able to act as an angiogenic factor in a variety of tissues,[6] including the retina,[7] and may promote neural differentiation[8] and cell survival.[9,10]

Opticin, or oculoglycan, is a 35- to 45-kDa proteoglycan, belonging to the small leucine-rich repeat proteoglycan (SLRP) family, which is abundant in several ocular tissues of the adult mouse, bovine, and human, including the iris, ciliary body, choroid, and retina, as well as in the vitreous.[11–13] There are species differences in molecular size, possibly due to differences in glycosylation and/or posttranslational modification.[12] The molecule consists of a signal peptide of 19 amino acids and two structural domains, of which one is the leucine-rich repeat (LRR) consisting of six such repeats.[12,13] Of relevance to the present study, the LRR domain contains variable repeat motifs forming potential binding sites for other molecules.[12] In addition, the N-terminal region contains a cluster of potential O-glycosylation sites, which appears to be unique among other known extracellular LRR proteins and which may be important to the binding function of opticin.[13] Within the different ocular tissues there are a number of splice variants of the opticin molecule,[14] but there has been no assessment of the potential physiological significance of these variants.

In the mammalian vitreous, opticin is a collagen fibril-associated macromolecule,[15,16] and in this location it is one of several proteoglycans that make up the bulk of the vitreous.[17] The function of opticin in the vitreous has thus been assumed to be associated with collagen fibrillogenesis and in the maintenance of collagen fibril spacing[15]; however, there has been speculation that the persistence of opticin expression in the adult may indicate that it has other, hitherto undefined functions.[16] It may also be pertinent that the opticin gene maps to the long arm of chromosome 1 in region 31-32, which is near the loci associated with age-related macular degeneration (1q25-q31) and retinitis pigmentosa (1q31-q32).[11]

In general, proteoglycans are known to perform a variety of biological functions,[18] including acting as binding factors for several different growth factors,[19] such as basic fibroblast growth factor[20] and transforming growth factor-β.[21] Therefore, because of the abundance of opticin in the vitreous and its presence in the retina itself, and because we speculate that GH derived from the retina acts as an embryonic growth factor in ocular tissues, we investigated the possibility that opticin binds and concentrates retinal GH in the vitreous of the chick embryo.

Materials and Methods

Fertilized White Leghorn hens' eggs were incubated at 37°C for 8 days, and the embryos were removed and washed in Tyrode's saline. Vitreous was taken from embryos at embryonic day (ED) 8 as well as from adult chickens. Retinas were taken from ED8 embryos only, as that is the time when GH is known to be present in the neural retina and vitreous[5] and when the retinal ganglion cells (RGCs) are undergoing differentiation and apoptosis.[22,23]

Animals were handled in accordance with the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

From the Departments of [1]Physiology and [2]Ophthalmology, University of Alberta, Edmonton, Alberta, Canada; and the [3]Department of Cellular and Molecular Neurobiology, Institute of Neurobiology, Universidad Nacional Autónoma de Mexico, Querétaro, Mexico.

Supported by an operating grant from the Canadian Institutes of Health Research and Grants 41218 from CONACYT and IN226002-3; PAPIIT University Nacional Autónoma de Mexico.

Submitted for publication May 21, 2003; revised July 7, 2003; accepted July 31, 2003.

Disclosure: **E.J. Sanders**, None; **M.A. Walter**, None; **E. Parker**, None; **C. Arámburo**, None; **S. Harvey**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Esmond J. Sanders, Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 1Y6; esmond.sanders@ualberta.ca.

Investigative Ophthalmology & Visual Science, December 2003, Vol. 44, No. 12
Copyright © Association for Research in Vision and Ophthalmology

Downloaded from iovs.arvojournals.org on 06/27/2019

Source of Reagents

A 15-kDa fragment of recombinant chicken GH (rcGH) was obtained after thrombin digestion of the hormone and was purified as described elsewhere.[24] This digest is similar to a 15-kDa GH variant present in the chicken pituitary gland.[24] Rabbit antibody to chicken pituitary GH was prepared as described previously.[25] Anti-opticin antibodies were raised in chickens against a 19-kDa peptide encompassing residues 24 through 42 of the human opticin protein.[26] The extent of homology of this human peptide to the chicken opticin molecule is not known. This particular 19-kDa peptide was originally selected because it distinguishes opticin from other proteoglycans, but is similar to other known opticins.

Immunoprecipitation and Western Immunoblotting

For immunoprecipitation, antibodies to opticin (dilution, 1:50) and chicken GH (dilution, 1:1000) were used, corresponding to 1 to 5 μg antibody protein. The antibody was mixed with 100 μL (200–500 μg protein) of vitreous in immunoprecipitation buffer, (in 0.5% sodium dodecyl sulfate, 1.0 mM sodium orthovanadate, 10 mM Tris buffer [pH 7.4], for denaturing conditions) with 400 μL water and 500 μL of 2$\times$ immunoprecipitation buffer. The mixture was vortexed and incubated at 4°C for 1 hour. Protein A-Sepharose (50 μL; Amersham Biosciences, Montréal, Québec, Canada) was added, vortexed, and incubated overnight with agitation. After centrifugation (16,000*g* for 4 minutes) and two washes in immunoprecipitation buffer, the pellet was resuspended in 30 μL sample buffer containing β-mercaptoethanol, boiled for 5 minutes, and centrifuged again.

For polyacrylamide electrophoresis, 30 μL of supernatant was added to each well of a 8% gel, and run for 48 minutes at 140 V. Retina lysates were prepared in protease-inhibitor buffer, containing 15 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL pepstatin, and 1.74 mg/mL phenylmethylsulfonyl fluoride (PMSF), and 20 μg of this lysate protein in 2$\times$ sample buffer containing bromophenol blue and β-mercaptoethanol was loaded in each lane. Samples were transferred from the gels onto a supported nitrocellulose membrane at 100 V for 2 hours. For immunoblot analysis, membrane blocking was performed with 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20. Primary antibodies were incubated for 18 hours at 4°C followed by four washes for 10 minutes each. For staining GH bands, biotinylated goat secondary antibodies (1:100 dilution; Sigma-Aldrich Co., St. Louis, MO) were used for 1.5 hours at room temperature (RT). Proteins were peroxidase labeled (Vectastain ABC Reagent; Vector Laboratories Inc., Burlington, Ontario) for 1.5 hours at RT. For staining opticin bands, peroxidase-conjugated goat secondary antibodies were used at a dilution of 1:100. In both cases, the chemiluminescence reaction (ECL; Amersham Biosciences) was used for color development. Negative controls were performed using the primary antibody after preabsorption with antigen.

Preabsorption of anti-GH antibody was performed by mixing 1 mg/mL recombinant chicken GH with the antiserum at a dilution of 1:3000 (Amgen, Thousand Oaks, CA). Preabsorption of the anti-opticin antibody was performed by mixing equal parts of the antibody with the peptide antigen used to raise the antibody.

Immunocytochemistry

RGCs were identified in formaldehyde-fixed paraffin sections of ED8 retina using the anti-islet1 mouse monoclonal antibody, 39.4D5,[22,27–28] obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Sections were labeled with undiluted 39.4D5 antibody overnight at 4°C, followed by the secondary antibody, goat-anti-mouse IgG conjugated to AlexaFluor 594 (1:200; Molecular Probes Inc., Eugene, OR), for 1.5 hours at room temperature. The blocking agent was 4% bovine serum albumin. For double labeling for opticin, sections were then incubated with anti-opticin antibody (dilution, 1:100) for 1 hour at room temperature, followed by the appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100) for 1 hour at room temperature. For double labeling for GH after RGC labeling, sections were incubated with the anti-GH antibody (1:500, overnight at 4°C) followed by the appropriate secondary antibody. The labeled sections were examined by confocal microscope (model LSM510; Carl Zeiss Meditec, Thornwood, NY) equipped with appropriate lasers. Control experiments, in which the antibodies were replaced by nonimmune serum, were negative.

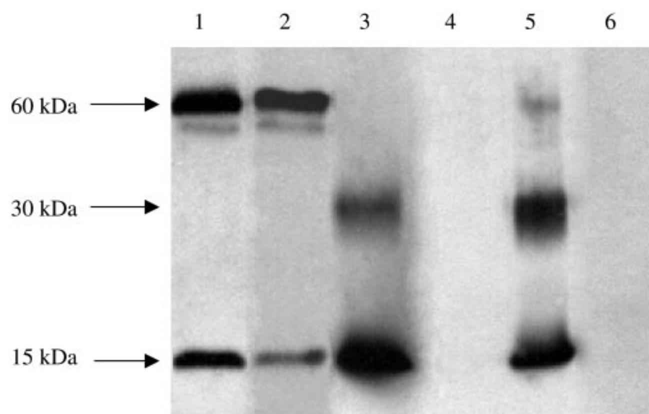

Figure 1. Growth hormone immunoreactivity of embryonic and adult vitreous. *Lane 1*: ED8 vitreous; *lane 2*: adult chicken vitreous; *lane 3*: a purified sample of 15-kDa growth hormone; *lane 4*: a sample of the 19-kDa fragment of the opticin molecule, used to generate the opticin antibody; *lane 5*: a mixture of 15-kDa growth hormone and opticin; *lane 6*: as *lane 3*, but immunoblotted with anti-growth hormone preabsorbed with 15-kDa growth hormone.

Results

Immunoblot Analysis for Growth Hormone and Opticin in the Vitreous

Figure 1 shows the result of Western immunoblot analysis with an antibody to GH on ED8 (lane 1) and adult (lane 2) chicken vitreous. Both samples showed a specific band at 15 kDa,[5] and higher molecular weight bands at approximately 60 to 62 kDa. The 15-kDa band corresponded with the reactivity of a purified sample of 15-kDa GH N-terminal fragment (lane 3), which has been isolated from a thrombin digest of rcGH.[24] This fragment apparently dimerized to form an immunoreactive band also at 30 kDa. The GH antibody did not react with the 19-kDa fragment of the opticin molecule (lane 4) that was used to generate opticin antibodies. Mixing 15-kDa GH with opticin (lane 5) in equal parts (overnight at 4°C) resulted in the expected bands at 15 and 30 kDa, and in addition a band at approximately 60 kDa, corresponding to those seen in lanes 1 and 2. Preabsorption of the GH antibody with chicken GH abolished the immunoreactivity of the antibody against the 15-kDa fragment (lane 6).

Figure 2 shows the result of Western immunoblot analysis with an antibody to opticin on ED8 (lane 1) and adult (lane 2) chicken vitreous. The embryonic vitreous showed immunoreactive bands at 60 to 62 kDa, whereas the adult vitreous showed immunoreactivity at 45 kDa. The antibody did not cross-react with the 15-kDa GH fragment (lane 3), but showed intense reactivity against the 19-kDa opticin fragment (lane 4) used to produce the antibody. Preabsorption of the antibody with the 19-kDa fragment abolished immunoreactivity (lane 5).

Coimmunoprecipitation of the Vitreous

Coimmunoprecipitation was performed both with anti-opticin antibodies and with anti-GH antibodies. Figure 3 shows the result of immunoprecipitation of ED8 vitreous with anti-opticin followed by immunoblot analysis with anti-GH. Samples run under denaturing (lane 1) and nondenaturing (lane 2) conditions both showed a single GH-immunoreactive band at 60

Downloaded from iovs.arvojournals.org on 06/27/2019

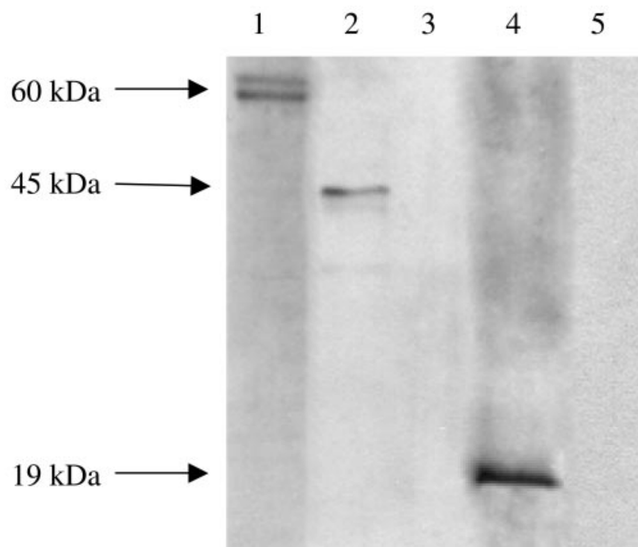

FIGURE 2. Opticin immunoreactivity of embryonic and adult vitreous. *Lane 1*: ED8 vitreous; *lane 2*: adult chicken vitreous; *lane 3*: 15-kDa growth hormone; *lane 4*: 19-kDa opticin fragment; *lane 5*: as *lane 4*, but immunoblotted with anti-opticin preabsorbed with the opticin fragment.

kDa, corresponding to a similar band in nonimmunoprecipitated vitreous run simultaneously (lane 3). Comparison of the intensity of the bands in lanes 2 and 3 indicates that some material was lost during immunoprecipitation, possibly accounting for the loss of the second band in this region. The nonimmunoprecipitated sample (lane 3) also showed a band at 15 kDa corresponding with a band at the same molecular weight for purified 15-kDa GH (lane 4). As shown also in Figure 1, the 15-kDa sample apparently dimerized.

Figure 4 shows the reciprocal experiment, in which vitreous samples were immunoprecipitated with antibody against GH and then immunoblotted against opticin. In this case also, the denatured and nondenatured ED8 vitreous showed the same immunoreactivity at 60 kDa (lanes 1 and 2), equivalent to the reactivity of nonimmunoprecipitated vitreous (lane 3). The effectiveness of the antibody was shown by its positive reactivity against the 19-kDa opticin fragment (lane 4).

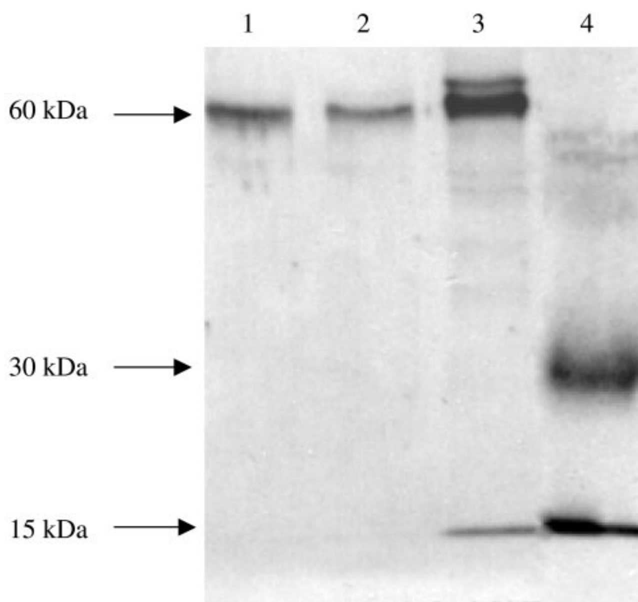

FIGURE 3. Immunoprecipitation with anti-opticin antibody and immunoblot with anti-growth hormone antibody. *Lane 1*: ED8 vitreous under denaturing conditions; *lane 2*: ED8 vitreous under nondenaturing conditions; *lane 3*: ED8 vitreous not immunoprecipitated; *lane 4*: 15-kDa growth hormone.

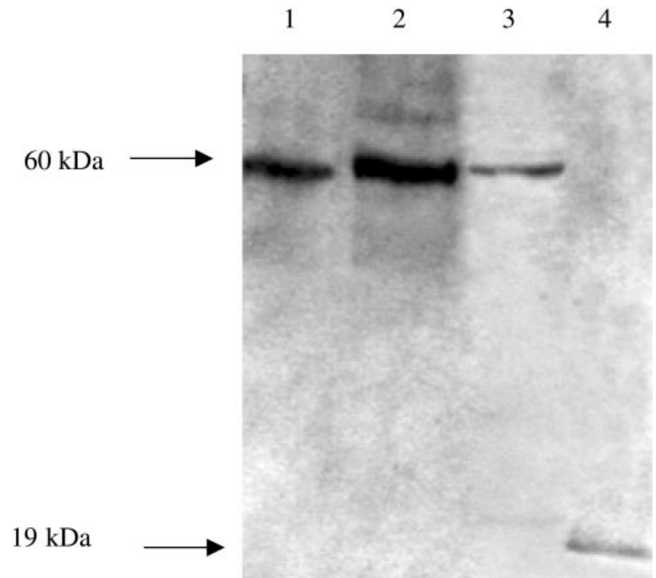

FIGURE 4. Immunoprecipitation with anti-growth hormone antibody and immunoblot with anti-opticin antibody. *Lane 1*: ED8 vitreous under denaturing conditions; *lane 2*: ED8 vitreous under nondenaturing conditions; *lane 3*: ED8 vitreous not immunoprecipitated; *lane 4*: 19-kDa opticin fragment.

Opticin Immunoreactivity in ED8 Retina

Lysates of ED8 retina showed multiple bands of immunoreactivity against the opticin antibody (Fig. 5, lane 1), which were all abolished by preabsorption of the antibody with the 19-kDa peptide (lane 2). The immunoreactivity at approximately 45 kDa corresponds with a reported size for the human opticin molecule,[12,13] whereas the reactivity at 60 to 62 kDa corresponds to that in the ED8 vitreous.

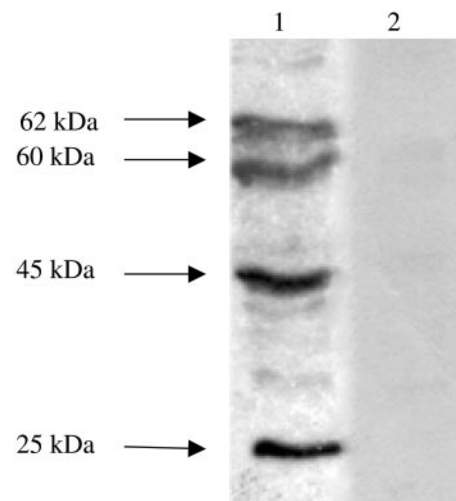

FIGURE 5. Opticin immunoreactivity of ED8 retina. *Lane 1*: ED8 retina lysate immunoblotted with anti-opticin antibody; *lane 2*: ED8 retina lysate immunoblotted with anti-opticin antibody preabsorbed with the 19-kDa opticin fragment.

Downloaded from iovs.arvojournals.org on 06/27/2019

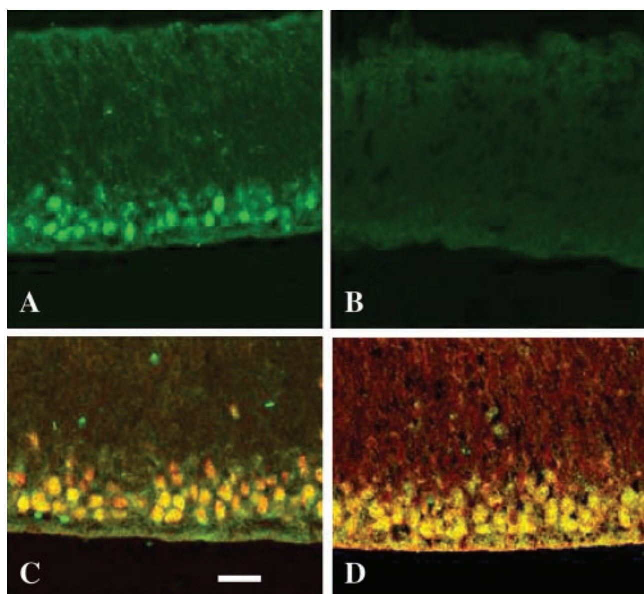

FIGURE 6. Confocal immunocytochemistry on sections of ED8 retina. (**A**) Labeling with anti-opticin antibodies indicated immunoreactivity primarily in a specific population of retinal cells. (**B**) Substitution of nonimmune serum for opticin antiserum resulted in no reaction. (**C**) Colocalization (*yellow*) of opticin (*green*) with a marker for RGCs (monoclonal antibody 39.4D5, *red*), indicates that opticin localizes to this cell population. (**D**) Colocalization (*yellow*) of growth hormone (*red*) and opticin (*green*), indicates that growth hormone also localizes to this cell population. Bar, 20 μm.

Immunocytochemistry on ED8 retina sections (Fig. 6) clearly showed that opticin localized to cells in the RGC layer, as judged by the colocalization of opticin immunoreactivity (green fluorescence, Fig. 6A) with the RGC marker antibody, 39.4D5 (Fig. 6C, red fluorescence; the yellow image indicates colocalization). Control experiments in which the anti-opticin antibody was replaced by nonimmune serum were negative for opticin immunoreactivity (Fig. 6B). Examination of the localization of GH in the retina (Fig. 6D, red fluorescence), showed that it colocalized with opticin (green fluorescence) in the RGCs (the yellow image indicates colocalization).

Discussion

It is now well-established that opticin constitutes a major proteoglycan component of the vitreous.[11–13] Although it is undoubtedly of structural importance in the formation and maintenance of the vitreous,[15] it remains to be determined whether opticin has any other significant physiologically functional roles in this location. Despite speculation that it does,[16] the only indication of other possible functions is the recent observation that opticin binds integrins, and might be a regulator of cellular functions such as ocular angiogenesis (Le Goff M., et al. *IOVS* 2003;44:ARVO E-Abstract 435). In the present work, opticin in the vitreous bound GH, suggesting that opticin may, similar to other proteoglycans, regulate growth factor function.[18,19] Although physiological roles for GH as an ocular growth factor are still speculative, it is clearly recognized as an embryonic growth factor,[29–33] and it has been implicated in neurogenesis in the teleost retina[34] and in retinal angiogenesis.[35,36] Retinal GH, of extrapituitary origin, has been considered to be a likely embryonic paracrine factor of significance in the regulation of ocular development,[3,5] with possible influences on the survival of RGCs and on the differentiation of the lens using the vitreous as a conduit for delivery.

In recent years, GH-like proteins and GH-like mRNA have been detected in the eyes of embryonic chicks.[3,5,37] Growth hormone immunoreactivity, labeled by the same antibody used in the present study, has previously been located in the neural retina,[3] but it has not previously been localized specifically to RGCs. Because pituitary somatotrophs are not present in chick embryos until at least ED12[38] and GH is not present in the plasma of embryonic chicks until ED17,[39] RGCs are thus likely to be the source of the GH-immunoreactivity in the vitreous of ED8 chick eyes. The persistence of GH immunoreactivity in the vitreous of adult chickens, in which the blood–ocular barriers[40] are likely to exclude pituitary-derived GH in the systemic circulation, suggests the continued production of GH in the neural retina after hatching and neonatal development. In the adult, we suggest that GH in this location probably acts as an angiogenic factor in the maintenance of the retinal microvasculature.[7]

Although several GH moieties were evident in the vitreous of ED8 embryos, a major part of the GH immunoreactivity was associated with a 15-kDa protein. This is consistent with the characteristics of the GH immunoreactivity previously shown in the neural retina,[5] whereas most of the GH immunoreactivity in the pituitary gland is associated with a coded protein with a deduced molecular size of 22 kDa.[41] The 15-kDa GH moiety has been shown to result from the proteolytic processing of the full-length protein[12] and hence is likely to be produced in the retina rather than in the vitreous.

It is now well established that pituitary GH is a family of proteins rather than a single moiety. Indeed, although full-length monomer GH is the most abundant GH moiety in the chicken pituitary gland, multiple forms of GH, due to posttranslational modifications, have been identified. Molecular weight variants result from deamidation, proteolytic cleavage, reduction, glycosylation, phosphorylation, and aggregation.[41,42] The 15-kDa GH moiety, which results from proteolytic cleavage of the full-length protein (191 amino acids) at Arg_{133}-Gly_{134},[17] is preferentially produced in the chick embryo[41] and is the main GH variant present in the vitreous of embryonic chicks.

It is important to note that this cleavage fragment is unlikely to reflect the 16.5-kDa protein (small chicken growth hormone [s-cGH]) coded by the novel GH transcript identified by Takeuchi et al.[37] in extracts of the whole ED17 chick eye. This transcript was not detected by those researchers in the retina, retinal pigmented epithelium, choroid, cornea or lens, and its cellular localization is currently unknown. Moreover, if this transcript is translated, s-cGH would not have a signal peptide, and it is thus unlikely to be a secretory product. Thus, although this N-terminally truncated protein might be expected to cross-react with our polyclonal antiserum raised against full-length pituitary GH, it is highly unlikely to be present in the vitreous.

The GH immunoreactivity in the vitreous also included moieties of approximately 60 to 62kDa. This probably reflects the 15-kDa GH bound to the 45-kDa opticin moiety in the vitreous of embryonic and adult chicken eyes, especially as a protein of approximately 60 kDa with GH and opticin immunoreactivity was generated after the incubation of opticin with purified 15-kDa GH. This strongly suggests that opticin acts as an ocular growth hormone-binding protein (GHBP).

Immunoreactive GHBPs, derived from the extracellular domain of the membrane-anchored growth hormone receptor (GHR) by proteolytic cleavage,[43–45] are present in the circulation of chickens.[46,47] Although these have a molecular mass of approximately 70 to 74 kDa, several uncharacterized 30- to 60-kDa proteins that bind radio-labeled GH have also been detected in the circulation before (Radecki SV, et al., unpublished observations, 1996), and after[46–49] hatching. As GHBPs are also in the plasma of species with dysfunctional GHRs,[50–52] some of these may be structurally unrelated to the membrane GHR. Indeed, a low-affinity GHBP in human serum, immuno-

Downloaded from iovs.arvojournals.org on 06/27/2019

logically unrelated to the tissue GHR, has been identified as a transformed α_2-macroglobulin.[53] Thus, although opticin is unrelated to the chicken GHR, it may act as a GHBP in vitreous because it has protein domains that could facilitate its binding with other proteins[12] and carbohydrate side chains that may also bind GH. The affinity, capacity, and specificity of opticin for 15-kDa GH and its potential to modulate GH activity and GH clearance are currently unknown. However, it might be envisaged that opticin could sequester and concentrate a GH reservoir in the vitreous and regulate the release of GH in response to specific developmental events in the retina and/or lens.

We have shown that in the vitreous at ED8, opticin immunoreactivity is associated with proteins at 60 to 62kDa, whereas in the adult this reactivity occurs at 45 kDa (Fig. 2). We suggest that in the embryo, opticin is heavily complexed with GH and probably with other factors also—thus, the higher molecular weight moieties. In the adult such associations may be weaker, so that the primary band detected by the immunoblot technique is the free opticin molecule at 45 kDa. It is also possible that there are alternative splice variants of opticin in the embryo and adult that give rise to complexes of different molecular size. The double band at 60 to 62 kDa in the embryonic and adult vitreous (Fig. 1) and in the retina (Fig. 5) may reflect the variable glycosylation of the opticin molecule, a not uncommon feature of proteoglycans.[18,19]

In the embryonic retina, opticin immunoreactivity was primarily associated with a 25-kDa molecule, but also with 45-, 60-, and 62 kDa moieties. Opticin immunoreactivity is primarily associated with molecules of 48 kDa and 62 kDa in human ocular tissue (cornea, iris, ciliary body, vitreous, choroid, and retina[12,26]); 45 and 25 kDa in bovine vitreous and retina[13] (Bishop PN, et al. *IOVS* 2003;44:ARVO E-Abstract 434); and 37 kDa in the rat iris.[12] The relationship between the opticins of different species is not yet known. It is possible that the large 62-kDa opticin moiety in human vitreous and retina is bound to GH, but this is uncertain. The smaller 25-kDa molecule that we show in the retina of embryonic chicks is, as in the bovine (Bishop PN, et al. *IOVS* 2003;44:ARVO E-Abstract 434), likely to be a cleavage fragment of the full-length opticin molecule.

In the retina, immunoreactivity for opticin was primarily found in the RGCs. This is the first determination of the cellular localization of opticin within retinal tissues. It is also evident that opticin is colocalized with GH in the RGCs, which are likely to be the sites of GH production in the neural retina. These results therefore suggest that opticin binds GH at its site of production, before its extracellular release into the vitreous, and may serve to modulate the activity of GH within the retina, to protect it from proteolytic degradation, or to facilitate its extracellular transport. It may therefore be pertinent that GHBPs are also present in pituitary somatotrophs in which they are thought to have similar roles in modulating GH action, clearance, or transport.[54]

The vitreous is a repository of a significant number of growth factors.[55,56] Opticin, similar to other proteoglycans in this location,[17] may thus serve a role in the sequestering and concentration of ocular growth and differentiation factors during development and in the adult. Retinal development, in particular, is dependent on the balance between cell proliferation and cell death,[57] and the RGCs, at least, are susceptible to the influence of local growth factors.[58] We speculate that GH, derived from the retina and secreted into the vitreous, may therefore serve to exert local influence as a cytokine over cell division and/or apoptosis in ocular tissues, as it does in other tissues.[59,60]

Because GH influences neurogenesis in the developing brain by its anti-apoptotic action,[8,61] it may similarly influence retinal neurogenesis in embryonic chicks by promoting RGC survival. The critical period in the development of the chick embryo retina is approximately between ED3 and ED9, when the undifferentiated neuroepithelial cells proliferate and migrate to their final stratified locations.[62] Phenotypic differentiation occurs within the strata of the retina. At ED8, the retina consists of a mixture of proliferating cells at different phases of the cell cycle and postmitotic cells that are at various points in their migration to their final strata and are variably differentiated. It is these differentiative events that we propose are influenced by GH. The possibility that the opticin–GH association could be exploited as a therapeutic option in the treatment of age-related macular degeneration remains to be explored.

In summary, these results demonstrate the colocalization of GH and opticin immunoreactivity in RGCs of embryonic chicks and in the vitreous, in which 15-kDa GH and 45-kDa opticin bind to form a 60-kDa complex. The opticin binding of this GH variant may modulate growth factor-like actions of GH in retinal development or ocular function.

Acknowledgments

The authors thank Martha Carranza for excellent technical work in purifying the 15-kDa fragment of rcGH.

References

1. Harvey S, Hull KL. Growth hormone: a paracrine growth factor? *Endocr J.* 1997;7:267–279.
2. Harvey S, Johnson CDM, Sanders EJ. Extra-pituitary growth hormone in peripheral tissues of early chick embryos. *J Endocrinol.* 2000;166:489–502.
3. Harvey S, Johnson CDM, Sharma P, Sanders EJ, Hull KL. Growth hormone: a paracrine growth factor in embryonic development? *Comp Biochem Physiol C.* 1998;119:305–315.
4. Harvey S, Johnson CDM, Sanders EJ. Growth hormone in neural tissues of the chick embryo. *J Endocrinol.* 2001;169:487–498.
5. Harvey S, Kakebeeke M, Murphy AE, Sanders EJ. Growth hormone in the nervous system: autocrine/paracrine roles in retinal function? *Can J Physiol Pharmacol.* 2003;81:371–384.
6. Corbacho AM, Martinez de la Escalera G, Clapp C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. *J Endocrinol.* 2002;173:219–238.
7. Rymaszewski Z, Cohen RM, Chomczynski P. Human growth hormone stimulates proliferation of human retinal microvascular endothelial cells *in vitro. Proc Natl Acad Sci USA.* 1991;88:617–621.
8. Ajo R, Cacicedo L, Navarro C, Sanchez-Franco F. Growth hormone action on proliferation and differentiation of cerebral cortical cells from fetal rat. *Endocrinology.* 2003;144:1086–1097.
9. Costoya JA, Finidori J, Moutoussamy S, Searis R, Devesa J, Arce VM. Activation of growth hormone receptor delivers an antiapoptotic signal: evidence for a role of Akt in this pathway. *Endocrinology.* 1999;140:5937–5943.
10. Scheepens A, Sirimanne ES, Breier BH, Clark RG, Gluckman PD, Williams CE. Growth hormone as a neuronal rescue factor during recovery from CNS injury. *Neuroscience.* 2001;104:677–687.
11. Friedman JS, Ducharme R, Raymond V, Walter MA. Isolation of a novel iris-specific and leucine-rich repeat protein (oculoglycan) using differential selection. *Invest Ophthalmol Vis Sci.* 2000;41:2059–2066.
12. Hobby P, Wyatt MK, Gan W, et al. Cloning, modeling, and chromosomal localization for a small leucine-rich repeat proteoglycan (SLRP) family member expressed in human eye. *Mol Vis.* 2000;6:72–78.
13. Reardon AJ, Le Goff M, Briggs MD, et al. Identification in vitreous and molecular cloning of opticin, a novel member of the family of leucine-rich repeat proteins of the extracellular matrix. *J Biol Chem.* 2000;275:2123–2129.
14. Wistow G, Bernstein SL, Wyatt MK, et al. Expressed sequence tag analysis of human RPE/choroids for the NEIBank Project: over 6000 non-redundant transcripts, novel genes and splice variants. *Mol Vis.* 2002;8:205–220.
15. Bishop PN. Structural macromolecules and supramolecular organization of the vitreous gel. *Prog Retin Eye Res.* 2000;19:323–344.

Downloaded from iovs.arvojournals.org on 06/27/2019

16. Takanosu M, Boyd TC, Le Goff M, et al. Structure, chromosomal location, and tissue-specific expression of the mouse opticin gene. *Invest Ophthalmol Vis Sci.* 2001;42:2202–2210.
17. Noulas AV, Theocharis AD, Feretis E, Papageorgakopoulou N, Karamanos NK, Theocharis DA. Pig vitreous gel: macromolecular composition with particular reference to hyaluronan-binding proteoglycans. *Biochimie* (*Paris*). 2000;84:295–302.
18. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. *Ann Rev Biochem*. 1998;67:609–652.
19. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J.* 1992;6:861–870.
20. Bernfield M, Sanderson RD. Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. *Phil Trans R Soc Lond B*. 1990;327:171–186.
21. Massague J, Andres J, Attisano J, et al. TGF-beta receptors. *Mol Reprod Dev*. 1992;32:9–104.
22. Pimentel B, Sanz C, Varela-Nieto I, Rapp UR, de Pablo F, de la Rosa EJ. c-Raf regulates cell survival and retinal ganglion cell morphogenesis during neurogenesis. *J Neurosci*. 2000;20:3254–3262.
23. González-Hoyuela M, Barbas JA, Rodríguez-Tébar A. The autoregulation of retinal ganglion cell number. *Development*. 2001;128: 117–124.
24. Aramburo C, Carranza M, Reyes M, et al. Characterization of a bioactive 15kDa fragment produced by proteolytic cleavage of chicken growth hormone. *Endocrine*. 2001;15:231–240.
25. Harvey S, Scanes CG. Purification and radioimmunoassay of chicken growth hormone. *J Endocrinol*. 1997;73:321–329.
26. Friedman JS, Faucher M, Hiscott P, et al. Protein localization in the human eye and genetic screen of opticin. *Hum Mol Genet*. 2002; 11:1333–1342.
27. Trousse F, Marti E, Gruss P, Torres M, Bovolenta P. Control of retinal ganglion cell axon growth: a new role for sonic hedgehog. *Development*. 2001;128:3927–3936.
28. Zhang X-M, Yang X-J. Regulation of retinal ganglion cell production by sonic hedgehog. *Development.* 2001;128:943–957.
29. Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ, Kaye PL. Functional growth hormone (GH) receptors and GH are expressed by reimplantation mouse embryos: a role for GH in early embryogenesis? *Proc Natl Acad Sci USA*. 1997;13:5125–5130.
30. Waters MJ, Shang CA, Behncken SN, et al. Growth hormone as a cytokine. *Clin Exp Pharmacol Physiol*. 1999;26:760–764.
31. Ishiwata I, Tokieda Y, Kiguchi K, Sato K, Ishikawa H. Effects of embryotrophic factors on the embryogenesis and organogenesis of mouse embryos *in vitro. Hum Cell*. 2002;13:185–195.
32. Waters MJ, Kaye PL. The role of growth hormone in fetal development. *Growth Horm IGF Res*. 2002;12:137–146.
33. Markham KE, Kaye PL. Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst. *Reproduction*. 2003;125:327–336.
34. Otteson DC, Cirenza PF, Hitchcock PF. Persistent neurogenesis in the teleost retina: evidence for regulation by the growth-hormone/insulin-like growth factor-I axis. *Mech Dev*. 2002;117:137–149.
35. Struman I, Bentzien F, Lee H, et al. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. *Proc Natl Acad Sci USA.* 1999;16:246–1251.
36. Hellstrom A, Carlsson B, Niklasson A, et al. IGF-I is critical for normal vascularization of the human retina. *J Clin Endocrinol Metab*. 2002;87:3413–3416.
37. Takeuchi S, Haneda M, Teshigawara K, Takahashi S. Identification of a novel GH isoform: a possible link between GH and melanocortin systems in the developing chicken eye. *Endocrinology*. 2001;142:5158–5166.
38. Porter TE. Regulation of somatotroph differentiation during chicken embryonic development: a review. In: Harvey S, Etches RJ, eds. *Perspectives in Avian Endocrinology.* Bristol, UK: Society for Endocrinology; 1998:47–56.
39. Harvey S, Davison TF, Chadwick A. Ontogeny of growth hormone and prolactin secretion in the domestic fowl (*Gallus domesticus*). *Gen Comp Endocrinol*. 1979;39:270–273.
40. Cunha-Vaz JG. The blood-ocular barriers: past, present and future. *Doc Ophthalmol*. 1997;93:149–157.
41. Aramburo C, Luna M, Carranza M, Reyes M, Martinez-Coria H, Scanes CG. Growth hormone size variants: changes in the pituitary during development of the chicken. *Proc Soc Exp Biol Med*. 2000;223:67–74.
42. Aramburo C, Carranza M, Martínez-Coria H, et al. Molecular and functional heterogeneity of growth hormone. In: Dawson A, Chaturvedi CM, eds. *Avian Endocrinology*. New Delhi, India: Narosa Publishing House; 2001:273–286.
43. Bingham B, Oldham ER, Baumbach WR. Regulation of growth hormone receptor and binding protein expression in domestic species. *Proc Soc Exp Biol Med*. 1994;206:195–199.
44. Monsonego E, Baumbach WR, Lavelin I, Gertler A, Hurwitz S, Pines M. Generation of growth hormone binding protein by avian growth plate chondrocytes is dependent on cell differentiation. *Mol Cell Endocrinol*. 1997;30:1–10.
45. Vleurick L, Kuhn ER, Decuypere E, Burnside J, Pezet A, Edery M. Generation of chicken growth hormone-binding proteins by proteolysis. *Gen Comp Endocrinol*. 1999;113:283–289.
46. Davis SL, Graf M, Morrison CA, Hall TR, Swift PJ. Identification and partial purification of serum growth hormone binding protein in domestic animal species. *J Anim Sci.* 1992;70:773–780.
47. Hull KL, Marsh JA, Harvey S. A missense mutation in the GHR gene of Cornell sex-linked dwarf chickens does not abolish serum GH binding. *J Endocrinol*. 1999;161:495–501.
48. Tobar-Dupres ET, Froman DP, Davis SL. Factors affecting circulating growth hormone binding protein in chickens. *Poult Sci*. 1993; 72:2337–2346.
49. Davis SL, Wehr NB, Laird DM, Hammond AC. Serum growth hormone-binding protein (GHBP) in domestic animals as measured by ELISA. *J Anim Sci*. 1994;72:1719–1727.
50. Baumann G, Shaw M., Merimee TJ. Low levels of high-affinity growth hormone-binding protein in African pygmies. *N Engl J Med*. 1989;320:1705–1709.
51. Hull KL, Janssens WCJ, Baumbach WR, Harvey S. Growth hormone (GH)-binding proteins in GH-resistant guinea pigs. *Growth Reg*. 1996;6:165–175.
52. Parks JS, Brown MR, Faase ME. The spectrum of growth hormone insensitivity. *J Pediatr*. 1997;131:S45–S50.
53. Kratzsch J, Selisko T, Birkenmeier G. Identification of transformed alpha 2-macroglobulin as a growth hormone-binding protein in human blood. *J Clin Endocrin Metab*. 1995;80:585–590.
54. Harvey S, Baumbach WR, Sadeghi H, Sanders EJ. Ultrastructural colocalization of growth hormone binding protein and pituitary hormones in adenohypophyseal cells of the rat. *Endocrinology.* 1993;133:1125–1130.
55. Tripathi BJ, Tripathi RC, Livingston AM, Borisuth NSC. The role of growth factors in the embryogenesis and differentiation of the eye. *Am J Anat*. 1991;192:442–471.
56. Hernández-Sánchez C, López-Carranza A, Alarcón C, de la Rosa EJ, de Pablo F. Autocrine/paracrine role of insulin-related growth factors in neurogenesis: local expression and effects on cell proliferation and differentiation in retina. *Proc Natl Acad Sci USA*. 1995;92:9834–9838.
57. Pimentel B, Rodriguez-Borlado L, Hernández C, Carrera AC. A role for phosphoinositide 3-kinase in the control of cell division and survival during retinal development. *Dev Biol*. 2002;247: 295–306.
58. Fischer AJ, Reh TA. Exogenous growth factors stimulate the regeneration of ganglion cells in the chicken retina. *Dev Biol*. 2002;251: 367–379.
59. Jeay S, Sonenshein GE, Kelly PA, Postel-Vinay M-C, Baixeras E. Growth hormone exerts antiapoptotic and proliferative effects through two different pathways involving nuclear factor-κB and phosphatidylinositol 3-kinase. *Endocrinology*. 2001;142:147–156.
60. Jeay S, Somenshein GE, Postel-Vinay M-C, Kelly PA, Baixeras E. Growth hormone can act as a cytokine controlling survival and proliferation of immune cells: new insights into signaling pathways. *Mol Cell Endocrinol*. 2002;188:1–7.
61. Isla A, Budke M, Cacicedo L, et al. Protective effects of growth hormone en cell cultures of the central nervous system. *Rev Neurol*. 2002;34:208–211.
62. Adler R. A model of retinal cell differentiation in the chick embryo. *Prog Retin Eye Res*. 2000;19:529–557.

Downloaded from iovs.arvojournals.org on 06/27/2019