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Analysis of basic fibroblast growth factor in rats with inherited retinal degeneration

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Abstract In RCS rats, photoreceptors degenerate between postnatal days 20 and 60, secondary to a genetic defect expressed in the neonatal retinal pigmented epithelium (RPE). Previous work has shown delay of the photoreceptor degeneration in this model by intraocular injection of basic fibroblast growth factor (bFGF). Evidence is presented here, from bFGF immunostaining and Northern analysis of bFGF mRNA, for reduced bFGF expression in uncultured RPE of dystrophic RCS pups. It is also shown that in the mutant eyes angiogenesis in the underlying choroid, which normally occurs between postnatal days 7 and 10, is markedly delayed, with irregular distribution of vessels, consistent with a reduction in this known angiogenesis factor. Mutational analysis of the bFGF transcript and gene by denaturing gradient gel electrophoresis and Southern analysis did not, however, reveal abnormalities in the coding sequence of this gene in RCS rats.

Key words: Basic FGF; RCS rat; Pigment epithelium of eye; Choroid; Angiogenesis; Mutational analysis

1. Introduction

The Royal College of Surgeons (RCS) rat is a well known animal model of inherited retinal degeneration, of unknown etiology, in which progressive loss of photoreceptors is evident from 20–60 days after birth [1]. The genetic defect was shown to be expressed in the retinal pigmented epithelium (RPE), but not the photoreceptors themselves, using experimental chimeras in which normal and mutant RPE could be distinguished on the basis of pigmentation [2]. The photoreceptor cell death in the RCS retina appears to be secondary to the RPE defect, which involves failure to phagocytize renewing photoreceptor membranes, which progressively accumulate in the subretinal space beginning about postnatal day 12 [1–8]. The reason for the death of the photoreceptors is at present unknown.

Basic FGF (bFGF), also known as FGF-2, is a widely distributed heparin-binding growth factor, with a very broad range of actions (reviewed in [9,10]). During embryogenesis, bFGF has been shown to mediate many fundamental processes including induction of mesoderm from endoderm, mitogenesis in mesoderm- and neuroectoderm-derived cells and angiogenesis. In most adult tissues, its distribution is primarily confined to extracellular matrix, where it is normally stored in an inactive form. Basic FGF is also a neurotrophic factor, capable of promoting neuronal cell survival in the visual system and other parts of the central nervous system [11–13]. Interest in this factor in relation to retinal degeneration has been greatly stimulated by the observation that injection of bFGF into eyes of 23-day-old RCS rats dramatically delays the progress of photoreceptor degeneration in these retinas, possibly by virtue of its neurotrophic effects [14]. Our focus has been to investigate whether bFGF might also be directly related to the primary RPE defect in this model. In this study we compared bFGF expression in the RPE of normal and dystrophic RCS rat pups and, finding evidence of reduced bFGF expression in the dystrophic RPE and delayed, abnormal angiogenesis in the underlying choroid, proceeded to examine the bFGF gene for mutations in this mutant.

2. Materials and methods

2.1. Experimental animals

Breeding colonies were maintained by brother×sister matings of four RCS rat strains, i.e. tan-hooded retinal dystrophic (RCS) and congenic control (RCS-rdy$^+$) and black-hooded (pigmented) dystrophic (RCS-p$^+$) and congenic control (RCS-rdy$^+$p$^+$). Retinal phenotype of breeders and offspring was verified histologically on a routine basis. Pups were used for experiments on postnatal days (P) 7–14. All animal procedures conformed to the Resolution on the Use of Animals in Research of the Association for Research in Vision and Ophthalmology.

2.2. Preparation of rat RPE-lined and choroid-lined eyecups, isolated RPE sheets and plated primary RPE cells

Eyecups used for immunohistochemistry were prepared from eyes of P7–14 tan-hooded normal and dystrophic rats. Under a dissecting microscope, eyes immersed in Hank's balanced salt solution (HBSS) were carefully trimmed of ocular adnexa, then cut open at the limbus. The anterior segment and lens were discarded, the neural retina was gently removed from the eyecup, and the exposed RPE monolayer was fixed in situ. All other procedures using RPE involved, prior to dissection, incubation of eyes in Dispase, which results in clean separation of the RPE monolayer from the choroid, as previously described [15–17]. The choroid-lined eyecups remaining after removal of the RPE were fixed in 2% paraformaldehyde and whole-mounted for observation of the choroidal vasculature. Freshly isolated RPE was prepared for immunostaining by plating numerous small patches of epithelium (average 5×10^3 cells/well) in 8-well chamber slides in minimum essential medium with Earle's salts (Gibco) + 20% fetal bovine serum (FBS) + 1% penicillin/streptomycin, and fixing them following attachment to the glass substrate (within 18 h of plating).

2.3. Immunofluorescent detection of bFGF in rat RPE cells

RPE-lined eyecups and sheets of freshly isolated RPE cells plated in multiwell slides were fixed at room temperature (RT) in 2% or 4% paraformaldehyde in 0.1M $NaPO_4$ buffer, pH 7.4, then washed extensively in buffer. For immunostaining, whole eyecups were processed through droplets of the various solutions, and plated RPE were processed in multiwell slides. Both types of RPE preparation were first incubated for 30 min in PBS + 3% bovine serum albumin (BSA) + 1% FBS, then reacted for 1 h at RT with primary antibody against bFGF diluted with PBS + 3% BSA, i.e. either (1) rabbit polyclonal antipeptide antibody raised against amino acids 1–24 of the human and bovine bFGF sequence (Sigma), 1:30 dilution or (2) mouse monoclonal antibody raised against purified bovine brain bFGF (Upstate Biotechnology Inc., type 1), 1:50–1:200 dilution. Following washing,

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bound antibody was detected by incubation with an appropriate FITC-labeled secondary antibody, as previously described [18]. Controls included (1) preadsorption of the antipeptide antibody with a 10-fold excess of the corresponding bFGF synthetic peptide (Sigma) for 24 h at 4°C prior to use and (2) incubation of samples in all groups for 1 h in PBS + 3% BSA alone, followed by secondary antibody. After the final washing step in PBS, all preparations were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL), and slides were observed and photographed by phase contrast and fluorescence microscopy using a Zeiss Photomicroscope III as previously described [18].

2.4. Isolation of RNA from uncultured rat RPE cells

Immediately following isolation, pooled RPE sheets obtained from a litter of P10 pigmented normal or dystrophic rat pups (10–20 eyes; $0.5–1.0 \times 10^6$ RPE cells) were lysed by addition of a 1 ml volume of 4 M guanidine thiocyanate solution, followed by vigorous vortexing and homogenization with a 7 mm Polytron generator. Homogenates from each batch of cells were stored at −20°C, then 5–7 batches, each obtained from pups of a different mother, were combined for RNA isolation. Low speed centrifugation was used to remove pigment granules and debris, then RNA was purified by CsCl centrifugation as described by Chirgwin [19]. Typically, 5–7 μg of purified total RNA was obtained from 10^6 uncultured neonatal rat RPE cells. To produce RNA samples representative of RPE from a large number of rat pups, total RNA samples obtained from 100–125 individual eyes were combined for use on Northern blots.

2.5. Preparation of RT-PCR-amplified bFGF probe from rat RPE

PCR primers (sense, nt 254–272; antisense nt 739–720) were designed from the published rat brain bFGF cDNA sequence [20] such that the entire coding sequence would be contained in the fragment analyzed. Total RNA isolated from cultured BPEI-1 normal rat RPE cell line [16] was used for the RT-PCR amplification (GeneAmp kit, Perkin-Elmer) with these primers, and the RT-PCR product of correct size (486 bp) was purified by gel electrophoresis, confirmed to be bFGF by DNA sequencing, and cloned into the pCR-Script SK(+) vector (Stratagene). Both the cloned and uncloned RT-PCR product were used as hybridization probes after labeling with ^{32}P by random oligonucleotide incorporation.

2.6. Northern analysis of bFGF expression in uncultured rat RPE cells

RNA (10 μg/lane) isolated from uncultured normal or dystrophic rat RPE or confluent cultures of BPEI-1 normal rat RPE cell line, grown with cobblestone morphology [16], was electrophoresed in formaldehyde gels [21], transferred to nylon filters, baked, and used for hybridization with the rat RPE bFGF cDNA probe, and a probe for β-actin. The size of the hybridizing species was approximated from the positions of the 28S and 18S ribosomal RNAs, and RNA size standards (Gibco BRL) co-electrophoresed with the samples. For standardization of the quantity and quality of RNA present on the blot, ethidium bromide staining of the ribosomal RNA and hybridization intensities of β-actin message were used as reference.

2.7. Isolation of DNA and genomic southern blot analysis

High-molecular-weight genomic DNA was isolated from liver of adult control (RCS-rdy$^+$) and RCS rats using proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation [22]. 10 μg of DNA was double digested with *Bam*HI and another restriction enzyme, i.e. *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I or *Pst*I, then electrophoresed, blotted onto a nylon filter, hybridized with ^{32}P-labeled RT-PCR bFGF probe, and autoradiographed [23].

2.8. Mutational analysis of the bFGF gene and transcript by denaturing gradient gel electrophoresis (DGGE)

The sequence of the rat bFGF gene is not known; in order to analyze this gene in the rat, the sequence of the published human gene [24] was used as a reference to determine the limits of the predicted three exons of the rat gene. As shown in Fig. 5a, PCR primers (20 bp) were designed from the rat bFGF cDNA sequence [20] to amplify the coding region of the gene sequence contained in the putative exons, i.e. nucleotide residues 274–408, 449–512, and 553–719. For the analysis of the rat bFGF transcript, the rat cDNA sequence was divided into two overlapping regions, i.e. R1 (residues 235–512) and R2 (residues 341–719) and primers were designed to amplify these regions from the RT product of RNA from BPEI-1 normal and MGM-1 dystrophic rat RPE cell lines [16]. The R1 and R2 products contained the 20 bp of exonic sequence flanking each of the introns, missed in the direct amplification of the gene (Fig. 5a). Each PCR product also contained a 40-bp GC-clamp at either the 5′ or 3′ end to increase the detectability of mutations by DGGE [25]. The PCR products predicted with the designed primers were also analyzed by the MELT, MU, and SQHTX computer programs to determine their suitability for DGGE analysis [26].

The bFGF-coding gene and transcript sequences were amplified by PCR from control and RCS genomic DNA or RT-product of RNA, respectively, examined by gel electrophoresis to confirm the size, and partial DNA sequencing to confirm the identity of the products, and analyzed by DGGE as we have previously described [27,28]. The denaturing gradient gels were routinely electrophoresed at 80 or 150 V for 6–16 h, depending on the condition specified by the computer analysis, stained with ethidium bromide, and photographed. To detect potentially hidden homozygous mutations involving guanine and cytosine, samples from dystrophic rats were mixed with corresponding normal PCR products and analyzed for heteroduplex formation by DGGE.

3. Results

The RPE and choroidal melanocytes are amelanotic in the pink-eyed, tan-hooded strains of normal and dystrophic RCS rats; we took advantage of the lack of pigment in the posterior eyecup to develop a simple new immunofluorescent technique to directly assess the distribution and staining intensity of bFGF-positive cells within the intact RPE monolayer, which could be viewed en face in whole mounts of RPE-lined eyecups. All data were obtained from normal and dystrophic RPE samples prepared and immunostained concurrently. Similar results were obtained with both bFGF antibodies. In eyecup preparations from 8-day-old normal rats, most of the RPE cells in the monolayer were brightly stained following reaction with anti-bFGF antibodies (Fig. 1a). Specificity of the reaction was shown by loss of immunostaining with the bFGF (1–24) peptide antibody following preadsorption with the corresponding peptide (Fig. 1b). Controls from all groups showed only weak background fluorescence upon omission of the primary antibody (Fig. 1c). In contrast, the RPE monolayer of 8-day-old mutant rats, when reacted with the bFGF antisera, showed very weak immunostaining (Fig. 1d) which was barely above control levels (Fig. 1e). In eyecups from 10-day-old normal rats, a mixed reaction was seen in which about 30–50% of the RPE cells showed a distinct positive reaction, whereas in adjacent cells within the epithelium, staining was closer to background levels (Fig. 1f; compare with control in Fig. 1h). In 12-day-old normal rats, fewer RPE cells were immunoreactive with bFGF antibodies (not shown). In all of the positively stained normal RPE cells, many of which are binucleate in rats, reaction product was diffusely distributed throughout the cytoplasm, revealing the hexagonal shape of the cells and the one or two unstained nuclei (Fig. 1f). In the dystrophic eyecups, as seen in the 7–8-day-old animals, almost no strongly positive cells were observed in RPE monolayers from 10–14-day-old RCS rats (P10 result shown in Fig. 1g).

To ensure that the immunostaining observed in the RPE in eyecup preparations was intrinsic to the RPE cells, bFGF immunostaining was also performed on small sheets of freshly isolated RPE cells from eyes of 7-day-old pigmented normal and dystrophic rats. Clumps of isolated RPE cells from normal rats of this age were brightly stained (Fig. 1i,j), whereas

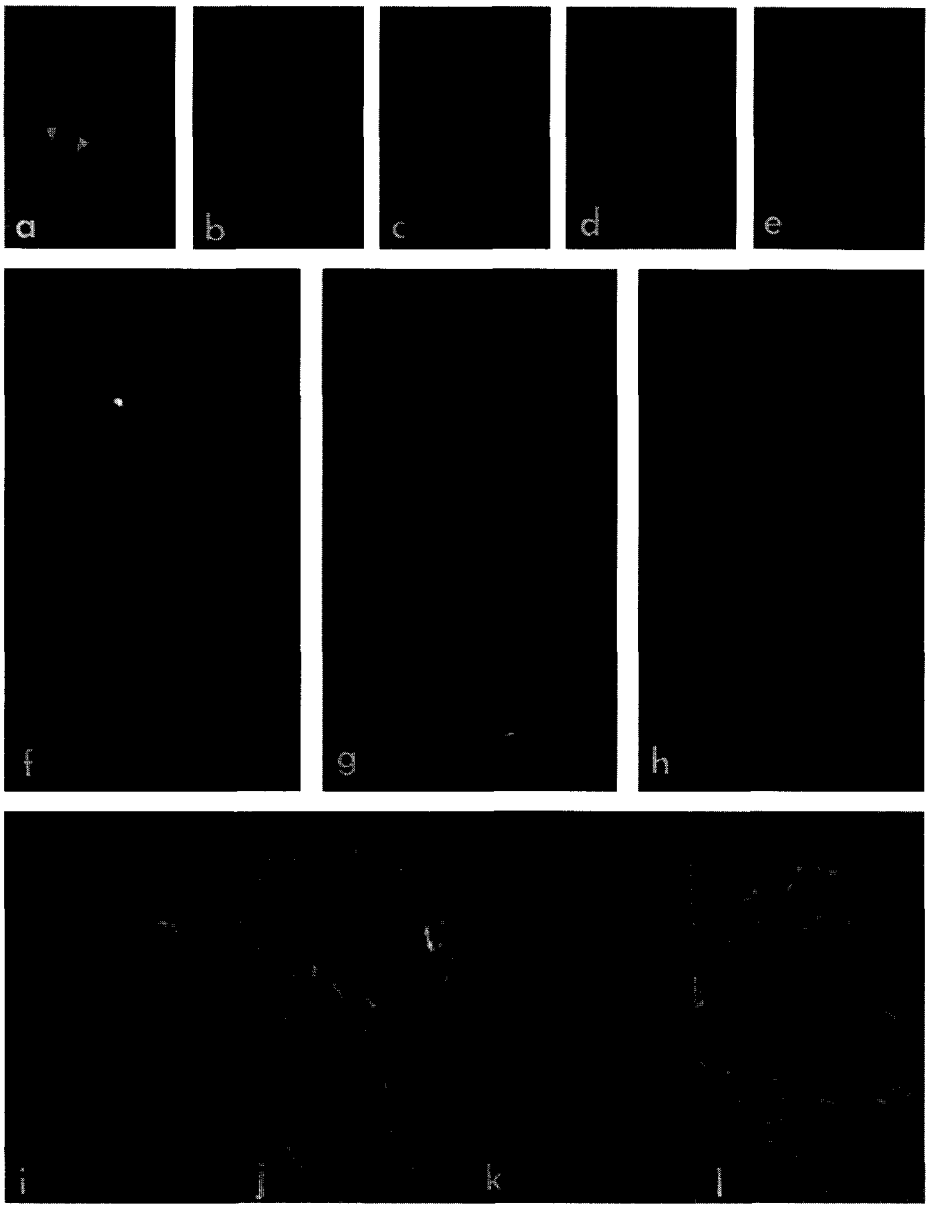


Fig. 1. Immunofluorescent detection of bFGF in control and dystrophic rat RPE cells. (a–e) Low power views of whole-mounted RPE-lined eyecups from tan-hooded P8 normal (a–c) and P8 dystrophic (d,e) rats. Two eyecups from one normal animal (a,b) and two samples from a mutant pup (d,e) are compared. Samples in (a,d) reacted with anti-bFGF (1–24) antipeptide antiserum; controls in (b,e) reacted with the same antiserum preadsorbed with bFGF (1–24) peptide; control in (c) reacted with secondary antibody alone. Arrowheads in (a) indicate sites (dark holes) where individual RPE cells have fallen out of the otherwise brightly fluorescent epithelium, revealing the underlying unreactive choroid. Note the weak bFGF immunoreactivity in the mutant RPE compared with control (a,d). Bar, 100 μm (a–e). (f–h) Higher magnification of eyecups from P10 normal (f,h) and dystrophic (g) rats. Bar, 40 μm (f–h). (f,g) Preparations reacted with monoclonal antibody against bovine brain bFGF; (h) control. (i–l) Clumps of isolated RPE cells from pigmented P7 normal (i,j) and P7 dystrophic (k,l) rats. Bar, 125 μm (i–l). Cells in (i,k) reacted with monoclonal anti-bFGF antiserum; (j,l) show phase contrast images of (i,k), respectively. Strong bFGF immunoreactivity is seen only in normal RPE cells in (i).

sheets of dystrophic cells of comparable appearance were minimally labeled with the anti-bFGF antibodies (Fig. 1k,l).

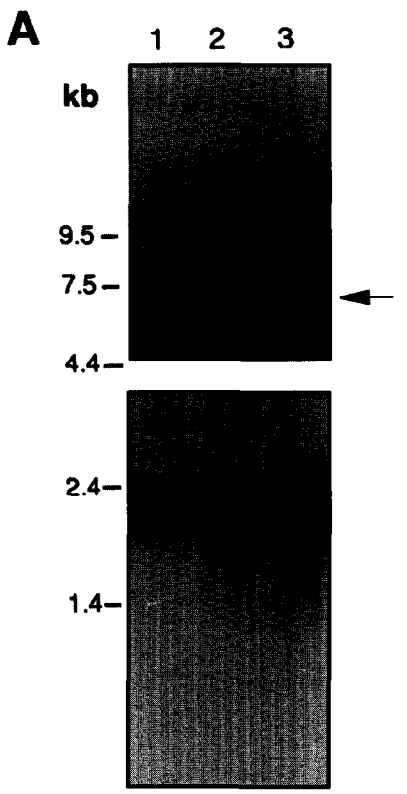
Northern analysis was used to evaluate the level of expression of bFGF mRNA in RPE of neonatal rat eyes, using 10 μg samples of pooled RNA from uncultured normal or dystrophic RPE (representative of approximately 125 eyes of each rat strain), and RNA from cultured BPEI-1 normal rat RPE cells for reference (Fig. 2a). The result revealed a faint but distinct 7.0-kb bFGF transcript in P10 uncultured RPE from both rat strains. A slightly stronger bFGF signal was seen in the cultured RPE cells. All of the signals, however, were much weaker than β-actin, a highly expressed transcript in these cells (Fig. 2a). Densitometric analysis of bFGF expression, normalized to actin, revealed an approximately two-fold reduction in bFGF levels in uncultured P10 mutant RPE, as compared with age-matched normal controls (Fig. 2b).

To determine whether the observed decrease in bFGF immunostaining, and mRNA levels were reflective of a biologically significant difference between normal and dystrophic RCS strains, we looked for evidence of functional impairment in the developing mutant eyes. Basic FGF is a very potent effector of biological processes in developing systems and con-

trol of angiogenesis is one of its well known functions [29]. In the eyes of normal neonatal rats, choroidal angiogenesis is known to occur during the second postnatal week [30,31]. We reasoned that if the observed decrease in bFGF expression were functionally significant, it might have detrimental effects on this process. This hypothesis was tested using histological sections of eyes of P7–12 pups fixed by perfusion with mixed aldehydes, and whole mounts of choroid-lined eyecups from 7–14-day-old pigmented animals (Fig. 3). In both normal and dystrophic eyes, the choroid and sclera were undifferentiated at P7 (Fig. 3a,b). In the normal pups, the choriocapillaris and the vortex venous system were seen to develop explosively within the subsequent 1–3 days (Fig. 3c). By contrast, choroidal tissue remained relatively undifferentiated in P10 dystrophic pups (Fig. 3d). In the developing choroid of pigmented rats, the connective tissue spaces become filled with darkly pigmented melanocytes which intimately surround the forming vasculature (Fig. 3c).

The distribution of the developing vasculature was observed by transmitted light and phase contrast microscopy of whole mounts of choroid-lined eyecups from 24 eyes sampled from six normal litters and 44 eyes from seven dystrophic litters, ranging in age from P7 to P11. In both normal and mutant rats, developing choroidal vessels arose as sprouts extending from three main vessels, surrounded by dense clusters of melanocytes. At all ages examined, the number of forming vessels was obviously reduced in the dystrophic eyecups relative to age-matched controls (compare Fig. 3e,f). Furthermore, the distribution of those vessels that formed was often markedly less organized than in normals. The number of branches sprouting from each of the main vessels was quantitated in choroids of 8- and 10-day-old normal and mutant rats (Fig. 4). As expected, results revealed increasing choroidal angiogenesis between P8 and P10 in the normal rat strain, but significantly, total counts of vessels in choroids of mutant P8 and P10 rats were only 21% and 27% respectively, of control values (Fig. 4).

In light of the above indications of reduced bFGF expression in the mutant neonatal RPE, and delayed or abnormal development of choroidal vessels in pups of this age, an analysis of the bFGF gene in the RCS rat was undertaken. The gene was first examined in DNA from control and dystrophic RCS rats by Southern analysis, to look for gross abnormalities such as a deletion or rearrangement. High-molecular-weight genomic DNA from control and RCS rats was digested with various combinations of restriction enzymes and subjected to Southern blot analysis with the RT-PCR-amplified bFGF probe. The results revealed identical bands in the two congenic rat strains (data not shown). Denaturing gradient gel electrophoresis (DGGE) was next used to search for single point mutations; this analysis was conducted at two levels, i.e. on the bFGF gene and the transcript. As described in Section 2, the selected PCR primers were predicted to amplify the putative three exons of the rat bFGF gene (Fig. 5a). Following PCR amplification of genomic DNA from dystrophic and control rats using these primers, products of expected size were obtained and partially sequenced, confirming the suspected similarity in structure of the rat and human gene sequences (data not shown). DGGE analysis of the PCR products representing the three exons revealed no differences in the bFGF exonic sequences of mutant and control RCS rat DNA (Fig. 5b, lanes 1–6). Since homozygous mutations involving guanine and cytosine residues can sometimes be missed by DGGE, the corresponding PCR products from normal and mutant DNA were mixed and analyzed for the production of potential heteroduplex molecules. The result again indicated absence of any mutation (Fig. 5b, lanes 7–9).



B

Hybridization Intensity	1	2	3
bFGF	1.00	0.90	5.19
actin	1.00	1.96	4.10
bFGF/actin x100 (%)	100	46	127

Fig. 2. Northern blot analysis of bFGF expression in uncultured neonatal rat RPE and normal RPE cell line. (a) 10 μg of total RNA from freshly isolated RPE sheets from P10 RCS-rdy^+p^+ normal rats (lane 1), P10 dystrophic RCS-p^+ rats (lane 2) or cultured BPEI-1 normal rat RPE cells (lane 3) was electrophoresed, blotted, and hybridized with ^{32}P-labeled RT-PCR-amplified bFGF probe (upper panel), or β-actin probe (lower panel) and autoradiographed. Arrow indicates a faint 7.0 kb bFGF message present in each lane, but most prominent in lane 3. (b) Densitometric analysis of relative hybridization intensity of bFGF and actin in autoradiographs shown in (a). Results show slightly more than twofold reduction in bFGF expression in lane 2 (P10 mutant RPE) vs. lane 1 (P10 normal RPE).

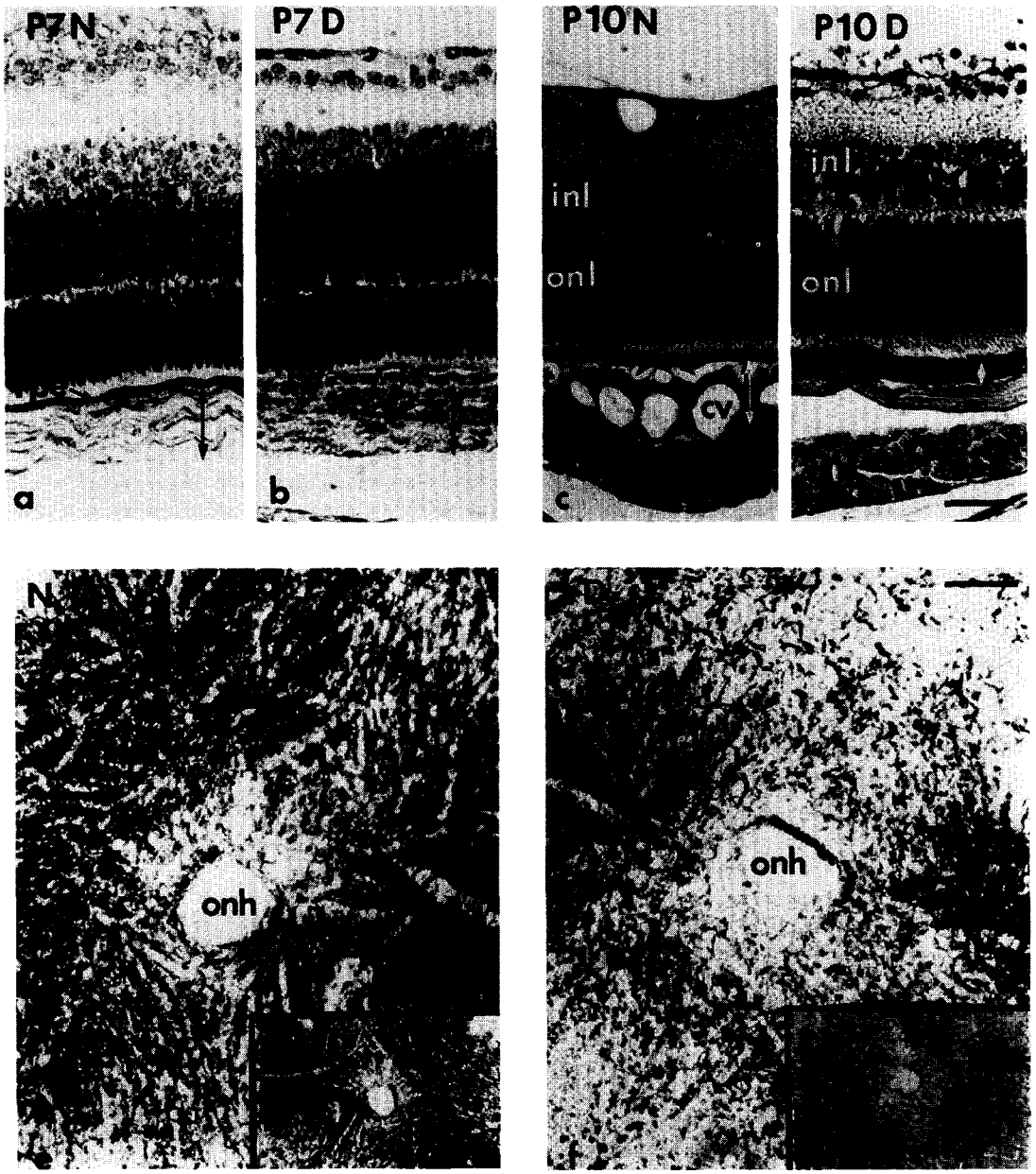


Fig. 3. Analysis of choroidal angiogenesis in developing eyes of pigmented normal (N) and dystrophic (D) RCS rat pups. (a–d) H+E-stained paraffin sections of eyes from normal and mutant rat pups perfusion fixed on the indicated postnatal (P) day, showing the appearance of the posterior wall of the globe close to the optic nerve head. Bar, 100 μm (a–d). Black vertical arrows in (a,b) indicate extent of the undifferentiated choroid+sclera on P7; white vertical arrows in (c,d) show the width of the differentiating choroid on P10. rpe, retinal pigment epithelium; inl, inner nuclear layer; onl, outer nuclear layer; cc, choriocapillaris; cv, choroidal vessel; s, sclera; m, extraocular muscle. In P10 normal pups (c), but not mutants (d), an extensive network of choroidal vessels has developed, intimately surrounded by stroma containing abundant heavily pigmented melanocytes. (e,f) Views of representative whole mounts of choroid-lined eyecups, prepared following enzymatic removal of the RPE layer from eyes of pigmented rat pups: (e) P8 normal, N; (f) P8 dystrophic, D. Insets show similar preparations (P8 N, P7 D) at low power. Bar, 250 μm (e,f) and 1100 μm (insets). The distribution of the developing choroidal vessels is revealed as light lines and streaks coursing through heavily pigmented stroma (dark areas). onh, optic nerve head. Choroidal vessels are abundant in the normal eyes (e) in contrast with the mutant eyes (f).

Because the PCR primers had to be designed from exonic sequences, the above analysis of the putative exons missed the 20 bp of exonic sequence flanking each intron (Fig. 5a). Thus mutational analysis was also conducted on the bFGF transcript by RT-PCR amplification of RNA isolated from control and dystrophic rat RPE cell lines [16], using primers designed to amplify fragments (R1 and R2) containing the missed sequences (Fig. 5a). RT-PCR products of expected size were obtained from RNA of both RPE cell lines and DGGE analysis again showed no difference between the RCS and control sequences (Fig. 5c, lanes 1–4). The R2 product was also subjected to potential heteroduplex formation and analyzed but showed no mutation (Fig. 5c, lane 5). Thus, the coding region of the bFGF gene in the RCS rat appeared to be intact.

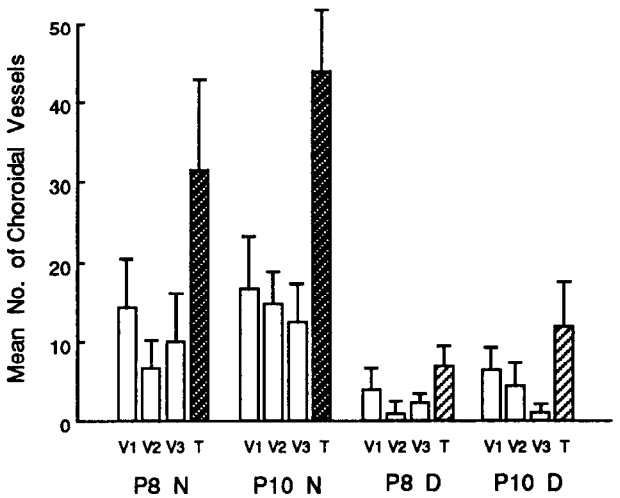


Fig. 4. Quantitation of angiogenesis in whole mounts of choroid-lined eyecups from pigmented normal (N) and dystrophic (D) RCS rat pups. Counts were made, at the indicated postnatal ages, of vessels sprouting from the three main vessels (V1, V2, V3) which form foci of angiogenesis in the differentiating choroid. T (hatched bars), total counts of vessels. The reduction in T in mutant pups, relative to normals, is highly significant ($P < 0.005$, Student's t-test) at both P8 and P10.

4. Discussion

The function of bFGF in the developing and mature retina, RPE and choroid is not yet well understood. However, the possibility of a defect in bFGF as the primary defect in this model is an attractive hypothesis for several reasons. First, the retinal dystrophy begins while the eye is still developing. During the second postnatal week, the photoreceptor outer segments begin to form, and phagocytosis and other normal interactions between RPE and photoreceptors are becoming established [32,8]. The RPE differentiates to its mature state, the underlying Bruch's membrane is elaborated and the choroid undergoes maturational changes, including angiogenesis involving development of the specialized fenestrated capillaries of the choriocapillaris, which supply the RPE and outer retina [30,31]. It is reasonable to hypothesize therefore that bFGF, an important factor in development, might play a central role in one or more of these processes. Secondly, from the original chimera experiment there was indication that the normal *rdy* gene product is a diffusible factor [2]. Finally, studies from our laboratory using primary cultures of neonatal normal and dystrophic rat RPE have shown that exogenously added bFGF can reverse the outer segment phagocytic defect, the hallmark of dystrophic rat RPE cells in vitro*.

Since the RPE is known to be the primary site of the genetic defect in RCS rats [2], in this study we evaluated the status of bFGF in normal and dystrophic RPE from P7 to P14, which spans the critical period in rat retinal development during which the RCS phagocytic defect first appears. Assessment of bFGF immunoreactivity in the transparent eyecup preparations was quite informative, in that first, the reaction of all RPE cells in the intact monolayer could be viewed en face and second, without the need to section the RPE cells, potential loss of this soluble factor during processing was greatly reduced. Previous studies have shown specific immunostaining of bFGF in rat RPE using a variety of antibodies including an antipeptide antibody recognizing the first 24 amino acids of the bovine and human bFGF sequence [33,34]. In our investigation, immunofluorescent staining of bFGF in RPE both in situ and in isolated sheets clearly demonstrated a marked reduction of immunoreactivity, relative to RPE cells from age-matched normals, in dystrophic RPE analyzed from P7-P14. Our results on bFGF immunolocalization in normal and dystrophic rats of this age differ from those of Connolly et al. [34], obtained using paraffin or frozen sections of paraformaldehyde-fixed retinas of 19 different ages from P0 to P90; in that study no staining differences between the two strains were noted at any age. Nevertheless, the authors suggested that the RCS retinas might possess a relative deficit in bFGF too small to detect by their immunohistochemical method. Indeed, in

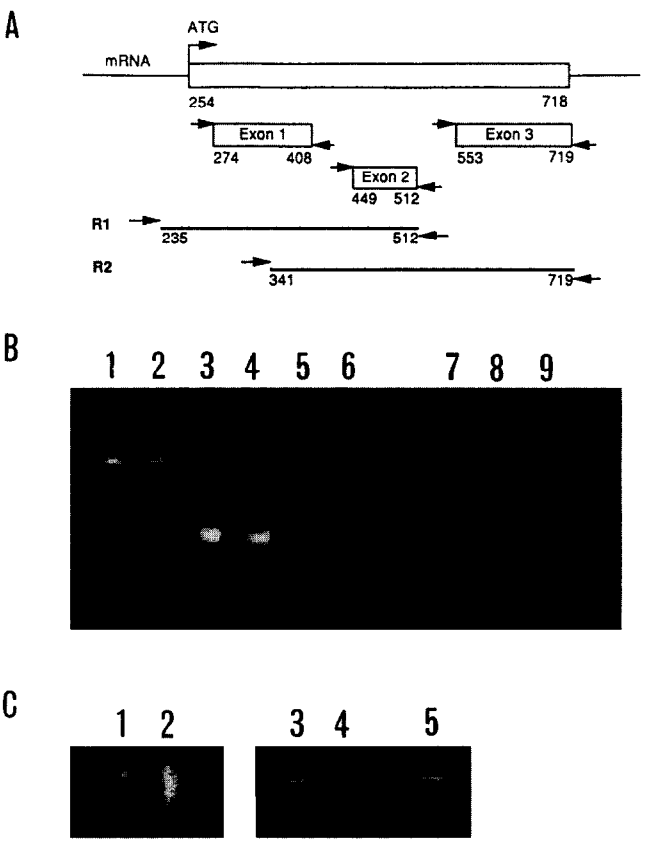


Fig. 5. PCR amplification of the rat bFGF gene and message, and analysis by DGGE. (a) Based on structure of the human gene, indicated PCR primers were designed for the amplification of the three predicted coding exons of the rat bFGF gene. Primers were also designed to amplify overlapping fragments (R1 and R2) of the rat bFGF cDNA. See text for details. (b) DGGE analysis of the PCR-amplified exonic sequences from normal (lanes 1,3,5) and dystrophic (lanes 2,4,6) rat DNA. To produce potential heteroduplex bands, normal and dystrophic products were mixed and analyzed (lanes 7,8,9). No differences in migration are seen (lanes 1–6) and heteroduplexes are not observed (lanes 7–9). (c) RT-PCR amplification and DGGE of normal (lanes 1,3) and dystrophic (lanes 2,4) RPE cell line RNA in two overlapping fragments i.e. R1 (lanes 1,2) and R2 (lanes 3,4). The R2 RT-PCR products were also mixed and analyzed for heteroduplex formation (lane 5). No differences are observed between the normal and dystrophic RT mRNA samples.

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the published figures showing a few cross-sectioned RPE cells, there is evidence of variable expression of bFGF from one RPE cell to the next. Such variations, which we observed in intact P10–12 RPE of normal rats, are of course more easily appreciated when viewing the entire RPE monolayer en face. Alternatively, the apparent differential bFGF expression we observed in RPE monolayers in situ and in freshly isolated RPE cells from P7 to P14 may be specific to the particular developmental window which we were studying in detail.

The level of bFGF mRNA is known to be quite low in most mature tissues, making it difficult to study bFGF expression at the message level [35-37]. This was certainly borne out in our experiments with uncultured RPE cells, since only a barely detectable signal was obtained by Northern analysis of RNA from 10 day old rats. Nevertheless, a twofold reduction in the level of message was observed in the RCS RPE compared to congenic controls, based on results from over 100 eyes from 5–7 litters per sample. Another group previously used RT-PCR to compare bFGF expression in cultured RPE from neonatal rat pups, following growth of the cells for 10 days in vitro, and in contrast to our results with freshly isolated RPE, found no difference between normal and dystrophic cells under these conditions [38]. It is known that bFGF levels can vary significantly once cells are removed from their natural environment and cultured [39-41]; thus the most likely explanation for our differing results might simply be the difference between uncultured and cultured RPE cells. Because of the possibility of culture-induced changes in bFGF expression, uncultured RPE cells were used here as the source of RNA for evaluation of expression of this gene in vivo, which, as shown in the results, appeared to be downregulated in the RPE of mutant rat pups.

A theoretical cause of a bFGF protein and mRNA deficit in the RCS RPE, as shown here, could be a mutation in the coding sequence of the bFGF gene: either gross, such as a deletion or rearrangement, or more subtle, such as a nonsense codon mutation resulting in a message with reduced stability or expression level, as shown for mutations in ornithine aminotransferase in gyrate atrophy [27]. Alternatively, a mutation in the promoter region could also result in reduced expression or loss of inducibility. The failure in this study to detect any differences between the normal and dystrophic bFGF genes by Southern analysis and between the exons and RT mRNAs by DGGE essentially rules out the possibility of a mutation in the coding sequence, although the possible existence of a promoter mutation has not been excluded. An alternate explanation for these results is that bFGF expression may be down regulated in the mutant RPE as a result of a mutation in another RPE-expressed gene, i.e. the as yet unknown *rdy* gene.

Although it may not be the primary genetic defect in the RCS rat, the reduction of bFGF expression shown here could have significant ramifications, occurring as it does during a critical developmental period for the outer retina and underlying choroid. The data presented here from the analysis of choroidal vasculature in perfusion-fixed eyes and whole mounts of choroid provide the first demonstration that choroidal angiogenesis is abnormal in neonatal RCS rats. Interestingly, the choroidal defect, evident from P8 or earlier, predates the phagocytic defect in these developing eyes by at least four days [8]; thus this is the earliest known abnormality to be detected in the differentiating eyes of these mutant rats. It has been previously proposed that maintenance of the choriocapillaris is under control of the RPE [42]. Recently it has been demonstrated using an in vitro model of choroidal angiogenesis that elongation of choroidal endothelial tubes is stimulated by coculture with RPE, and inhibited by neutralizing antibodies against angiogenic factors including bFGF [43]. We have shown in this study that abnormal choroidal angiogenesis in RCS rat pups is coincident with reduced bFGF expression by the RPE. This result is consistent with the hypothesis that bFGF expression by the RPE may play a central role in the normal postnatal development of the choroidal blood supply in the rat eye. Further studies are required to test the involvement of this pluripotential growth and differentiation factor in choroidal angiogenesis, RPE phagocytosis, and other processes that are becoming established at this critical stage of ocular differentiation.

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