Bone morphogenetic proteins promote neurite outgrowth in retinal ganglion cells

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Purpose: The purpose of the present study is to test the ability of members of the transforming growth factor/bone morphogenetic protein family to influence retinal ganglion cell (RGC) survival and neurite outgrowth in primary cell culture using a high throughput analysis.

Methods: Primary cell cultures were generated using immunoselection of Thy-1 positive cells from dissociated postnatal rat retina and grown on poly-L-lysine/laminin coated 96 well culture dishes in the presence or absence of members of the transforming growth factor/bone morphogenetic protein family. High throughput analysis was performed following fluorescence staining with Hoechst, Calcein AM, and TOTO-3. Outcomes included overall cell survival, survival of cells with neurite outgrowth, and a variety of parameters of neurite outgrowth.

Results: Immunomagnetic selection led to an enrichment of cell cultures for RGCs (79%±6.8%). While no significant effect on overall survival was observed with any of the factors tested, members of the bone morphogenetic protein (BMPs) family (BMP2, BMP13, and GDF8 (growth differentiation factor 8)) and BDNF (brain derived neurotrophic factor) increased the number of surviving RGCs with neurite extension in a dose dependent manner. As a group, BMPs increased the number of neurites, length of neurites, and the number of branch points, while BDNF primarily increased neurite length and branch points.

Conclusions: We have developed an efficient system that allows for high throughput analysis of cultures enriched for RGCs. Using this assay system, we found that BMPs promote the survival of outgrowth neurons and neurite development in RGC culture.

Retinal ganglion cells (RGCs) are the projection neurons of the retina, and their axons transmit neural signals generated in the retina to visual centers in the brain via the optic nerve. Damage to RGC axons and death of RGCs contributes to loss of vision associated with optic neuropathies and glaucoma. The identification of factors that can enhance RGC survival and promote axonal growth or regeneration could contribute to the development of novel therapies. Bone morphogenetic proteins (BMPs), originally discovered for their capacity to promote osteogenesis, are members of the transforming growth factor (TGF)-β superfamily that are known to regulate a variety of cell functions in multiple tissues including proliferation, apoptosis, differentiation, and morphogenesis [1,2]. Based on amino acid homologies, BMPs may be categorized into six groups [3]. They signal responding cells via different hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors and activate intercellular signaling through the SMAD proteins [4,5].

BMPs and their receptors are expressed in the eye during embryogenesis and play critical roles in ocular development. BMP4 [6] and BMP7 [7] expression in the developing optic vesicle and lens is essential for early morphogenesis of the optic cup and lens vesicle [6,8-12]. BMP4 is required for lens induction [6] and lens fiber elongation in chick [13]. Monoclonal antibodies against BMP7 arrest eye development at the optic vesicle stage and block lens and optic cup formation in cultured rat embryos [7], and targeted deletion of BMP7 in mice results in microphthalmia and anophthalmia [5,6]. Many of the BMPs and their receptors are expressed in gradients within the developing eye. Similar to the Drosophila TGFβ homolog Dpp [14,15], they may act as morphogens to provide positionally dependent differentiation cues. Ectopic expression of BMP4 in Xenopus results in defects in dorsal/ventral and proximal/distal patterning in the developing eye characterized by expansion of dorsally expressed genes including pax6 and tbr2/3/5 [16]. Similarly, overexpression of BMP4 and BMP5 in chick results in loss of midline structures in both the brain and eye leading to holoprosencephaly and cyclopia [17,18].

TGFβ and BMP signaling has been implicated in retinal neurogenesis, RGC differentiation, and axonal pathfinding. TGFβ3 is expressed in differentiated RGCs and is a mitogen for rat retinal progenitor cells in vitro [19]. The BMP receptor BmpRIB/Alk6 is preferentially expressed in the ventral retina during embryonic development and is required for normal axon pathfinding of ventral RGCs at the optic nerve head. In mice with a targeted deletion of BmpRIB, many of the axons arising from the ventrally located ganglion cells fail to enter the optic nerve and instead make abrupt turns at the optic nerve.
head [20]. BMP2, BMP4, BMP5, BMP7, and all three BMP receptors (BmpRIA/Alk5, BmpRIB/Alk6, and BmpRII) are expressed in cells cultured from the human optic nerve head [21]. In the chick, ventroptin, an antagonist of BMP4, is mainly expressed in the ventral retina, not only with a ventral high/dorsal low gradient but also with a nasal high/temporal low gradient at later stages [22]. Misexpression of ventroptin alters expression patterns of several topographic genes in the retina, including Thbx5 and eVax, and results in abnormal projection of retinal axons to the tectum along both the dorsal/ventral and rostral/caudal axes. Microarray and quantitative reverse transcription PCR (rt-PCR) analyses of gene expression in Brn3b knockout mice, which have a 70% reduction in the number of RGCs [23], show a 12 fold reduction in expression of the BMP related myostatin/GDF8 (Growth and differentiation factor 8) [24]. Based on the accumulated data suggesting a role for Bmps in retinal neuronal development and pathfinding, we have been examining the effects of Bmps on the behavior of cultured RGCs. We report here the results of a high throughput analysis of the effects of members of the TGFβ family on cell survival and neurite outgrowth in primary retinal cell cultures, enriched for RGCs.

METHODS

Cell culture: The study protocol for rat retinal culture was approved by and is in accordance with the Johns Hopkins Animal Care and Use Committee and Statement for the Use and Care of Animals in Ophthalmic and Visual Research of the Association for Research in Vision and Ophthalmology. Retinas were dissected from postnatal day 3 to 5 Sprague Dawley rats, digested with activated papain (Worthington Biochemical Corporation, Lakewood, NJ) and DNase (Sigma-Aldrich, St. Louis, MO) prior to dissociation by trituration. Immunomagnetic selection of RGCs was performed by incubating the cell suspension with magnetic microbeads (about 50 nm in size) conjugated to Thy-1 antibodies (Miltenyi Biotec Inc., Auburn, CA) followed by passage over a magnetized metal column (MS Column, Product Number 130-042-201, Miltenyi Biotec Inc.). Cells bound by the Thy-1 antibody conjugated microbeads were retained within the column, whereas the remaining retinal cells passed through the column. After removal of the column from the magnet, the purified Thy-1 immunoreactive cells were eluted and resuspended in growth media [Neurobasal media, B27 supplement, glutamine (2 mM), and Penicillin/Streptomycin (1 U/ml), supplemented with various growth factors (Peprotech, Inc. Rocky Hill, NJ) 2 h after seeding]. It was not necessary to remove the anti-Thy-1 beads from the surface of the immunoselected cells. Selection of RGCs with this procedure was previously shown to be successful [25]. Cells were seeded at a density of 5000 cells/well (150 cells/mm²) in 96 well culture dishes (Falcon, BD Biosciences, Bedford, MA) that had been sequentially coated with poly-d-lysine (0.01 mg/ml) and laminin (0.01 µg/µl, Sigma). About two thirds of the media was replaced after 72 h and analysis was performed at 120 h after initial seeding. For analysis, cells were triple stained with Hoechst 33342 (5 µM), Calcein AM (10 µM, cell stain; Molecular Probes, Eugene, OR), and Toto-3 (1 µM, dead cell stain; Molecular Probes). RGCs were retrograde labeled in vivo by application of 1'-dioctadecyl-3, 3, 3’,3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) suspended in N,N-dimethylformamide (15 mg/ml) to the superior colliculus three days prior to sacrifice and culture. Efficiency of immunomagnetic purification for Thy-1 binding was assessed by immunostaining cultured cells, fixed briefly in 4% paraformaldehyde, with mouse anti-rat Thy-1 antibody (Chemicon MAB1406; Temecula, CA) diluted 1:100 and secondary labeling with FITC conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA).

Analysis: In a standardized fashion that was identical for each well, twenty nonoverlapping fields, approximately half of the surface area of each well, were imaged on a Cellomics KSR instrument (Cellomics, Inc., Pittsburgh, PA) using epifluorescence and filter sets corresponding to each of the three dyes. The entire scan takes just under 6 h in a fully automated and unattended fashion and collects 5760 images which are analyzed using Extended Neurite Outgrowth software package (Cellomics, Inc.). While rescanning individual wells demonstrates reproducible images and fields, a complete rescan of the same plate is not performed because increasing background calcein fluorescence. Live cells were defined as Hoechst positive, TOTO3 negative cells that surpassed a threshold level of Calcein AM staining that was determined empirically. Clusters of two or more cells (based on size of the Hoechst stained image), cells stained with both Calcein AM and TOTO3, and cells that stained with neither dye were excluded from analysis. Using the Cellomics Extended Neurite Outgrowth program, neurites were traced and the neurite length, neurite count, number of branch points, crosspoints, and “outgrowth” (a product of neurite length and neurite count) determined. In addition, the number of cells with neurite features (i.e., neurite length, neurite count, outgrowth) surpassing a threshold of one standard deviation above the mean neurite characteristic of a set of reference wells containing growth medium without additional growth factors was determined. These data were normalized to counts or various neurite parameters from wells with cells containing growth media alone and compared using a 2 tailed t-test, with a p<0.007 considered significant to account for multiple comparisons. Data are reported as the average ±standard deviation (SD).

RESULTS

Purity and efficiency of Thy-1 immunoselection: To analyze the effects of growth factors on RGC survival and neurite outgrowth, we used immunomagnetic selection of primary retinal cells to enrich for RGCs. To assess the efficiency of our selection technique, RGCs were retrograde labeled by bilateral transcranial injection of DiI into the superior colliculus three days prior to sacrifice (Figure 1). Following dissection, dissociation, and immunopurification of 20 retinas, 79%±6.8% of the cells that were retained by the magnetized column were DiI positive RGCs. In contrast, only 0.6%±0.86% of cells in the flow-through were DiI positive. As a secondary test of the efficacy of the magnetic purification, cells were
immunostained with anti-Thy-1 antibodies on the day after plating. Greater than 99% of cells retained by the column stained with Thy-1 antibody, whereas <1% of cells passing through the column were Thy-1 immunopositive (Figure 2).

Survival and neurite outgrowth: RGC enriched cultures were derived from 16 retinas from postnatal day three to five rats and seeded into poly-L-lysine/laminin coated, 96 well plates. Initial viability of purified cells was determined by counting the number of cells stained with TOTO3 (dead cell stain) 2 h after initial seeding. Based on an initial plating density of 5000 per well, viability was routinely greater than 90%. Each growth factor was added to 12 wells 2 h after plating, with 7 growth factors tested per plate. For each plate, 12 control wells did not receive additional growth factors. After 120 h in culture, cells were imaged (Figure 3), and the number of live cells and number of cells with neurite outgrowth were determined and compared. Based on the number of living cells present 2 h after seeding, the 120 h average survival without additional growth factors was 44.0%±6.6%. Based on the analysis of five experiments, the addition of BMP2, BMP13, TGFα, TGFβ1, or TGFβ2 (50 ng/ml) had no statistically significant effect on overall cell survival, although addition of 50 ng/ml BDNF and GDF8 (myostatin) resulted in a slight increase in the number of surviving cells (Table 1). However, the number of outgrowth neurons, defined as those cells with total neurite length and neurite count exceeding one standard deviation above the mean in control wells, was significantly increased for BDNF, and all of the BMPs as a group (BMP2, BMP13, GDF8 [myostatin]; Figure 4). The most robust effect

Figure 1. Purity of Thy-1 immunoselection. Three days following retrograde labeling of RGCs by DiI in postnatal day 2 rats, dissociated retinal cells were purified using immunomagnetic selection of RGCs with Thy-1 antibody conjugated microbeads and photomicrographed using DIC optics (A), epifluorescence illumination of DiI labeling (B), and Thy-1 immunostaining (C). This illustrates the enrichment of cultures for DiI labeled, Thy-1 immunopositive RGCs (Figure 2) as most of the cells after immunoselection (A) are positive for both diI (B) and Thy-1 immunoreactivity (C); whereas prior to immunoselection, only 1-5% of the cells were diI and Thy-1 immunopositive.

Figure 2. Efficient Thy-1 immunoselection. Both the cells passing through the magnetized columns (represented as open squares in the figure) and the cells retained by the magnetized column and later eluted (represented as filled triangles in the figure) were plated, immunolabeled with Thy-1 antibody and secondary FITC conjugated antibody, and imaged with the Cellomics KSR instrument. The intensity of fluorescence for each cell is plotted, demonstrating two distinct populations of cells (p<0.00001, Student’s t-test) and highly efficient immunomagnetic purification. The fluorescence intensity of the cells passing through the magnetized columns (represented as open squares in the figure) can be considered background.
Figure 3. Neurite outgrowth of cultured RGCs. At 120 h, neurites were visualized after staining with Calcein AM. RGCs were grown in the standard media alone (A), in the presence of BDNF (B), or in the presence of BMP2 (C). In the presence of BDNF, 15 to 30% of cells were “outgrowth neurons,” having neurite features surpassing a threshold of one standard deviation above the mean neurite characteristic of RGCs cultured in growth medium alone. The scale bar represents 40 µm.
was observed with BMP2 (outgrowth count ratio 1.61±0.80). No significant change in the number of outgrowth neurons was observed for TGFα, TGFβ1, or TGFβ2.

As expected, based on the significant increase in the number of neurite bearing cells, parameters of neurite development (neurite length, neurite count, and branch point count) were significantly increased for BDNF, BMP2, BMP13, and GDF8 (Figure 5, Table 2). While BDNF had a larger impact on neurite length and branch point count than neurite count, the BMPs tend to promote increased neurite count and branch point count to an extent comparable to their effect on neurite length (Figure 3). In contrast, TGFα had no significant impact on overall survival or the number of neurite bearing cells, but it did promote a modest increase in branch count and neurite length.

**Dosage effect, combinatorial effect, and inhibition:** To test for a dose dependent effect, cells were cultured in the presence of 0, 10, or 50 ng/ml demonstrating a dose response relationship (Figure 6). Although no change in the number of outgrowth neurons was observed for BMP13 and GDF8 at a concentration of 10 ng/ml, there was a statistically significant increase at 50 ng/ml. Previous studies have shown BDNF can potentiate the effects of growth factors including CNTF [26] and BMP2 [27,28]. To look for synergistic effects of BDNF with the BMPs, cultures were analyzed following addition of each BMP in the presence of BDNF at 50 ng/ml (Figure 7). While an increased number of outgrowth neurons was observed in the presence of both factors relative to controls lacking growth factors, the overall effect did not exceed that of BDNF alone. For BMP2, the addition of the BMP antagonist noggin reduced the effect of BMP2 on the number of outgrowth neurons (data not shown).

**DISCUSSION**

We have developed a system that allows for robust and high throughput analysis of cultures enriched for RGCs in a 96 well format. At our maximum capacity, we are able to culture, image, and analyze eight 96 well plates per week. This method has been developed in order to screen a variety of molecules for the ability to promote RGC survival and neurite outgrowth. In addition to assaying for survival, our system makes possible the analysis of a variety of parameters of neurite out-
growth. Furthermore, the system can distinguish those cells with robust neurite development (referred to as outgrowth neurons). We believe these to be RGCs based on their morphologic appearance in terms of cell body size and neurite outgrowth, response to BDNF, DiI retrograde labeling, and Thy1 immunopositivity. The high throughput format allows for objective assessment and increased statistical power in the analysis of a cell’s response to various growth conditions. The culture system does not offer the high purity of the two step procedure of immunodepletion of macrophages followed by immunoselection of RGCs [29]; however, it offers the advantage of being faster, a desirable quality for screening.

Previous studies have shown that BDNF promotes both neurite outgrowth and survival of RGCs [17,18]. We found that BDNF increased neurite development and the number of outgrowth neurons, although it had little effect on total cell survival in our assay system. There are several possible explanations for the lack of an effect on overall cell survival. First, under our growth conditions, total cell survival may be robust enough to make it more difficult to observe the survival promoting effects of molecules. Our overall cell survival without additional growth factors at five days was 44%, notably higher than previous studies where survival at three days ranged from 15 to 20% [30,31]. Second, the lack of an effect on overall cell survival may reflect the presence of Thy-1 positive cells other than RGCs (for example, macrophages) [29].

Figure 6. Dosage effect on survival of outgrowth neurons. In two experiments, cells were cultured in the presence of 0, 10, or 50 ng/ml demonstrating a positive dose response relationship for BDNF and the BMPs. Statistical comparisons are made to control wells without growth factors. For statistical comparisons to media only, an asterisk (*) denotes p<0.002; double asterisks (**) denote p<0.0001. The outgrowth count ratio is the number of outgrowth neurons (neurons with neurites) normalized to the number of outgrowth neurons in media only.

Figure 7. Combinatorial effects of BMP and BDNF on outgrowth neuron survival. To look for synergistic effects of BDNF with the BMPs, cultures were analyzed following addition of each BMP in the presence of BDNF at 50 ng/ml. Although an increased number of outgrowth neurons was observed in the presence of both factors relative to controls lacking growth factors, the overall effect did not exceed that of BDNF alone. The outgrowth count ratio is the number of outgrowth neurons (neurons with neurites) normalized to the number of outgrowth neurons in media only. Statistical comparisons are made to control wells without growth factors. Asterisk (*) denotes p<0.05; double asterisks (**) denote p<0.0001.

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<th>Treatment</th>
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<th>Neurite count ratio</th>
<th>SD</th>
<th>p value</th>
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The table summarizes the average normalized neurite length, neurite count, and branch point count from five experiments. For each factor, the parameter was normalized to the media alone. The p values correspond to the statistical comparisons of neurite length ratio, neurite count ratio, and branch point count made to the respective parameter for media only. While BDNF had a larger impact on neurite length and branch point count than neurite count, the BMPs tend to promote increased neurite count and branch point count to an extent comparable to their effect on neurite length (Figure 5). There were 59 wells used for each sample.
in our system which would have reduced our ability to detect an overall significant change in cell numbers in the presence of BDNF. Surviving macrophages, which would be counted as live cells (without neurites), would diminish our ability to detect changes in the number of surviving RGCs. By counting cells with neurite outgrowth, we believe that we were able to minimize the contribution of non-RGCs to our analysis. Our ability to detect an effect on neurite outgrowth suggests that the presence of macrophages did not have an impact on the BDNF signaling per se, rather it may have reduced the sensitivity of our assay to detect surviving RGCs in the basic measurement.

We found that addition of BMP2, BMP13, and GDF8 increased the number of outgrowth neurons in our cultures. While BMP2 had the largest effect in our assay, BMP13 and GDF8 had qualitatively similar effects. Variation in potency within a family of growth factors in the responsiveness of RGCs has also been observed. In the neurotrophin family, BDNF has the greatest effect whereas others (e.g., CNTF) have a similar, although less robust effect on RGC survival and neurite outgrowth. Previous studies have shown that BMPs can enhance both survival and neurite outgrowth of multiple types of cultured primary neurons, including developing GABAergic neurons from rat striatum [32,33] and serotonergic neurons isolated from the embryonic rat raphe [28]. As noted above, our assay may be less sensitive to changes in survival because of the overall robust survival in our culture system and the possible indirect contribution of surviving non-RGCs. Therefore, we cannot exclude the possibility that these growth factors are able to promote survival and neurite development.

BMPs are known to mediate neuron induced differentiation of CNS stem cells into neurons [34] and may modulate axonal outgrowth and dendritic arborization during neuronal development and plasticity [35] as well as in response to hypoglycemia [36]. Consistent with this, we found that BMP2, BMP13, and GDF8 all increased the number of neurites, length of neurites, and branch points to a similar magnitude. In contrast, BDNF had a larger effect on neurite length and branchpoints in comparison with neurite count. Thus, BDNF produced long, branching neurites while BMPs produced short, numerous, and branching neurites. In cultures treated with saturating concentrations of BDNF in combination with BMP2 or GDF8, we observed an increase in the number of outgrowth neurons compared to either factor alone. It is notable that the BMP effect was observed in the presence of an excess concentration of BDNF demonstrating that the maximal potential of RGCs to survive and differentiate in culture is not reached in the presence of BDNF alone. The combinatorial effect may be due to the activation of independent intracellular signaling pathways: BDNF though CREB and BMP via the SMADs.

BMP signaling appears to play a fundamental role in dorsal-ventral patterning in the retina and influences RGC axon pathfinding to the optic nerve head and the subsequent establishment of the retinotopic organization of RGC axon terminals in the optic tectum [16,20,22]. Blocking of BMP signaling by noggin, a binding protein with high affinity for TGFβ family members BMP4 and BMP2, results in multiple developmental anomalies in the ventral retina including retinal coloboma, ventral displacement of the optic nerve head, and variable defects of the optic nerve associated with abnormal projection of nerve fibers into the vitreous [37]. The observations that targeted deletion of the BmpR1b gene leads to defects in axon pathfinding of ventral RGCs at the optic nerve head [20], the reduction in myostatin/GDF8 transcripts in Brn3b knockout mice [24], and our finding that BMPs support survival and outgrowth in culture is consistent with a role for BMP signaling in RGC development and axon outgrowth.

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