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Vascular Endothelial Cell Growth Factors Promote the *In Vitro* Development of Rat Photoreceptor Cells

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We have identified and characterized a novel trophic effect of vascular endothelial cell growth factor (VEGF) on photoreceptor cells. Treatment of retinal cultures, derived from postnatal day 1 (P1) rats, with VEGF-2 resulted in a dose- and time-dependent increase in the level of rhodopsin protein, as determined by ELISA assay. After 7–9 d of treatment the VEGF-1 or VEGF-2, at a concentration of 10 ng/ml, induced a 200–300% increase in rhodopsin protein and a 220% increase in the number of rhodopsin-immunopositive cells. Treatment with VEGF-2 induced a 250% increase in the number of syntaxin-immunopositive cells and a 67% increase in high-affinity GABA uptake, both markers for amacrine cells. In contrast, there was no increase in the non-neuronal cell populations. VEGF-2 induced an ~300% increase in the number of bromodeoxyuridine-labeled (BrdU) retinal cells within 48 hr of treatment. After 3 d in culture both the basal and stimulated levels of BrdU incorporation were reduced, suggesting that the proliferative effect of VEGF was restricted developmentally. Furthermore, there was a developmentally dependent increase in the mitogenic response to VEGF-2, with retinal cultures derived from E15, E20, or P1 animals demonstrating a 50, 100, and 300% increase in thymidine incorporation, respectively. However, VEGF treatment resulted in an increase in the number of rhodopsin-immunopositive cells only when the cultures were derived from P1 animals. Therefore, retinal progenitor cells appear to be targets for VEGF, and thus VEGF may be involved in the regulation of the early developmental program of retinal neurogenesis.

Key words: retina; rhodopsin; CNTF; amacrine cells; photoreceptor progenitor cell; vascular endothelial cell growth factors

The retina has proven to be an advantageous experimental model for studying the role of intrinsic and extrinsic factors in the regulation of the development of neuronal and non-neuronal cell types from a more primitive neuroepithelial cell. The differentiated retina is composed of at least seven cell types: sensory (rod and cone photoreceptors), glia (Müller cells), and two types of neurons, interneurons (horizontal, bipolar, and amacrine) and projection neurons (ganglion cells) (for review, see Dowling, 1987). The development of the various cell types in the retina does not occur synchronously with the majority of the cones and ganglion and horizontal cells developing before birth (for review, see Altshuler et al., 1991; Harris, 1991; Reh, 1991). In contrast, differentiation of a majority of the rods, the main cell type in the rat retina, occurs postnatally. Clonal analysis of the progeny of retinal precursor cells has demonstrated that the progenitor cells can produce various combinations of retinal cell types, indicating that the progenitors are either totipotent or multipotent, depending on the developmental age that is examined (Turner and Cepko, 1987; Wetts and Fraser, 1988; Turner et al., 1990). Furthermore, findings from both *in vivo* and *in vitro* studies demonstrate that the final phenotype of the retinal cells is mainly lineage-independent, which suggests that the changing microenvironment within the retina has a role in determining the cellular potential of the progenitor cells as well as the differentiated phenotype of the progeny (Watanabe and Raff, 1990, 1992; Harris and Hartenstein, 1991; Reh, 1991; Ezzeddine et al., 1997).

In vitro, retinal cell proliferation and differentiation are regulated by a variety of factors—for example, basic fibroblast growth factor (FGF-2; Hicks and Courtois, 1992), ciliary neurotrophic factor (CNTF; Fuhrmann et al., 1995; Ezzeddine et al., 1997), leukemia inhibitory factor (LIF; Ezzeddine et al., 1997), transforming growth factor-β (TGFβ; Lillien and Cepko, 1992), retinoic acid (Kelley et al., 1994), and epidermal growth factor (EGF; Lillien, 1995). Recently, Yang and Cepko (1996) have identified and characterized the expression pattern of vascular endothelial cell growth factor receptor-2 (VEGFR-2/FLK-1) in developing and adult retina. VEGFR transcripts are detected first at embryonic day 11.5 (E11.5) in association with the developing retinal vasculature and with the central region of the neural retina. By developmental day E15 the VEGFR-2 expression extends to the periphery of the retina, consistent with the outward gradient of retinal development (Young, 1985; LaVail et al., 1991). VEGFR-2 expression is localized mainly to the ventricular zone during the perinatal period when neurogenesis is at its peak and a large number of postmitotic neurons are being formed.

We are interested in determining what biological significance the VEGF receptors have in regulating the development and homeostasis of retinal cells. We determined that VEGF is mitogenic for retinal progenitor cells. The proliferative response increases with age from E15 to postnatal day 1 (P1). In retinal cultures that are derived from P1 animals, VEGF treatment increases the number of rhodopsin- and syntaxin-immunopositive cells. Results from double-labeling immunohistochemical studies for rhodopsin and bromodeoxyuridine (BrdU) suggest that VEGF-2 also enhances the survival of photoreceptor cells. Furthermore, CNTF inhibits the VEGF-2 induced increase in the level of rhodopsin protein, but not the early proliferative response.

MATERIALS AND METHODS

Animals. Timed pregnant animals were obtained from Harlan Sprague Dawley (Indianapolis, IN). All animal-related procedures were conducted in strict compliance with approved institutional protocols and in accordance with provisions for animal care and use as described in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23, 1985).

Retinal cultures. The retinal tissue was obtained from either embryonic or neonatal rats. The dissociated primary cells were prepared by incubating the tissue in 0.25% trypsin for 6 min at 37°C. After the inactivation of the trypsin by a 5 min incubation in growth medium [F12: DMEM containing 1% fetal bovine serum, 1% hormonal supplements (N2; Bottenstein, 1983), 1% glutamine, and 0.5% penicillin–streptomycin (10,000 U/ml and 10 mg/ml, respectively; Life Technologies, Grand Island, NY)]

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containing 50 μg/ml of deoxyribonuclease type I (Sigma, St. Louis, MO), the tissue fragments were passed repeatedly through a Pasteur pipette with a constricted tip of a diameter of ~1 mm. The dissociated cells were collected by centrifugation (800 × *g*, 5 min) and resuspended in growth medium. The cells were seeded in 96-well plates precoated with poly-L-lysine (50 μg/ml; Sigma) and laminin (10 μg/ml; Life Technologies) at a density of 425 cells/mm^2 unless stated otherwise. The cultures were shifted gradually to a serum-free growth medium by changing one-half of the medium every other day. The trophic factors were replenished with each medium change.

Rhodopsin, syntaxin immunohistochemical staining, and cell-counting procedures. For the immunohistochemical staining the cultures were fixed overnight in 4% paraformaldehyde containing 4% sucrose. For rhodopsin and syntaxin staining the cultures were permeabilized with 0.05% saponin in PBS for 30 min. The nonspecific IgG binding was inhibited by incubating the cells in PBS containing 5% horse serum and 2% BSA for 3 hr at room temperature. Then the cultures were incubated overnight at 4°C with anti-rhodopsin (1:10,000, Rho 4D2; obtained from Dr. Molday, University of British Columbia) or anti-syntaxin (1:10,000; Sigma) diluted in PBS containing 5% horse serum and 2% BSA (Molday, 1989). After the removal of the primary antibody the cultures were incubated with a biotinylated anti-mouse antibody (1:2500; Vector Laboratories, Burlingame, CA) for 90 min. Then the avidin–biotin–peroxidase complex, diluted 1:50 in PBS containing 5% horse serum, was added for 60 min. To visualize the bound peroxidase, we used diaminobenzidine at a final concentration of 0.4 mg/ml in a 0.1 M acetate buffer containing 2.5% nickel sulfate. The number of immunopositive cells per well was determined by counting the labeled cells in an area representing 7% of the total surface area of the well and then correcting the count for the total surface area.

Rhodopsin–BrdU double-labeling immunohistochemistry. After various treatment paradigms the retinal cells were incubated with BrdU for 4 hr and, subsequently, were washed twice with PBS. Growth medium was added back to the cultures, which then were maintained *in vitro* for various time intervals. For the rhodopsin–BrdU double-labeling studies the cultures were fixed in 70% ETOH at −20°C for 18 hr. Then the cultures were permeabilized, and the nonspecific IgG binding was inhibited as described above. Subsequently, they were incubated with the anti-rhodopsin antibody (1:2500) for 3 hr at room temperature. After the removal of the primary antibody the cultures were incubated with a biotinylated anti-mouse antibody (1:1000; Vector Laboratories) for 60 min at room temperature and then subsequently with fluorescein–avidin (30 μg/ml; Vector Laboratories) for 1 hr. Next the cultures were post-fixed with 70% ETOH containing 20% glacial acetic acid for 20 min at room temperature and incubated with the anti-BrdU antibody (1:10) for 30 min 37°C per the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). The anti-BrdU was detected with an anti-mouse antibody labeled with rhodamine (30 μg/ml; Boehringer Mannheim). In the experiments in which the cultures were stained only immunohistochemically for BrdU, the staining was conducted per the manufacturer's instructions. In experiments in which the cultures were double-labeled for rhodopsin and with propidium iodide, the photoreceptor cells first were labeled for rhodopsin by using the method described above. To determine the total number of cells, we then incubated the cultures for 1 min in PBS containing 1 μg/ml of propidium iodide and subsequently rinsed them twice with PBS.

Rhodopsin, vimentin, and glial fibrillary acidic protein (GFAP) ELISAs. For the rhodopsin ELISAs the cultures were rinsed with PBS and fixed overnight with 4% paraformaldehyde containing 4% sucrose. Then the cultures were rinsed with PBS and permeabilized with 0.05% saponin in PBS for 30 min at room temperature. The nonspecific protein-binding sites were suppressed by incubating the cells in PBS containing 5% horse serum and 2% BSA (blocking buffer) for at least 3 hr. The cultures were incubated overnight with the mouse anti-rhodopsin antibody (diluted 1:5000 in blocking buffer) or for 3 hr with anti-vimentin (1 μg/ml; Boehringer Mannheim) and subsequently with goat anti-mouse IgG conjugated to horseradish peroxidase (diluted 1:2500) in blocking solution. The cultures were washed extensively with PBS; then the substrate (3,3′,5,5′-tetramethylbenzidine) was added to the wells, and the plates were incubated in the dark for 30 min. The reaction was stopped by adding 2 M H_2SO_4, and the amount of product formed was quantitated by measuring the absorbance at 450 nm. The absorption of the reagent blank ranged from 0.1 to 0.15 and was not subtracted from the indicated values.

The GFAP ELISA was conducted essentially as previously described (Greene et al., 1998).

High-affinity GABA uptake and calcein AM assay. The level of high-affinity GABA uptake was determined as previously described (Greene et al., 1998). As a relative measurement of the total cell number, the level of calcein AM emission was determined in the cultures after treatment. The medium was removed, and the cultures were rinsed once with Ham's F12. Then Ham's F12 containing 2 μM calcein AM was added, and the cultures were incubated for 1 hr at 37°C. Subsequently, the level of emission was determined at 530 nm.

[^{3}H]thymidine incorporation. After treatment with factors the cells were labeled for 6 hr with [^{3}H]thymidine at a final concentration of 0.33 μM (25 Ci/mmol; Amersham, Arlington Heights, IL). The incorporated [^{3}H]thymidine was precipitated with ice-cold 10% trichloroacetic acid for 24 hr. Subsequently, the cells were rinsed with ice-cold water. After lysis in 0.5 M NaOH the lysates and PBS rinses (500 μl) were pooled and counted.

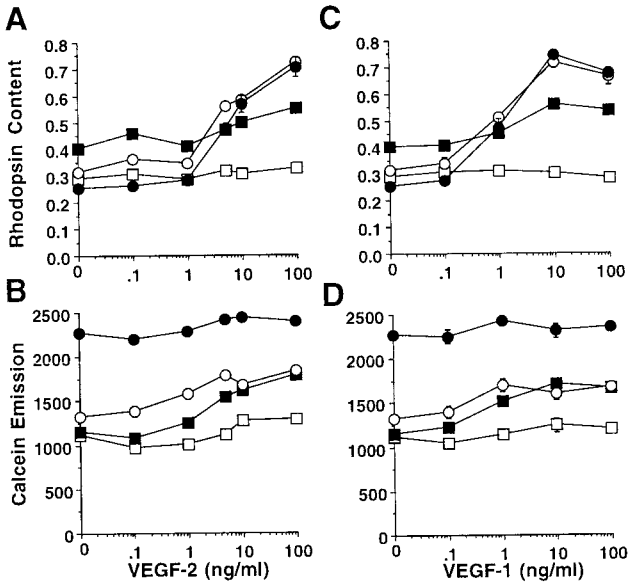

Figure 1. Treatment with VEGF increases the number of cells and the level of rhodopsin protein in the retinal cultures. Dissociated retinal cells were prepared from P1 animals, plated at a density of 425 cells/mm^2, and treated with VEGF-2 (*A, B*) or VEGF-1 (*C, D*). After 2 d (*open squares*), 5 d (*filled squares*), 7 d (*open circles*), or 9 d (*filled circles*) the total number of cells in the cultures was estimated by measuring the calcein emission. Then the cultures were fixed, and the levels of rhodopsin protein were quantitated by ELISA and expressed in terms of optical density. The experiment that is shown is representative of three to four independent experiments. The *data points* are the mean of five to six determinations ± SEM.

RESULTS

VEGF-2 treatment increases the level of rhodopsin protein and the number of photoreceptor cells

The regulatory role of VEGFs on photoreceptor cell development initially was investigated by using cultures derived from P1 animals. Previous reports have demonstrated that multipotent progenitors are present during this developmental period and retain their capacity to differentiate into photoreceptor cells as well as other retinal cell types *in vitro* (Morrow et al., 1998). Treatment with VEGF-2 (Hu et al., 1997) or VEGF-1 (R & D Systems, Minneapolis, MN) induced a dose- and time-dependent increase in the level of rhodopsin protein in the retinal cultures (Fig. 1*A*). The time course of the VEGF-induced increase in rhodopsin was relatively slow, consistent with the known developmental profile of photoreceptor cells. After 5 d of treatment a 25–40% increase in rhodopsin protein was noted with 10–100 ng/ml of VEGF-2. However, by 7–9 d of treatment these same concentrations of VEGF-2 produced a 200–250% increase in rhodopsin protein. Furthermore, at these later time points concentrations of VEGF-1 as low as 1 ng/ml significantly increased rhodopsin levels. To ascertain whether VEGF treatment affected the total number of retinal cells, we monitored the level of emission of calcein AM. An ~25–50% increase in calcein emission was observed after a 5–7 d treatment with at least 10 ng/ml of VEGF-2 (Fig. 1*B*). A pronounced increase in the basal level of calcein emission was noted in the retinal cultures between 7 and 9 d, indicating that retinal cell proliferation that is independent of exogenous VEGF had increased by this later time point. The presence of VEGF-2, even at concentrations as high as 100 ng/ml, for 9 d did not increase the number of retinal cells further. However, there was not a concomitant increase in the basal level of rhodopsin protein after 9 d in culture, suggesting that the proliferation of other cell types accounts for the increase in the level of calcein emission and that these cells were not affecting photoreceptor cell development indirectly (Fig. 1*A*). Treatment with VEGF-1 induced changes in the level of rhodopsin and

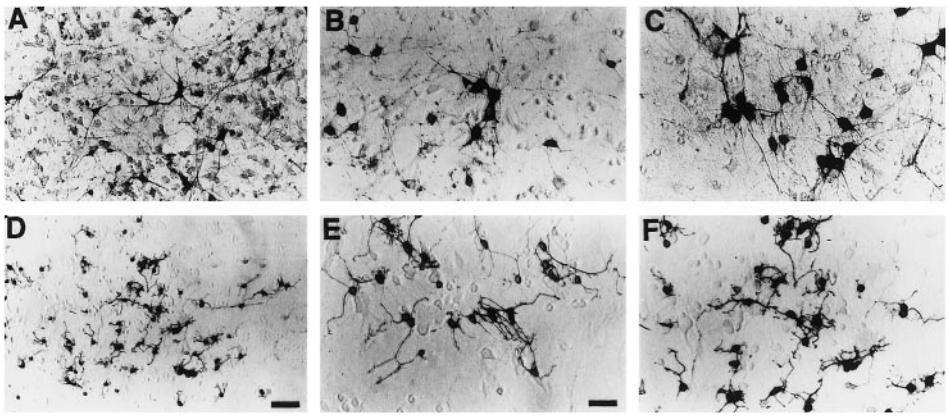

Figure 2. Photomicrographs of immunohistochemically stained retinal cultures. The cultures were derived from P1 animals and maintained *in vitro* for a period of 8 d in the presence of 100 ng/ml of VEGF-2. Then the cells were fixed and immunohistochemically stained for rhodopsin (*A–C*) or syntaxin (*D–F*). Scale bars: *A, D*, 25 μm; *B, C, E, F*, 50 μm.

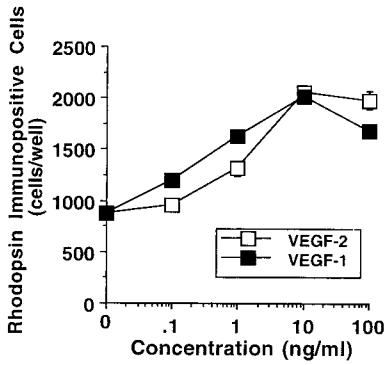

Figure 3. The number of rhodopsin-immunopositive cells is increased after treatment with VEGF. The retinal cells were maintained *in vitro* for 8 d in the presence of either VEGF-1 or VEGF-2. Then the cultures were fixed and immunohistochemically stained for rhodopsin. The experiment that is shown is representative of the three experiments that were conducted, and the *data points* are the mean of five to six determinations ± SEM.

calcein emission similar to those described for VEGF-2 (Fig. 1*C,D*, respectively).

To determine whether the increase in rhodopsin content reflected an increase in the number of photoreceptor cells, we treated cultures with VEGF-1 or VEGF-2 for 8 d and then immunohistochemically stained them for rhodopsin. The morphology of the photoreceptor cells at this *in vitro* developmental stage is depicted in the photomicrographs in Figure 2*A–C*. To quantitate the effects of VEGF treatment, we made cell counts. The number of rhodopsin-immunopositive cells increased as a function of concentration, with the response having an EC_{50} value of 0.25 and 1.5 ng/ml for VEGF-1 and VEGF-2, respectively (Fig. 3). At a saturating concentration of VEGF-2, a 2.4-fold increase in the number of rods was observed. Furthermore, the response was stable in the presence of concentrations of VEGF-2 as high as 100 ng/ml, suggesting that VEGF-2 does not readily induce a desensitization of the biological response. When the cultures were treated on the day of plating and then maintained in the presence of VEGF-2 (100 ng/ml) for 8 d, the number of rhodopsin-immunopositive cells represented 15% of the total number of propidium iodide-positive retinal cells.

Proliferative and survival effects of VEGF

The mechanism by which VEGF-2 induces an increase in photoreceptor cell number may involve an increase in the proliferation of precursor cells, enhanced survival of differentiated photoreceptor cells, and/or the redirection of the rod lineage pathway. To investigate whether VEGF-2 is mitogenic for retinal cells, we treated the cultures with factors 4 hr after plating and subsequently labeled them with BrdU after 24, 48, or 72 hr. At the end of the final labeling period (72 hr) the cultures were fixed, and the incorporated BrdU was detected immunohistochemically. A significant increase in the number of BrdU-labeled cells was observed after 48 hr of treatment, when 10 ng/ml of VEGF-2 or VEGF-1 induced a two- to threefold increase (Fig. 4*A,B*, respectively). The EC_{50} value for the response was calculated as 1 ng/ml. The 48 hr time point appeared near-maximal because, after an incubation of 72 hr, the level of BrdU incorporation had declined in the VEGF-treated cultures regardless of concentration. However, a decrease in the number of immunopositive cells also was observed in the vehicle-treated wells. Similar results were observed in studies in which [^{3}H]thymidine incorporation was used as an endpoint (Fig. 4*C*).

The effect of VEGF treatment on the number of rhodopsin and BrdU double-labeled cells was investigated. The retinal cultures were treated 2–4 hr after plating with VEGF-2 (100 ng/ml) and subsequently were labeled with BrdU after 48 hr. Then the cultures were maintained in the presence of VEGF-2 for 7 d. Although VEGF-2 treatment induced a threefold increase in the number of double-immunopositive cells, this population was only 3% of the total rhodopsin-immunopositive cells. Thus it appears that the major effect of VEGF on photoreceptor cells under the culture conditions used in this study may be the enhancement of their survival or the level of differentiation.

The potential role of VEGF-2 in regulating the survival of retinal cells early in the *in vitro* culture period was investigated by treating the cells at the time of plating with VEGF-2 and then after the cell survival by taking calcein AM measurements at 0, 6, 18, 24, or 48 hr after plating (Table 1). After 6 hr in culture in the vehicle-treated wells the level of calcein AM emission was unchanged from the level noted at the time of plating. This was followed by a gradual rise in the level of calcein AM from 18 to 48 hr. In the presence of VEGF-2 the profile of calcein AM emission was similar to that observed for the vehicle-treated controls, with the exception of a somewhat greater increase at the 48 hr time point.

To characterize further a role for VEGF during the early *in vitro* culture period, we examined the effect that delaying the addition of the factor to the cultures had on the level of rhodopsin protein. The initial addition of VEGF was made 4, 24, or 48 hr after plating, and the cultures subsequently were maintained for 9 d and then prepared for the rhodopsin ELISA. The loss of the response to VEGF-2 or VEGF-1, as a function of the time that lapsed between the isolation of the cells and the initial addition of the factors, is depicted in Figure 5, *A* and *B*, respectively. The addition of factors within 4 hr of the plating of the cells resulted in a threefold increase in the level of rhodopsin. However, delaying the initial treatment with VEGF by 24 or 48 hr resulted in a reduction of the maximal response by 28 and 43%, respectively. Furthermore, after a delay of 48 hr, only treatment with 100 ng/ml of VEGF-2 induced a significant increase in rhodopsin content, thus suggesting that the VEGFs are mitogenic for retinal progenitor cells and that the proliferative response is restricted developmentally.

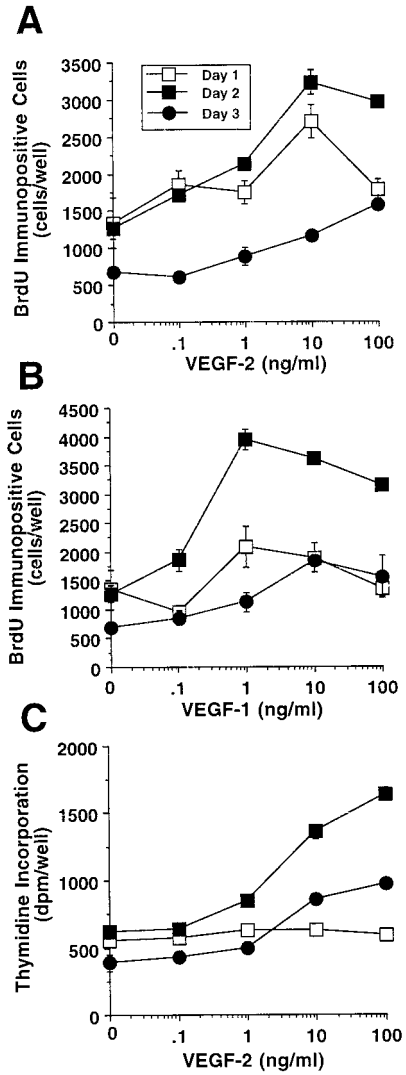

Figure 4. Treatment with VEGF increases the number of BrdU-immunopositive cells and [^{3}H]thymidine incorporation in retinal cultures. The cells were isolated from P1 animals and plated at a density of 425 cells/mm^2. The cultures were treated initially at 4 hr after plating with either VEGF-2 (*A*) or VEGF-1 (*B*). After 1, 2, or 3 d the cultures were labeled for 4 hr with BrdU. Then the cells were fixed and processed for BrdU immunohistochemistry. *C*, The cultures were treated with the factors as described above for the BrdU labeling. After the appropriate time interval the cells were labeled with [^{3}H]thymidine for 6 hr. The experiment shown is representative of the three experiments that were conducted, and the *data points* are the mean of five to six determinations ± SEM.

Treatment with VEGF increases the number of amacrine cells, but not Müller cells, astrocytes, or endothelial cells

The possibility that VEGF affects other retinal cell types that are born postnatally, e.g., amacrine and Müller cells, also was investigated. The morphology of amacrine cells, identified on the basis of their expression of syntaxin, is depicted in the photomicrographs shown in Figure 2*D–F*. Treatment with either VEGF induced a dose-dependent increase in the number of syntaxin-immunopositive cells, with 10 ng/ml inducing a maximal increase of ~2.4-fold as compared with the vehicle-treated controls (Fig. 6*A*). In contrast to the results with the rhodopsin ELISA and cell counts, 100 ng/ml of VEGF-1 or VEGF-2 induced a smaller increase in the number of syntaxin-immunopositive cells than was observed with 10 ng/ml. To characterize further the effect of VEGF-2 treatment on the phenotype of amacrine cells, we measured the level of high-affinity GABA uptake. Figure 6*B* depicts the dose-dependent increase in GABA uptake induced by VEGF. The dose–response curve is similar to that observed when the number of immunopositive syntaxin cells is used as the endpoint, with a significant increase and saturation in GABA uptake occurring with 1 and 10 ng/ml of VEGF-2, respectively.

Table 1. Retinal cell survival in the presence or absence of VEGF-2 during the first 48 hr in culture

	Treatment	
Time (hr)	Vehicle	VEGF-2
0	1272.0 ± 32.6	1195.0 ± 43.4
6	1207.3 ± 55.8	1294.8 ± 37.1
18	1678.0 ± 61.1	1789.3 ± 64.2
24	1728.2 ± 53.1	1831.4 ± 44.2
48	2061.2 ± 58.7	2395.2 ± 74.7

The retinal cells were plated at a density of 425 cells/mm^2 and treated with VEGF-2 at the time of plating. The level of calcein emission was determined at time zero by plating the cells in Ham's F-12 in the presence of calcein. For the later time points, the emission levels were determined as described in Materials and Methods. The data points are the mean of five or six determinations ± SEM.

Müller glial cells were identified on the basis of their expression of vimentin. After an 8 d treatment with VEGF-2 at a concentration of 100 ng/ml, the level of vimentin protein was 93 ± 2% of the level observed in the vehicle-treated controls. Similarly, VEGF-2 treatment resulted in a small decrease in the number of vimentin-immunopositive cells (data not shown). To determine whether VEGF-2 has an effect on the number of astrocytes or on the level of astrocyte differentiation, we measured the amount of GFAP protein by ELISA. After 7 d in culture, treatment with VEGF-2 or FGF-2 did not change the level of GFAP significantly as compared with the vehicle-treated controls (Fig. 6*C*). Furthermore, treatment with VEGF-2 did not increase the number of endothelial cells, PECAM-1-immunopositive cells, in the retinal cultures (data not shown).

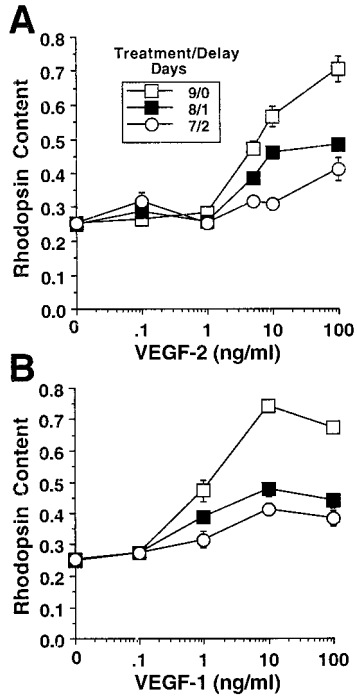

Figure 5. Delaying the addition of VEGF-2 to retinal cultures results in a time-dependent decrease of the VEGF-induced response. The retinal cultures were prepared as previously described in Figure 1. One set of cultures was treated initially with factors at 4 hr after plating (*9/0*); subsequently, additional sets were treated after 24 or 48 hr (*8/1* or *7/2*, respectively). After 9 d in culture the cells were fixed, and the level of rhodopsin protein was quantitated by ELISA assay.

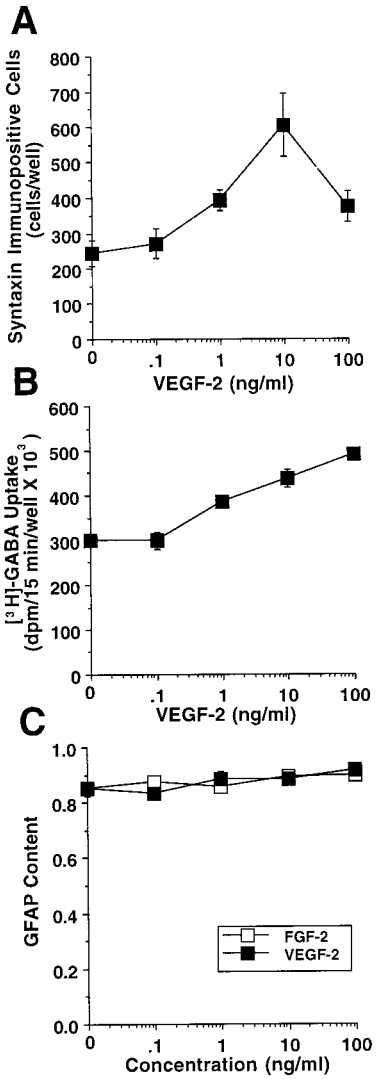

Figure 6. VEGF treatment increases the number of amacrine cells and the level of high-affinity GABA uptake, but not the level of GFAP protein. Retinal cells were treated for 8 d with the indicated concentrations of VEGF-2. Then the cells were fixed, immunohistochemically stained for syntaxin, and counted (*A*), analyzed for the level of high-affinity GABA uptake (*B*), or analyzed for GFAP protein (*C*). The data that are shown are representative of three to four experiments and are the mean of five to six determinations ± SEM.

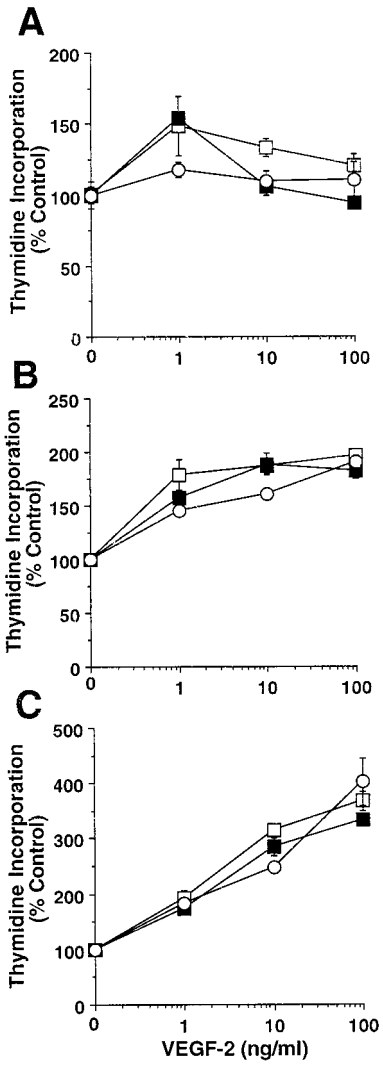

Figure 7. Effect of developmental age on the response of retinal cells to VEGF. Retinal cells derived from E15 (*A*), E20 (*B*), or P1 (*C*) animals were plated at a density of 212 (*open squares*), 318 (*filled squares*), or 425 cells/mm^2 (*open circles*). At 4 hr after plating the cultures were treated with the indicated concentrations of VEGF-2. After 24 hr the cultures were switched to serum-free medium, and the factors were added again. Then the cultures were labeled with [^{3}H]thymidine after 24 hr. The *data points* are the mean of five to six determinations ± SEM. At the density of 212, 318, or 425 cells/mm^2 the basal values (dpm/well) for [^{3}H]thymidine incorporation at E15 were 1599.7 ± 95.3, 4133.9 ± 409.2, and 6315.6 ± 154.7; at E20 they had risen to 3361 ± 192.8, 4936 ± 271.9, and 6377.5 ± 313.9, respectively. At P1 the basal values were 479.7 ± 33.3, 754.7 ± 70, and 744.7 ± 11.7, respectively.

Effect of developmental age on the response to VEGF-2

To characterize further the developmental pattern of the VEGF response, we isolated retinal cells at different developmental stages, and we quantitated the mitogenic response to VEGF-2 after 48 hr by labeling the cultures with [^{3}H]thymidine. In addition, because it has been noted previously that the differentiation of photoreceptor cells *in vitro* is density-dependent, the effect that plating density has on the response to VEGF also was investigated (Sparrow et al., 1990; Watanabe and Raff, 1990; Altshuler and Cepko, 1992).

When the cultures were derived from E15 animals and plated at a density of 212 cells/mm^2, the basal level of [^{3}H]thymidine incorporation was 1589 ± 94 dpm/well, and treatment with VEGF-2 induced a maximal increase of 50% (Fig. 7*A*). In contrast to the dose–response that was observed with P1 cultures in which saturation occurs at 10 ng/ml, the proliferative response in the E15 cultures saturates at a concentration of 1 ng/ml. Furthermore, there was an inverse relationship between the plating density and the mitogenic response to VEGF-2. At a density of 318 cells/mm^2 a leftward shift in the dose–response curve was noted, with concentrations higher than 1 ng/ml causing a desensitization of the response. At the highest density that was tested (425 cells/mm^2), the retinal cells were unresponsive to VEGF-2. It is interesting to note that FGF-2 (10 ng/ml), which has a biological activity similar to VEGF-2 in the P1 cultures (see below), inhibited proliferation in the higher-density E15 cultures by as much as 62% (data not shown).

In cultures that were derived from E20 animals, the basal level of [^{3}H]thymidine incorporation at a plating density of 212 cells/mm^2 was 3361 ± 192 dpm/well. The level of stimulation of [^{3}H]thymidine incorporation with VEGF-2 treatment was generally greater, ranging up to 180–200%, as compared with the vehicle-treated controls. Furthermore, the overall level of stimulation was greater than that observed in E15 cultures (Fig. 7*B*). There was still a trend toward VEGF-2 having less of an effect in cultures plated at the highest density. However, the inhibitory effect was much less pronounced. By P1, when the basal level of [^{3}H]thymidine incorporation was 478 ± 33 dpm/well at a density of 212 cells/mm^2, there was

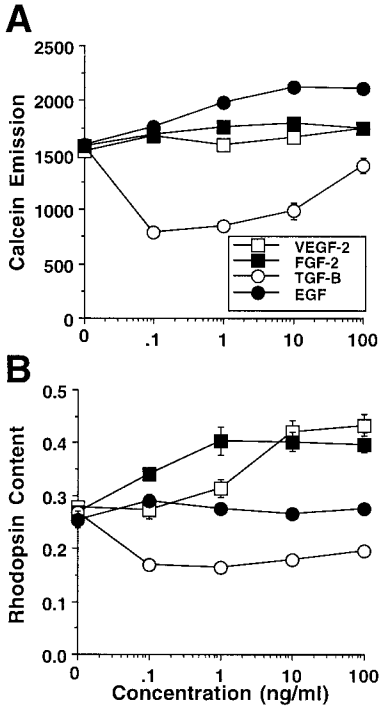

Figure 8. The response profile of P1 retinal cells to VEGF-2 as compared with EGF, FGF-2, or TGFβ-1. The cultures were seeded at a density of 425 cells/mm^2 and treated for 9 d. *A*, The total number of cells in the cultures was estimated by using calcein. *B*, The level of rhodopsin protein was determined by ELISA assay. The reagent blanks in this experiment had an optical density of 0.1. The experiment that is shown is representative of the four independent experiments that were conducted; the *data points* are the mean of five to six determinations.

a leftward shift in the dose–response with saturation occurring at 10 ng/ml, and the extent of the maximal increase was greater, in the range of 300% (Fig. 7*C*). Furthermore, there was no discernible effect of plating density on the response to VEGF-2.

Comparison of the response of retinal cells to VEGF-2 and other factors

To characterize more fully the responsiveness of the rod or retinal progenitor cells, we compared the effect that EGF, FGF-2, or TGFβ-1 has on the number of retinal cells and on the level of rhodopsin protein with that achieved with VEGF-2. EGF, a mitogen for various cell types, induces a 31% increase in the number of retinal cells, with the response saturating at 10 ng/ml and remaining stable up to 100 ng/ml (Fig. 8*A*). However, there was no concomitant increase in the level of rhodopsin protein in the EGF-treated cultures (Fig. 8*B*). FGF-2 in a concentration range of 1–100 ng/ml induces a small but significant increase (13%) in the number of retinal cells. Furthermore, FGF-2, which activates a number of the FGF receptors, induces an increase in the level of rhodopsin protein. A 45% increase in the level of rhodopsin was observed with concentrations of FGF-2 as low as 1 ng/ml, resulting in an EC_{50} value for the response in the range of 0.5 ng/ml. Treatment with TGFβ-1 resulted in a decrease in both the number of retinal cells and the level of rhodopsin protein. At a concentration of 0.1 ng/ml, TGFβ-1 maximally decreased calcein emission and the level of rhodopsin protein by 50 and 65%, respectively.

CNTF inhibits the response of photoreceptor cell progenitors to VEGF-2

The results from the BrdU-labeling experiments demonstrate that VEGF-2 enhances the rate of proliferation of retinal progenitor cells. Because the developmental pathway of photoreceptor cells is thought to be lineage-independent and thus under the regulation of environmental factors (Ezzeddine et al., 1997), VEGF also may modulate photoreceptor cell development at additional downstream sites. It has been determined previously that CNTF inhibits the differentiation of photoreceptor cells relatively late in their developmental pathway by redirecting their phenotype toward the bipolar cell lineage. We investigated the potential interaction of the two factors by cotreating retinal cultures with VEGF-2 at a concentration that is saturating for the induction of photoreceptor cells and various concentrations of CNTF. The increase in rhodopsin protein induced by VEGF-2 was inhibited by CNTF in a dose-dependent manner (Fig. 9*A*). The inhibitory response had an IC_{50} value of 0.4 ng/ml, and treatment with 100 ng/ml of CNTF resulted in the complete inhibition of the VEGF-2 response. However, treatment with CNTF did not alter the total number of retinal cells in the cultures (Fig. 9*B*). To determine whether the inhibitory effect of CNTF was an early or late event, we tested the effect that coadministration of CNTF had on the increased level of [^{3}H]thymidine incorporation induced by VEGF-2. In contrast to the previous results, the addition of CNTF did not inhibit the VEGF-induced proliferative response (Fig. 9*C*). These findings further substantiate that these two factors regulate photoreceptor cell development at different points in the lineage pathway.

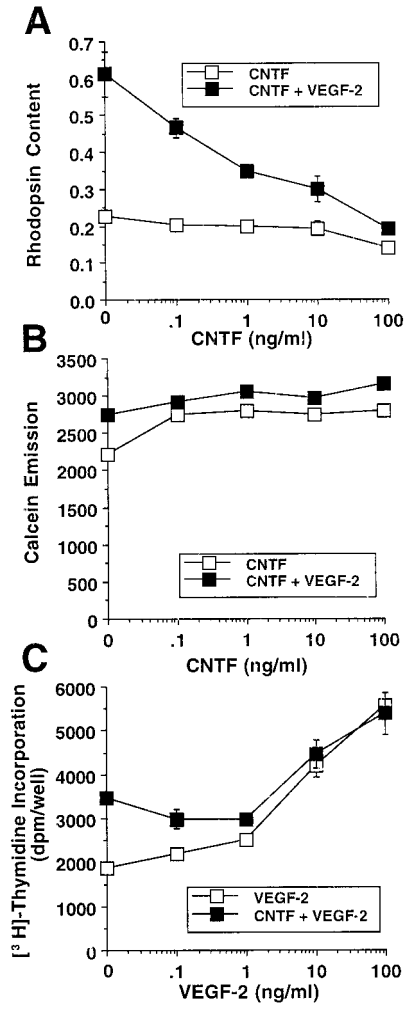

Figure 9. CNTF inhibits the VEGF-induced increase in rhodopsin, but not the early proliferative response. Retinal cultures were treated 24 hr after plating with the indicated concentrations of CNTF in the presence or absence of 150 ng/ml of VEGF-2. After 8 d *in vitro* the amount of rhodopsin was quantitated (*A*), and the total number of cells in the cultures was determined (*B*). *C*, To determine the effect of CNTF treatment on the early proliferative response induced by VEGF, we treated the cultures with the indicated concentrations of VEGF-2 in the presence or absence of 100 ng/ml of CNTF. After 48 hr the cultures were labeled for 6 hr with [^{3}H]thymidine.

DISCUSSION

We have identified and characterized the effect of VEGF-1 and VEGF-2 on retinal cells *in vitro*. Treatment with VEGF in the sub-nanomolar range induces an increase in the number of photoreceptor and amacrine cells and increases the level of rhodopsin protein and high-affinity GABA uptake. Time course studies demonstrate that VEGF induces a maximal increase in [^{3}H]thymidine incorporation within 48 hr of its addition, and delaying the treatment of the cultures by 24–48 hr results in the loss of the proliferative and differentiation responses. The mitogenic response was regulated developmentally, with VEGF-2 inducing an increase in [^{3}H]thymidine incorporation with cells derived from E15, E20, and P1 animals. When BrdU was added to the cultures 48 hr after the addition of VEGF-2, a minority of the rhodopsin-immunopositive cells were double-labeled. These results suggest that VEGF-2 also may affect the survival of photoreceptor cells. In comparison with members of other trophic factor families, the responses to treatment with VEGF-2 and FGF-2 were similar in that both factors increased the level of rhodopsin protein without inducing an increase in the total number of cells after 9 d in culture. The coadministration of CNTF with VEGF-2 resulted in the inhibition of the VEGF-induced increase in the level of rhodopsin, but not in the proliferative response.

The VEGF receptor family currently is composed of four members (for review, see Klagsbrun and D'Amore, 1996; Wen et al., 1998). The receptors demonstrate distinct yet overlapping ligand specificity. VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) bind the various forms of VEGF-1, whereas VEGFR-2 and VEGFR-3 (Flt-4) bind VEGF-2. Thus both VEGF-1 and VEGF-2 activate VEGFR-2 (Joukov et al., 1998), and both ligands have similar biological activities in the retinal cultures. Recently, Yang and Cepko (1996) described the developmental expression pattern of VEGFR-2 in the retina. In addition to the expected expression of the receptor on the newly forming vasculature, receptors were also present on components of the neural retina. This expression pattern is maintained during development as the retina grows in a centripetal manner.

The effect of developmental age on the response of the retinal progenitor cells to VEGF is consistent with the developmental expression pattern of the receptor (Yang and Cepko, 1996). Mitogenic effects of VEGF, based on [^{3}H]thymidine incorporation studies, were noted at the earliest developmental time point that was examined, E15, as well as at E20 and P1. The magnitude of the proliferative effect increased with age, reaching a peak by P1. VEGF-2 is more efficacious on E15 and E20 cultures than at P1 because that response saturated at 1 ng/ml as opposed to 10 ng/ml, respectively. Furthermore, the basal level of proliferation *in vitro* also changed with developmental age, with the highest levels observed at E20. The finding that the basal level of proliferation was relatively low at E15 but increased fourfold with a twofold increase in cell density, a greater proportional increase than was observed at the other developmental ages, suggests that endogenous mitogens may underlie the desensitization that occurs with VEGF-2 treatment in the E15 cultures (see legend for Fig. 7). Moreover, these data indicate that increased levels of VEGF during early development may have a negative impact on the differentiation of photoreceptor cells. The influence of developmental age on the response of retinal progenitor cells to other growth factors also has been observed (Altshuler and Cepko, 1992). Lillien and Cepko (1992) reported that the proliferative response of retinal cells in monolayer cultures to FGF-1 and FGF-2 was greater at earlier gestational ages (e.g., E15 and E18), and by E21 or P0 a rightward shift in the dose–response curve was apparent.

Previous studies in goldfish and frog have suggested that amacrine cell development is regulated by cell–cell contact (Negishi et al., 1982; Reh and Tully, 1986). More recently, the importance of cell–cell contact for the *in vitro* development of photoreceptor cells also was described by Watanabe and Raff (1990, 1992) in reaggregated cultures and then later by Altshuler and Cepko (1992) with dissociated retinal cells plated in collagen gels. In the former study, when E15 retinal cells were reaggregated with a 50-fold excess of neonatal retinal cells, there was no change in the developmental time when the rhodopsin-immunopositive cells were observed. However, there was a significant increase in the proportion of the E15 cells that eventually differentiated into photoreceptor cells. In the case of the monolayer cultures that were used in this study, there is a dissociation between the VEGF-2-induced early proliferative response and the later differentiation of photoreceptor cells. For example, VEGF-2 increases [^{3}H]thymidine incorporation by three- to fourfold in cultures seeded at densities as low as 212 cells/mm^2, and treatment for 7 d resulted in cell densities equivalent to those achieved at the higher plating densities (e.g., 425 cells/mm^2). However, there was no detectable rhodopsin protein or immunopositive cells in the cultures plated at the lower densities. These results suggest that there is not only a critical cell–cell interaction necessary for the development of photoreceptor cells but also a time frame during which the stimulus produced via cell contact is probably necessary.

Comparing the time course of the VEGF-induced proliferation with the developmental time course of the appearance of rhodopsin protein indicates that there is an ~5 d lag between the two events. The appearance of rhodopsin protein likely reflects the induction of gene transcription, because the two events have been shown to be closely correlated (Treisman et al., 1988). This time interval is similar to that observed by Morrow et al. (1998) in *in vivo* and *in vitro* studies when progenitor cells derived from animals within an age range of E20 to P3 are considered. Furthermore, between 5 and 9 d *in vitro* we observed the greatest increase in the level of rhodopsin protein, and this time period is within the postnatal developmental period (days 6–10) *in vivo* during which there is a pronounced appearance of rhodopsin-immunopositive cells (Morrow et al., 1998).

In comparison to members of other trophic factor families, the response to VEGF-2 resembled that of FGF-2 in that both factors increased the level of rhodopsin protein while inducing relatively small increases in the total number of retinal cells after 9 d *in vitro*. In addition, a proliferative response, based on [^{3}H]thymidine incorporation and cell counts, to FGF-2 was noted by Lillien and Cepko (1992) as late as P3, suggesting that FGF-2 retains some mitogenic activity in postnatal cultures. In contrast to our findings with VEGF-2, Fontaine et al. (1998) demonstrated that FGF-2 also has a survival effect on photoreceptor cells derived from P5 animals (data not shown). TGFβ-1 treatment resulted in a decrease in both the number of retinal cells and the level of rhodopsin protein. Kimchi et al. (1988) reported similar observations using human fetal retinal cultures, with the exception that maximal inhibition with the human cells required 0.5 ng/ml of TGFB-1 as compared with the <0.1 ng/ml that is required in the rodent cultures.

CNTF, a member of the neuropoietic family of cytokines, is known to affect the development of photoreceptor cells *in vitro* and *in vivo* and to enhance the survival of photoreceptor cells after light-induced damage (Unoki and LaVail, 1994; Fuhrmann et al., 1995; Ezzeddine et al., 1997; Cayouette et al., 1998). In contrast to CNTF, VEGF-2 did not rescue photoreceptor cells in the constant light-induced damage model (LaVail et al., 1992; Wen et al., 1995; R. Wen and R. Alderson, unpublished data). Treatment of postnatal rat retinal explant cultures with CNTF results in an increase in the number of cells expressing bipolar cell markers, with a loss in the population of cells expressing rhodopsin. Analysis of the effect of CNTF on the fate of [^{3}H]thymidine-labeled P0 retinal cells suggests that the cytokine does not induce the proliferation nor increase the survival of this cell population (Ezzeddine et al., 1997). Furthermore, the initiation of the effect of CNTF occurred at approximately the time that the cells became postmitotic and began to express rhodopsin. These data are consistent with the findings reported here demonstrating that CNTF inhibits the VEGF-2-induced increase in rhodopsin protein observed between 5 and 7 d in culture, but not its mitogenic activity observed between 1 and 2 d.

During the course of development in the retina, oxygen levels

control the microarchitecture of retinal vessels that in turn matches the pattern of differentiation of retinal neurons (Chan-Ling et al., 1990; Phelps, 1990). Stone et al. (1995) have demonstrated that, in the retina, astrocytes and microglia respond to hypoxia by synthesizing and secreting VEGF, which in turn induces vessel formation. The studies reported here suggest that the early differentiation events regulated by VEGF not only involve vessel formation but also may induce retinal progenitor cell proliferation. This ultimately may result in the coordinated development of numerous cell types in the retina.

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