Molecular Vision 3: 10, 1997 <http://www.emory.edu/molvis/v3/schwegler>
Received 11 Dec 1996 | Accepted 11 Sep 1997 | Published 15 Oct 1997

Basic, not Acidic Fibroblast Growth Factor Stimulates Proliferation of Cultured Human Retinal Pigment Epithelial Cells

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Purpose: In this study, we evaluated a possible effect of acidic and basic fibroblast growth factor (aFGF, bFGF) on the proliferation of human retinal pigment epithelial (RPE) cells in culture. As the RPE is the primary source for bFGF in the retina, such an effect would suggest autocrinic actions of FGFs.
Methods: Primary cultures of human and porcine RPE and an established human RPE cell line (D407) were subjected to aFGF and bFGF at different culture conditions. Cell proliferation was determined using the BrdU non-radioactive nucleotide analogue assay, and total protein was measured colorimetrically. The cells were subjected to aFGF and bFGF from 0.1 to 100 ng/ml for 1 to 14 days.
Results: In the presence of 100 ng/ml bFGF, cell proliferation doubled from day 2 (143±12 units) to day 6 (227±17). This effect was neither seen without bFGF nor with aFGF at the same concentration. The stimulating effect of bFGF on cell proliferation was dose-dependent, the ED50 being around 1-10 ng/ml. The bFGF effect was markedly greater at high fetal calf serum concentration (10% vs. 1%). No bFGF effect was seen on cells of the established human RPE cell line D407 nor on primary cultures from porcine RPE**.**
Conclusions: bFGF, in contrast to its analogue aFGF, stimulates cell proliferation in cultured human RPE cells. It may act as an autocrinic agent (secretion by and action on the same cell) and thus be a specific regulator for cell proliferation in repair and replacement of the RPE cell monolayer.

The retinal pigment epithelium (RPE) is increasingly looked upon as playing functional parts in both the visual cycle and the regeneration of retinal photoreceptors (1). One of the most intriguing functions is the interplay between the RPE, the Müller cells and the rod and cone outer segments in the micromilieu of the interphotoreceptor matrix. Many cell types of retinal and choroidal origin are capable of producing fibroblast growth factors (FGF). These FGFs probably play a key role in the maintenance of visual function at the photoreceptor level (2). Basic and acidic fibroblast growth factor (bFGF and aFGF) are two of a family of mitogenic polypeptides that stimulate proliferation in a variety of cell types. The name "fibroblast growth factor" results from early observations that they can increase the cell number of cultured 3T3 cells, an established fibroblast cell line (3). There is ample evidence for the participation of FGF in regeneration and repair, as well as pathologic neovascularization of the retina. Little is known about the action of these growth factors on the RPE cells producing them. We therefore analyzed the action of human recombinant aFGF and bFGF on primary cultures of human retinal pigment epithelium and two RPE models: the established human D407 RPE cell line and primary cultures from porcine RPE.

Basic fibroblast growth factor (bFGF) is an 18 kD polypeptide (4) that was identified in the bovine retina in 1985 (5). Its tertiary structure closely resembles that of interleukin 1ß (6), suggesting a similar mechanism of action. bFGF is secreted by different cell types of the retina and choroid into the interphotoreceptor matrix, and may be involved in the regulation of photoreceptor outer segments functions and the retinal pigment epithelium (RPE) (7). Concerning a possible future clinical use, this growth factor is particularly interesting as it delays photoreceptor decay in two different experimental models for macular degeneration (8,9) and stimulates retinal regeneration (10). On the other hand, members of the FGF family are hyperexpressed in vitreoretinal membranes and choroidal neovascularization (11,12), and thus may contribute to hypoxia induced neovascularization in diabetes.

Primary cultures of human RPE cells have been shown to express bFGF in vitro (13-16). Gene transcription depends on cell density and the adhesion to a solid substrate, and decreases when the cells become confluent (17,18). Although normal and dystrophic retinal pigment epithelium both express bFGF and its receptor (19), bFGF receptor deficiency may contribute to early photoreceptor degeneration in the Royal College of Surgeons (RCS) rat model of macular degeneration (20)..

MATERIALS AND METHODS

The primary human RPE cultures were obtained from eyes enucleated for corneal transplantation with the permission of the patients or their close relatives. After confirmation of brain death, the circulation was maintained until one hour before enucleation. The anterior segments were removed by circular scleral incision at the pars plana. The vitreous and neuroretina were mechanically dispatched and the eyecup was cut meridionally. The sclera was stripped of RPE cells under

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Dulbecco's phosphate buffered salt solution (Sigma, Munich) with the help of a rubber policeman. Trypsinization did not enhance the efficacy of the preparation but lead to more cell detritus in the initial culture medium. RPE cells were transferred to 25 cm^2 culture dishes and maintained for 10 days in Dulbecco's modified eagle medium (DMEM) containing fetal calf serum (FCS) and 1% standard penicillin/ streptomycin solution (Sigma, Munich) in a humidified 5% CO_2 atmosphere at 37° C. As growth of the initial preparation from human RPE shows a more prolonged lag phase before proliferation than porcine primary cultures and cell lines, we provided the initial passage of human RPE cells with 20% FCS. After the first split, all cells were routinely grown in the presence of 10% FCS. Because all reported experiments were done with later passages (minimum of 4 weeks on 10% FCS) it seems unlikely that different culture conditions would have affected our results.

Porcine eyes were obtained from the local abattoir 1-2 hours after slaughtering. The preparation of primary cultures was the same as described above, except for growing the cells in the presence of 10% FCS from the beginning. All cells were passaged before or immediately after having reached complete confluence by 3 min trypsinization and mild centrifugation in a cryocentrifuge. This procedure was duplicated with the established cell culture D407.

Proliferation of RPE cells was tested using a non-radioactive nucleotide analogue, 5-bromo-2'-deoxy-uridine (BrdU, Boehringer, Mannheim). Cells were seeded at a density of 12000/cm^2 on flat-bottom 96 well microtiter plates in culture medium with or without lymphocytes (BrdU product information, Boehringer, Mannheim). The results of the BrdU measurements are expressed in arbitrary units (ratio x 1000).

Total cellular protein was determined after 20 min of cell cleavage with Triton X100 1% v/v using a phenol reagent microprotein method (Sigma, Munich). Standards were prepared by dilution of bovine serum albumin. All data are expressed as µg protein per single well. The number of cells per well was determined by counting cell nuclei on photomicrographs of methylene blue stained cells on a standardized portion of the well bottom. The size of the counting area was chosen to have at least 50 cells in each area counted. Statistical significance was calculated using Student's t-test (two tailed) for unpaired samples; all data are given as arithmetic means±standard error.

RESULTS

In order to investigate whether fibroblast growth factors stimulate cell proliferation, we incubated human primary cultures with 100 ng/ml aFGF and bFGF in standard culture medium containing 10% FCS. This concentration is 10 to 50 fold greater than needed for the half-maximal effect (ED_{50}) of both growth factors. Figure 1 shows the respective results: One and two days after plating, incorporation of BrdU was identical whether or not growth factors were present. The cells were still in their lag phase after plating as the total cell protein did not show any significant rise from day 1 to day 2. However, 6 days after plating, BrdU incorporation in the bFGF group was twice that of controls (206±19 vs. 101±8.2 units; n=6). Total protein was also significantly increased (4.31±0.13 vs. 3.45±0.05 µg/well). aFGF did not increase BrdU incorporation or total protein.

As the observed increase in total protein after bFGF application could be the result of either cell enlargement (hypertrophy), or of cell proliferation, we counted the cells after application of bFGF at 2 and 6 days after plating. On day 2, total cell number without FGF (2.48±0.15 x 10^4 cells/well) did not differ from cells treated with aFGF (2.23±0.15 x 10^4 cells/well) and bFGF (2.43±0.10 x 10^4 cells/well). On day 6, bFGF treated cells numbered 3.81±0.19 x 10^4 cells/well (n=3), an increase compared to the 3.20±0.10 x 10^4 cells/well (n=3) of the controls ($p<0.05$); cells treated with aFGF numbered 3.40±0.15 x 10^4 cells/well (n=3) which was not significantly different from controls. Total protein per cell was not affected by 6 days of bFGF treatment (bFGF: 136±7, control: 137±4 pg/cell, n=3). Total protein per cell rose from 95±6 to 136±4 pg/cell from the second to sixth day after plating (n=3).

Following these first experiments, we measured the time course and dose-dependence of bFGF action (Figure 2, left panel). The data indicate a dose-dependency of bFGF on protein incorporation over the entire range from 0.1 to 100 ng/ml. The effect was detectable at all plotted time intervals

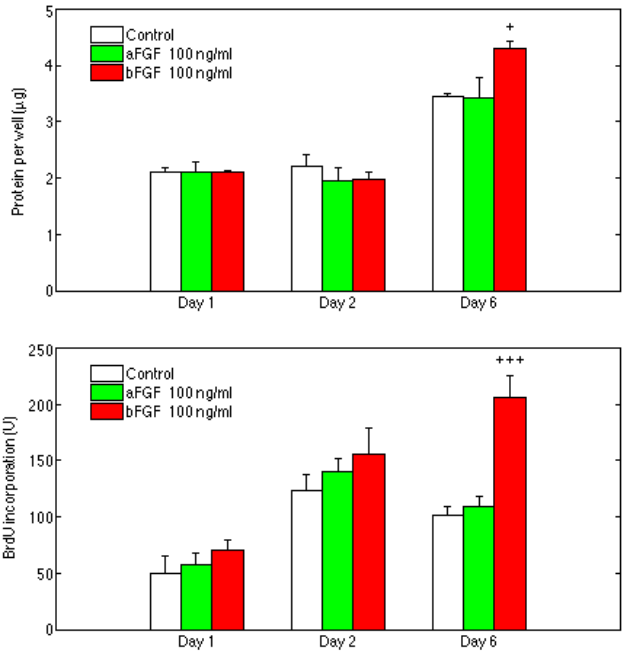

Figure 1. Effect of aFGF and bFGF on proliferation of human RPE cells. The effect of acidic (aFGF) and basic (bFGF) fibroblast growth factor on cultured human retinal pigment epithelial (RPE) cells. RPE cells of the second and third passage were cultured in the presence of 100 ng/ml of each growth factor. The upper panel shows the total protein content of a single well of a 96 well microtiter plate, the lower panel depicts cell proliferation measured by BrdU uptake in arbitrary units (U = ratio x 1000). Both parameters were determined in the presence of 10% fetal calf serum. Statistically significant differences in protein content or BrdU uptake between a treatment and control group are marked with a plus (+) for $p<0.05$ and a triple plus (+++) for $p<0.001$ (two tailed, unpaired t-test, n=6).

from 4 to 14 days, but was most pronounced at an incubation period of 7 days. At this time, total protein doubled on 100 ng/ml bFGF (8.48±0.50 vs. 4.82±0.44 µg/well). The concentration needed for half-maximal effect (ED_{50}) is probably between 1 and 10 ng/ml. Unfortunately, the scatter of the data preclude any more detailed dose-response analysis. When we simultaneously cultured the cells in a 1% FCS medium, the absolute protein values were lower, but also the relative increase upon bFGF was smaller than with 10% FCS (Figure 2, right panel). Nevertheless, in qualitative terms the dose- and time-dependency were similar to the 10% FCS group. Maximum bFGF effect on total protein incorporation only reached 119±6% of controls.

Established cell cultures from human RPE would be a welcome alternative to primary cultures because of their ready availability and the stability of their features in prolonged cultivation. We therefore checked whether we could duplicate the bFGF effect on the human RPE cell line D407. As depicted in Figure 3, bFGF concentrations up to 100 ng/ml did not stimulate D407 cells to proliferate. Independent of the treatment with FGF, D407 cells showed increasing polymorphism and a partial loss of contact inhibition with multilayered growth especially at the dish edges. However, neither aFGF nor bFGF could influence DNA synthesis measured by BrdU uptake or change in total protein. Also, neither could affect DNA synthesis or total protein in primary porcine cultures (Figure 4). The cells showed a regular increase of protein but no effect of aFGF or bFGF.

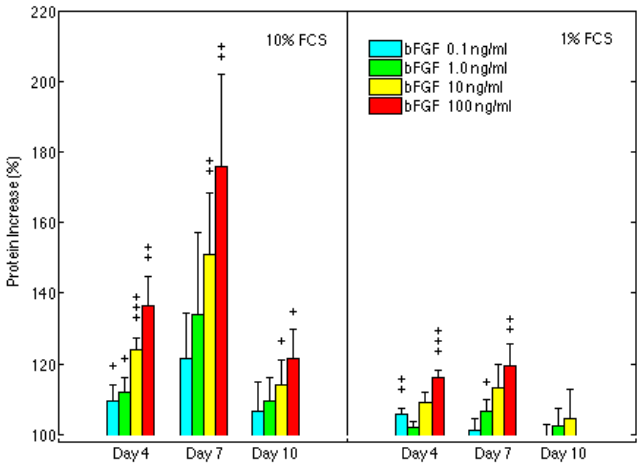

Figure 2. Dose-dependence of the bFGF effect on proliferation of human RPE cells in 10% and 1% FCS. The influence of different concentrations of basic fibroblast growth factor (bFGF) at varying time in culture and at high and low fetal calf serum (FCS) concentration on primarily cultured human retinal pigment epithelial (RPE) cells. Cells of the third and fourth passage were grown in 10% FCS (left) and 1% FCS (right), respectively. The vertical axis shows the relative increase of total protein content in a single well of a 96 well microtiter plate. A value of 100% is equivalent to the protein content at each given time in culture without the addition of bFGF. Statistically significant differences in protein content between a treatment and control group are marked with a plus (+) for $p<0.05$, a double plus (++) for $p<0.01$, and a triple plus (+++) for $p<0.001$ (two tailed, unpaired t-test, n=3).

DISCUSSION

FGFs have been studied with respect to their proliferation enhancing effect on anterior segment epithelial cells (21), but little is known about the biological actions of growth factors on RPE cells. As FGFs are secreted both by the RPE and adjacent retinal and choroidal cells, an FGF effect on the RPE would be either a paracrine or autocrine action. In bovine RPE, Esser and coworkers (22) report a 50% increase in cell numbers by bFGF in concentrations of 10 ng/ml and greater. Similar results were recently published about primary cultures of human RPE cells (23). The authors report an increase in the stimulatory effect of bFGF at low oxygen tension, which is consistent with the notion of bFGF contributing to hypoxia-induced neovascularization.

In contrast to the publications mentioned above, we measured the proliferative effect not only by cell counting but also by non-radioactive nucleotide incorporation. This procedure is advantageous as it assays proliferation at a defined and early stage, i.e. DNA synthesis. With this method we found a twofold increase of proliferation upon bFGF together with a concomitant rise in protein content. The effect was significant at as low a concentration as 0.1 ng/ml and was dose-dependent up to 100 ng/ml, the highest bFGF concentration tested.

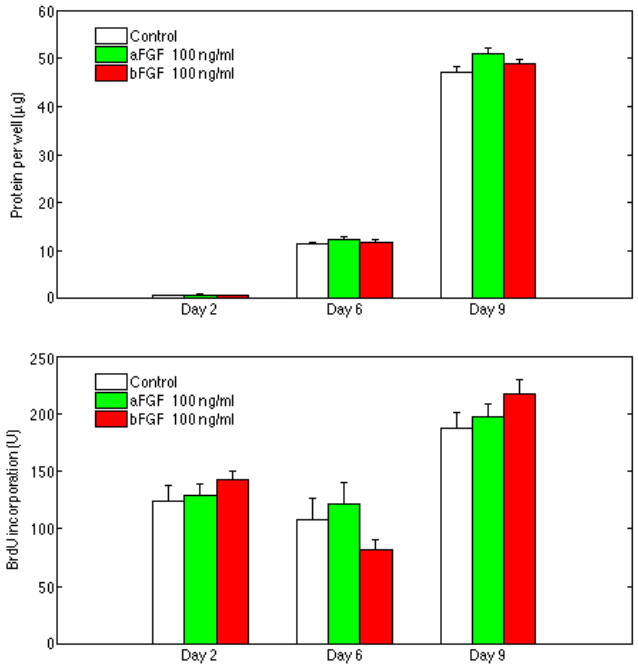

Figure 3. Lack of effect of FGF on proliferation of the cultured human cell line D407. The effect of acidic (aFGF) and basic (bFGF) fibroblast growth factor on the established human RPE cell culture D407. The cells were grown in the presence of 100 ng/ml of each growth factor under the same conditions as primary cultures (see Figure 1). The upper panel shows the total protein content of a single well of a 96 well microtiter plate, the lower panel depicts cell proliferation measured by BrdU uptake in arbitrary units (U = ratio x 1000). Both parameters were determined in the presence of 10% fetal calf serum (FCS). No statistically significant differences were detected between the treatment and control groups (alpha=0.05, two tailed, unpaired t-test, n=8).

With respect to the methods used, BrdU incorporation, cell counting, and protein measurements, all three have advantages and disadvantages. As total protein comprises the time integrated result of both translation and proliferation, it is not as specific as a true proliferation assay. This lack of specificity, however, can be advantageous for three reasons: (1) A time integrated parameter dampens possible oscillations in proliferation assays by accidental synchronization of the cell cycle (e.g. by feeding fresh medium). Therefore, the data show the result of FGF action over the whole incubation time and not only at one individual point in time. (2) Because of its low specificity, total protein measurement is a fairly good negative control. If a given agent does not affect total protein, it is very unlikely that the cells respond that agent. (3) In human cultures, the method is the advantage of a highly reproducible. Therefore, a sample size of 3 was sufficient to obtain values with a low enough standard error to allow a statistical analysis. In view of the problems obtaining readily proliferating human RPE cells, this point is of great practical importance.

The dose-dependency of bFGF action on RPE cells found in this paper differs from that obtained by cell counting in a recent study (23), where the authors show a significant increase from 0 to 0.1 ng/ml bFGF, but no further increase up to 70 ng/ml. Therefore, it must be concluded that the bFGF effect on cell division was already saturated at 0.1 ng/ml, whereas in our study DNA synthesis shows a flatter dose-response at higher concentrations. This discrepancy could possibly be explained by a shift in receptor affinity during different cultivating procedures.

We found that a 10% FCS is a prerequisite rather than an obstacle to bFGF action on RPE cells. This finding was somewhat surprising as our initial expectation was that in higher concentrations, growth factors intrinsic to FCS would mask any effect of bFGF. Furthermore, as FCS contains endogenous proteases, bFGF could have been deactivated by proteolysis before exerting its action. The respective experiments, however, showed that at 10% FCS both the absolute and relative protein content (normalized to higher absolute proliferation at high FCS) was enhanced by bFGF compared to 1% FCS. Similar results were reported from bovine RPE (22) where a minimum of 10% was required for bFGF to exert its full action on rising cell numbers. Obviously, bFGF depends on some soluble serum constituent for its biological activity. It may be speculated that this constituent is a proteoglycan because the FGF receptor requires a secondary cooperative binding site to heparan sulphate (24). However, up to now there can be only speculation about the possible bFGF action, especially as we were not able to demonstrate a reversal of the bFGF effect on cell proliferation in the presence of heparin (unpublished experiments).

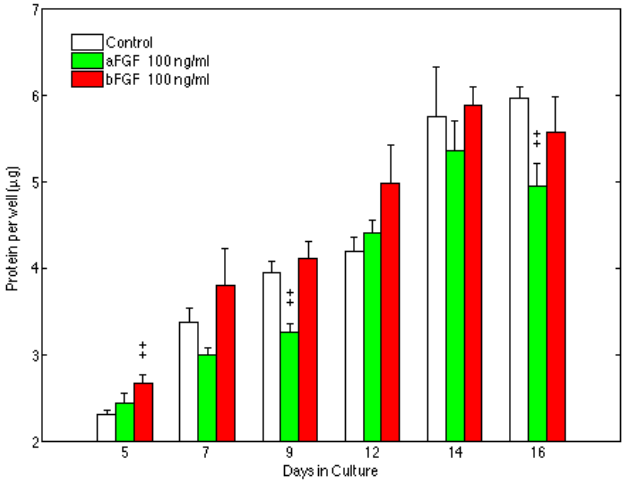

Figure 4. Lack of effect of FGF on proliferation of porcine RPE cells. Total protein content of primarily cultured porcine retinal pigment epithelial (RPE) cells at different times cultured with human acidic (aFGF) and basic (bFGF) fibroblast growth factor. The experiments were performed in the same manner as those with primary human cultures (see Figure 1). Statistically significant differences in protein content between a treatment and control group are marked with a double plus (++) for $p<0.01$ (two tailed, unpaired t-test, n=6).

Both bFGF and aFGF are members of the FGF gene family with similar proliferation enhancing effects. aFGF is associated with proliferative vitreoretinal diseases as well (11,25). It has been shown to occur together with bFGF in the retinal pigment epithelium in vivo (26,27). In primary cultures of human RPE cells, aFGF mRNA production and protein expression is down-regulated at increasing cell density (28) as in bFGF. In our experiments we were unable to show any proliferation enhancing effect of aFGF on cultured human RPE cells. All experiments were performed in parallel to those with bFGF, thus ruling out the possibility of potential receptor down regulation during the cultivating procedures. This finding supports the notion of a specific action of bFGF on RPE cells. Since RPE cells produce bFGF for themselves, bFGF may be an autocrinic factor for maintenance and proliferation of retinal pigment epithelial cells in physiologic and pathophysiologic conditions.

We showed that bFGF response upon RPE cells reaches its maximum one week after plating. This finding corresponds to data obtained with bovine RPE cells (22). In contrast, bFGF production is highest in low density cultures (early after plating) (17). Therefore, cell-to-cell contact may switch RPE cells from producing and secreting bFGF to expressing bFGF receptors. Whether such a mechanism contributes to retinal wound healing is yet unclear.

Primary cultures of human RPE cells are difficult to obtain and show a tendency to deteriorate after four to six passages. Therefore, an established RPE cell line would be extremely helpful as a ready-to-use model of the RPE monolayer. The recently established D407 cell line is a spontaneous mutation of primarily cultured human RPE cells. It contains most of the prominent RPE features such as cobblestone morphology, expression of retinoid binding protein and some of the key enzymes in the visual cycle (29). Therefore this cell line was a candidate for assaying FGF induced proliferation. Our results did not detect any influence by either aFGF or bFGF on D407 cells, even in a confluent culture.

ACKNOWLEDGEMENTS

We thank Ms. Sylvia Weber for her excellent technical assistance in performing the proliferation assays and running the daily lab work. We are grateful to Dr. Richard Hunt for his generous gift of the D407 cell line. We also thank the "Stiftung zur Foerderung der Augenheilkunde e. V. Mannheim" for funding a vital part of the cell laboratory equipment.

This study was published in abstract form at the 1996 meeting of the Association for Research in Vision and Ophthalmology in Ft. Lauderdale, FL (Schwegler JS, Nau-Staudt K, Knorz MC, Seiberth V, Vardarli I, Liesenhoff H. bFGF stimulates proliferation of cultured human retinal pigment epithelial cells: Evidence for an autocrinic loop. Invest Ophthalmol Vis Sci 1996; 37:S790).

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