Characterization of release of basic fibroblast growth factor from bovine retinal endothelial cells in monolayer cultures

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Release of basic fibroblast growth factor (bFGF) was investigated in bovine retinal endothelial cells (BREC) maintained in monolayer culture. Confluent cells released bFGF into serum-free culture medium or medium containing 5% serum at rates of up to 105.2 and 61.3 pM/day respectively. bFGF release coincided with a decrease in monolayer cell number and increases in lactate dehydrogenase (LDH) concentration and cells and cell-debris particles in the medium, which suggested that cell damage and lysis were responsible for growth-factor release. Maximum bFGF release at 24 h (230 ± 10 pM) occurred when the cells were treated with lipopolysaccharide ($10 \mu\text{g/ml}$), which also produced the greatest changes in parameters of cell damage. Sub-confluent cells showed little overt damage at 24 h, but released bFGF (78 ± 20 pM) along with LDH, indicating that some cell lysis had occurred. Insulin-like growth factor 1 (IGF-1) was also released into serum-free culture medium at a rate of 0.34 nM/day, but not into medium containing serum or when the cells were treated with lipopolysaccharide. This implies that the mechanism of IGF-1 release is different from that of bFGF and is not related to cell damage. Culture medium conditioned by BREC stimulated the proliferation of these cells, as measured by an increase in their incorporation of [*methyl*- ^3H]thymidine from 7550 ± 479 to 10467 ± 924 d.p.m. These results demonstrate that bFGF is released from damaged BREC and that medium conditioned by these cells can stimulate retinal-endothelial-cell proliferation. This strengthens the case for an involvement of this growth factor in retinal neovascularization.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a polypeptide originally isolated from bovine pituitary and brain [1,2]. It is one member of a family of growth factors characterized by their strong affinity for heparin, which includes acidic fibroblast growth factor [3] and some tumour-cell-derived factors [4,5]. It was originally isolated independently from many cells and tissues and was given several names, e.g. eye-derived growth factor I [6], heparin-binding growth factor β [7] and macrophage-derived growth factor [8], all of which have been shown to be homologous to bFGF [9].

Retinal extracts have been shown to contain bFGF [10], and purified bFGF is a potent mitogen for bovine retinal endothelial cells (BREC) [11]. bFGF is also angiogenic in several models [12,13]. Considerable interest has therefore developed as to its possible role in proliferative diabetic retinopathy. This condition is characterized by the growth of abnormal new blood vessels arising from existing retinal vessels [14].

bFGF is present in the extracellular matrix of cells, where it is bound to heparan sulphate proteoglycans [15,16], and early reports suggested that it was not present in media conditioned by bovine aortic and corneal endothelial cells, although it could be extracted from these cells and from the matrix [17,18]. When the gene for bFGF was cloned [19], it was realized that the transcribed sequence did not contain a classical signal-peptide region allowing secretion from cells, and that in most tissues mRNA levels were very low, suggesting little bFGF production [20].

The release of bFGF from extracellular matrix or from cells is required if it is to be implicated in either physiological or pathological angiogenesis. Two theories have been advanced, the first that extracellular matrix acts as a pool of bFGF which can be released in response to heparinase enzymes [15,21,22] and

the second that cell damage could cause the release of this growth factor from within the cell. Recently, injury-mediated release of bFGF has been demonstrated from bovine aortic endothelial cells [23], and bFGF has been shown to be present in medium conditioned by BREC [24], although it was not clear how release from these cells was mediated.

The aims of this study were (1) to examine the pattern of bFGF release from BREC with time, by using a radioimmunoassay, (2) to relate this release to parameters of cell damage and lysis, (3) to characterize biochemically the bFGF released, and (4) to measure its bioactivity with [*methyl*- ^3H]thymidine uptake as a marker of cell proliferation.

MATERIALS AND METHODS

Cell culture

BREC were isolated and cultured as previously described [25]. Retinas were dissected from bovine eyes, homogenized in Minimum Essential Medium (Eagle's), filtered through an $85 \mu\text{m}$ -pore-size filter (Henry Simon, Stockport, U.K.), and the blood vessels trapped on the filter were digested in a 1 mg/ml collagenase-Dispase (Boehringer Mannheim, Mannheim, Germany) solution for 90 min at 37 °C. Vessel fragments were plated on to a fibronectin substrate (New York Blood Centre, New York, NY, U.S.A.) in 75 cm² tissue-culture flasks. Culture medium was 8% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tryptose phosphate broth and 10% plasma-derived serum (DMEM + 10% PDS) [26]. All culture media were purchased from Gibco, Paisley, Scotland, U.K., and tissue-culture plastics were from Becton Dickinson, Oxford, U.K. Cells grew to confluence in 5 days, when they were passaged by trypsin treatment. Experiments were carried out on first- or second-subculture cells.

Abbreviations used: bFGF, basic fibroblast growth factor; rbFGF, recombinant bFGF; BREC, bovine retinal endothelial cells; IGF-1, insulin-like growth factor 1; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hanks Balanced Salt Solution; PDS, plasma-derived serum; LDH, lactate dehydrogenase.

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The cells were characterized by their cobblestone morphology and by uniform staining for Factor VIII-related antigen.

Growth-factor release from BREC monolayers

Subconfluent BREC were treated with trypsin (0.25 %, w/v) (ICN Flow, Rickmansworth, Herts., U.K.), and a single-cell suspension was produced by passing cell clumps through a 21-gauge needle. An equal volume of this suspension was then added to each of 24 25 cm² tissue-culture flasks previously treated with fibronectin, and the cells were grown in DMEM + 10 % PDS. At confluence the medium was removed and the monolayer washed with Hanks Balanced Salt Solution (HBSS). Then 2 ml of serum-free DMEM containing 0.01 % (w/v) CHAPS [27] was added to each flask, and the flasks were sealed and incubated at 37 °C; 21 flasks were used for measuring cumulative growth-factor release, and 3 for measuring daily release from the same monolayer, and subsequent results and discussion will refer to either serum-free cumulative or daily release. Photographs were taken of the cells every day (Fig. 1). A further series of experiments was carried out on 21 25 cm² flasks containing cells prepared identically with those above. At confluence the medium was removed, the monolayer washed with HBSS and 2 ml of DMEM containing 5 % (v/v) human serum (North London Blood Transfusion Centre, London N.W.9, U.K.) was added to each flask, and the flasks were sealed and incubated at 37 °C. These flasks are referred to as serum-containing cumulative-release flasks. At zero time and every day for 6 days, three of the serum-free cumulative-release flasks, three of the serum-containing cumulative-release flasks and the three flasks used for measuring daily release had their medium removed and centrifuged at 670 g for 10 min. The medium from each flask was divided into four 0.5 ml portions, and these were stored frozen at -20 °C before growth-factor assays. Samples for growth-factor bioassay were prepared under sterile conditions by using medium removed from the serum-free cumulative-release flasks. The remaining pellet of debris and cells was resuspended in 0.75 ml of HBSS and counted with a Coulter Counter (Coulter Electronics, Luton, U.K.). A 2 ml portion of fresh serum-free DMEM + 0.01 % CHAPS was added to each of the three flasks used for measuring daily release. Medium and debris were removed from these flasks each day and fresh serum-free DMEM + 0.01 % CHAPS was added. The cell monolayer in the three serum-free and serum-containing cumulative-release flasks was washed with HBSS and treated with 1 ml of 0.25 % trypsin until all cells had detached. The cells were resuspended in 1 ml of HBSS and counted.

Growth-factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

Cell growth conditions were chosen in order to achieve minimum (sub-confluent growing cells) and maximum (addition of lipopolysaccharide) cell damage. BREC were plated into 25 cm² flasks at either high density (600 000 cells per flask) or low density (200 000 cells per flask). The cells in the flasks seeded at high density were grown to confluence, whereas those seeded at low density were allowed to attach overnight at 37 °C (sub-confluent flasks). Medium was then removed and the cells were washed with HBSS. A 2 ml portion of serum-free DMEM + 0.01 % CHAPS or of DMEM containing 5 % human serum was added to the flasks containing the confluent cells, and lipopolysaccharide from *Escherichia coli* 055:B5 (10 µg/ml; Sigma, Poole, Dorset, U.K.) was added to half of the flasks in each group. A 1 ml portion of serum-free DMEM + 0.01 % CHAPS or of DMEM containing 5 % human serum was added to the flasks containing the sub-confluent cells to achieve similar medium/cell ratios to those used for the confluent cells. All flasks

were incubated at 37 °C for 24 h. The medium was then removed and divided into batches, and the cells were trypsin-treated and counted as in the cumulative growth-factor-release experiments above except that the medium from the sub-confluent flasks was divided into 0.25 ml portions. Samples were stored at -20 °C before growth-factor assays.

Iodination of bFGF

Recombinant bFGF (rbFGF) (Amersham, Aylesbury, Bucks., U.K.; 5 µg) was iodinated with 1 mCi of Na¹²⁵I by using chloramine-T, and free ¹²⁵I separated on a heparin-Sepharose affinity column as previously described [28]. The tracer was repurified on a heparin-Sepharose column immediately before use.

Radioimmunoassay for bFGF

The antiserum used was raised against a synthetic peptide bFGF-(1-24) and was given by Dr. A. Baird (The Whittier Institute, La Jolla, CA, U.S.A.). The antibody was used at a final dilution of 1:20 000. rbFGF iodinated as above was used as the label and rbFGF as the standard (9.8-5000 pM). Standards were diluted in serum-free DMEM + 0.01 % CHAPS or serum-containing DMEM and all other reagents in 50 mM-phosphate buffer containing 0.15 M-NaCl, 0.025 M-EDTA and 0.2 % (w/v) gelatin, pH 7.2. The assay sensitivity was 18 pM, the intra-assay variation 8 % and the inter-assay variation 18 % at 500 pM. Cross-reactivity in the assay was seen only with acidic FGF (FGF Co. Ltd., La Jolla, CA, U.S.A.) at concentrations greater than 2 nM.

Radioimmunoassay for IGF-1

IGF-1 was measured, after acid/ethanol stripping of samples, as previously described [29]. The polyclonal antiserum UBK 487 was donated by Dr. L. Underwood and Dr. J. J. van Wyck (University of North Carolina, Chapel Hill, NC, U.S.A.) through the National Hormone and Pituitary Program of the National Institute of Diabetes, Digestive and Kidney Diseases. The assay had a sensitivity of 0.3 nM and the inter-assay variation was 8 % at 14 nM.

Assay for lactate dehydrogenase (LDH)

LDH was measured kinetically on an RA-XT Analyser (Technicon Instrument Corp., Basingstoke, Hants., U.K.) by a spectrophotometric method optimized to Scandinavian conditions (pyruvate to lactate) at 340 nm [29a].

Bioassay for growth factors

Subconfluent BREC were treated with 0.25 % trypsin and a single-cell suspension was prepared. The cells were counted with a Coulter Counter and seeded into each well of 24-multiwell tissue-culture plates, pre-coated with fibronectin, at 2500 cells per well. After the cells had attached overnight in 0.5 ml of DMEM + 10 % PDS per well, they were washed once with HBSS and the medium was changed to 0.5 ml of DMEM + 0.1 % PDS. After 24 h the cells were again washed with HBSS, and 0.5 ml of DMEM + 0.1 % PDS containing 10 µl of [*methyl*-³H]thymidine solution {600 µl of [*methyl*-³H]thymidine (185 GBq/mmol; Amersham), 60 µl of 10 mM-thymidine and 1340 µl of sterile water}/ml was added. Wells were then treated individually by the further addition of either 100 µl of serum-free BREC-conditioned medium harvested from BREC monolayers at days 0-6 as described above, or bFGF (5 pM), and the cells were incubated for a further 48 h. The monolayer was then washed twice with Dulbecco's A phosphate-buffered saline (PBSA) (Oxoid, Basingstoke, Hants., U.K.), and the incorporated [*methyl*-³H]thymidine was precipitated by addition of 5 % (w/v) trichloroacetic acid for 5 min, followed by 100 % (v/v) ethanol.

Fig. 1. Changes in the appearance of confluent monolayers of BREC in serum-free DMEM with time

Flasks of confluent BREC used for the serum-free cumulative growth-factor release experiments were photographed daily over the period of the experiment. Results are shown for days 0 (a), 2 (b), 4 (c) and 6 (d) after addition of serum-free medium. Magnification $\times 50$.

The ethanol was removed, and the precipitated material was solubilized with 0.5 ml of 25 M-formic acid per well and then placed in 3.5 μ l of scintillation fluid (Optiphase Safe) (LKB/Pharmacia, Milton Keynes, U.K.). Each well was rinsed with 0.5 ml of PBSA, which was added to the appropriate scintillation vial. Vials were counted for radioactivity in a Beckman LS 1801 liquid-scintillation counter for 1 min per sample.

Identity of bFGF in BREC-conditioned medium

bFGF in BREC-conditioned medium was characterized by its elution profile on a heparin-Sepharose column compared with that of rbFGF. Heparin-Sepharose was prepared by rehydration and washing in distilled water, followed by pre-equilibration with 0.05 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl. A column (1.0 cm $\times$ 6.0 cm) was packed with heparin-Sepharose, and either 1 ml of rbFGF (305 pM) or 12 ml of BREC-conditioned medium (containing 4.7 pM-bFGF as measured by radioimmunoassay) was run on to the column. Heparin-binding growth factors were eluted from the column with a linear 0.15–3 M gradient of NaCl in 0.05 M-Tris/HCl, pH 7.4, in a total of 120 ml (6 ml/fraction) for rbFGF, or 30 ml (1.5 ml/fraction) for BREC-conditioned medium in a total of 20 fractions. Fractions eluted from the column run with rbFGF were diluted

1:20 with assay buffer for bFGF radioimmunoassay, whereas fractions from the column run with BREC-conditioned medium were dialysed overnight at 4 °C against 0.05 M-Tris/HCl, pH 7.4, and assayed neat against rbFGF standards prepared in 0.05 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl which had been run through the heparin-Sepharose column before sample application and dialysed.

RESULTS

Effect of serum-free and serum-containing DMEM on BREC monolayers

A confluent monolayer of BREC contained a mean of $6.8 (\pm 0.4) \times 10^5$ cells per 25 cm² flask ($n = 3$). After 24 h in serum-free medium this number had fallen to $5.2 (\pm 0.6) \times 10^5$ cells per flask (Fig. 2). This decrease continued to $4.1 (\pm 0.5) \times 10^5$ cells per flask by day 6. The photographic record of the BREC monolayer at zero time and at days 2, 4 and 6 after addition of serum-free DMEM + 0.01 % CHAPS is shown in Figs. 1(a)–1(d). At zero time the confluent monolayer shows closely packed cells with some cells rounded up on the surface of the monolayer; these appear as bright beads in the phase-contrast micrographs. By day 2 there is an increase in the number of rounded cells in the monolayer, and some of these are seen floating in the

Fig. 2. Change in BREC cell number with time

Cell number per flask in serum-free (○) or serum-containing (▲) culture medium, measured with a Coulter counter (see the text for details), against time. Each point represents the mean $\pm$ S.E.M. for three separate experiments.

medium. Also several smaller beads and many small dark particles can be seen; these are the components of cell debris. On day 4 the monolayer appears severely disrupted, with many dead cells and cell-debris particles; however, at this stage it is predominantly intact. Finally, on day 6 there is the complete break-up of the monolayer, with large gaps between the endothelial cells, seen on the bottom left of the micrograph (Fig. 1d), and an increased amount of cellular debris.

Confluent cell monolayers in medium containing serum showed a different pattern of changes in monolayer cell number compared with that in serum-free medium. There was an initial fall from $6.5 (\pm 0.1) \times 10^5$ to $6.1 (\pm 0.4) \times 10^5$ cells per flask, but the number then increased to $7.8 (\pm 0.4) \times 10^5$ cells per flask after 48 h before falling to the values seen in serum-free medium by day 5. The early increase could be explained by unsustained cell proliferation in response to released growth factors. Monolayer disruption, as viewed through the microscope, was similar to that seen in serum-free medium.

Release of cells from the monolayer and increase in cell debris in serum-free and serum-containing culture medium

There was an increase in the number of cells present in serum-free culture medium as measured with the Coulter Counter (Fig. 3), which coincided with a decrease in cell number in the BREC monolayer. There was a greater rate of increase in particles counted in the medium, $5.2 (\pm 0.2) \times 10^5$ counts per flask per day over the first 4 days compared with the decrease in cells in the monolayer of $0.57 (\pm 0.15) \times 10^5$ cells per flask per day over the same period. There was also no further decrease in monolayer cell number over the last 2 days, whereas the cell and debris count in the medium continued to increase by $2.3 (\pm 0.9) \times 10^5$ counts per flask per day. These results could be partly explained by cell proliferation in the monolayer, but there is also considerable break-up of cells into smaller fragments, as seen in the photographic record (Fig. 1). Cells and cell-debris particles measured in serum-containing culture medium increased at a lower rate [$2.6 (\pm 0.6) \times 10^5$ counts per flask] than in serum-free medium, and this could be explained by less cell damage. Monolayer cell number, however, eventually decreased to the levels seen in serum-free medium, indicating that similar monolayer damage did occur in serum-containing medium, and a better explanation is that there was less break-up of released cells into smaller fragments. The number of cells remaining in the BREC monolayer in those flasks where serum-free medium had been removed and replaced daily was $4.0 (\pm 0.7) \times 10^5$ cells per flask.

Fig. 3. Cells and cell debris released into the culture medium

Cells and cell debris released as a function of time in serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Each point represents the mean $\pm$ S.E.M. for three separate experiments.

Fig. 4. Release of bFGF into the culture medium

Results are shown as a percentage of the maximum release for each experiment in serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Each point represents the mean $\pm$ S.E.M. for three separate experiments, except for those for the daily-release flasks which show the means of two experiments.

Fig. 5. Release of IGF-1 into the culture medium

Results are shown for serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Results are expressed as means $\pm$ S.E.M. for three separate experiments.

bFGF release into serum-free and serum-containing culture medium

bFGF release from BREC monolayers is shown in Fig. 4. The results are expressed as a percentage of the maximum release of bFGF in each experiment, since the release of bFGF from BREC monolayers varied depending on the batch of cells used. The rate

Fig. 6. Release of LDH into the culture medium

LDH release into the medium by confluent cultures of BREC as a function of time is shown for serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Results are expressed as means $\pm$ S.E.M. for three separate experiments.

of release of bFGF over the first 4 days in each of three experiments was 31.8, 71.0 and 105.2 pM/day in the serum-free cumulative-release flasks, reaching a maximum of 149, 476 and 467 pM respectively. bFGF was released into serum-containing culture medium at rates of 49.0, 61.3 and 54.3 pM/day over the first 4 days, and reached a maximum of 224, 280 and 315 pM respectively. bFGF was released at rates of 49 and 177 pM/day in the daily-release flasks.

IGF-1 release into serum-free and serum-containing culture medium

The results for IGF-1 release are shown in Fig. 5; all results are means $\pm$ S.E.M. of data from three separate experiments. IGF-1

was released into serum-free DMEM by BREC monolayers at a rate of 0.34 ± 0.02 nM/day, reaching a maximum of 2.56 nM. The release rate of IGF-1 in the daily-release flasks was 0.75 ± 0.05 nM/day. A baseline level of IGF-1 of 2.50 ± 0.28 nM, owing to the presence of IGF-1 in serum, was seen in serum-containing culture medium, and there was no significant increase in IGF-1 concentration over the time course of the experiment.

LDH release into serum-free and serum-containing culture medium

The results for LDH release are shown in Fig. 6; all results are means $\pm$ S.E.M. of data from three separate experiments. LDH release into serum-free DMEM by BREC followed a rate of 105 units/litre per day, to reach a maximum of 628 units/litre. A constant release of 90 ± 7 units/litre per day was seen in the same monolayer when fresh medium was added daily. LDH was released into serum-containing medium at a rate of 110 units/litre per day, reaching a maximum of 689 units/litre. This was similar to the pattern of release seen in serum-free medium, indicating a comparable amount of cell lysis in cell monolayers maintained with or without the addition of serum.

Growth-factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

bFGF release after 24 h from sub-confluent BREC and confluent cells with or without lipopolysaccharide (10 μ g/ml) into both serum-free and serum-containing medium is shown in Fig. 7. Also shown are the corresponding changes in IGF-1 concentration, LDH concentration, cell and cell-debris particles in the medium and monolayer cell number. All columns represent means $\pm$ S.E.M. of data from at least three experiments, except where indicated. In confluent flasks at 24 h, bFGF concentrations were 91 ± 24 pM in serum-free medium and 161 ± 35 pM in

Fig. 7. Growth-factor release and changes in parameters of cell damage in sub-confluent BREC monolayers and confluent monolayers treated with lipopolysaccharide

Results are shown as the means for (a) monolayer cell number, (b) cell and cell-debris-particle numbers, (c) bFGF concentration, (d) IGF-1 concentration and (e) LDH concentration in serum-free medium (□) or serum-containing medium (▨) at zero time (t = 0) and at 24 h in flasks containing sub-confluent cells (Sub. 24 h), confluent cells (Con. 24 h) or confluent cells treated with lipopolysaccharide (10 μ g/ml) (LPS 24 h). Error bars represent the S.E.M. for at least three separate experiments, except for results from serum-containing confluent cells at 24 h and treated with lipopolysaccharide where two and one experiments were carried out respectively.

Table 1. Bioactivity of BREC-conditioned medium

[methyl-³H]Thymidine uptake into BREC was measured in 24-well tissue-culture plates (see the text for details) in the presence of serum-free BREC-conditioned medium removed from BREC monolayers at zero time and every day for 6 days. Uptake in response to rbFGF (5 pM) is shown as a control. Results are means $\pm$ S.E.M. of data from six separate experiments. Values were compared by one-way ANOVA ($P < 0.01$) and then each value was compared with that at zero time by an unpaired *t* test ($P < 0.05$ in every case).

Addition to cell-culture well	[methyl- ³ H]Thymidine uptake (d.p.m.)
100 μ l of BREC-conditioned medium harvested at:	
0 days	7550 $\pm$ 479
1 day	10467 $\pm$ 924
2 days	11307 $\pm$ 783
3 days	10210 $\pm$ 543
4 days	11171 $\pm$ 985
5 days	12075 $\pm$ 760
6 days	10073 $\pm$ 417
rbFGF (5 pM)	10678 $\pm$ 343

Fig. 8. Heparin-Sepharose profile of rbFGF and BREC-conditioned medium

rbFGF (●) and BREC-conditioned medium (○) were eluted from a heparin-Sepharose column (see the Materials and methods section for details) with a gradient of increasing NaCl concentration (—; 0–3 M). bFGF in the fractions was measured by radioimmunoassay.

medium containing serum. These were increased to 230 ± 14 and 238 pM respectively when the cells were treated with lipopolysaccharide ($10 \mu\text{g/ml}$). The decrease in monolayer cell number and increase in cell and cell-debris particles measured in the medium were also greatest when the cells were treated with lipopolysaccharide. The number of cell and cell-debris particles measured in serum-containing medium after 24 h was less than that seen in serum-free medium. This was also observed in the cumulative-release experiments, and was probably due to less cell break-up in the presence of serum. LDH release, however, was similar into both serum-free (116 ± 13 units/litre) and serum-containing (109 ± 5 units/litre) medium, and was substantially increased after addition of lipopolysaccharide, to 360 ± 56 and 428 units/litre respectively. These results suggest that, although the amount of cell debris was lower in serum-containing medium, cell lysis, as measured by LDH release, was just as great. IGF-1 concentrations did not show any change in serum-containing

medium from the baseline level of 2.50 ± 0.28 nM and, although there was a small increase in serum-free medium, from 0.32 to 0.6 ± 0.3 nM, there was no increase when the cells were treated with lipopolysaccharide.

bFGF was released into serum-free and serum-containing culture medium by sub-confluent BREC, reaching levels of 85 ± 23 and 70 ± 16 pM respectively. Cell number at 24 h was similar, $3.7 (\pm 1.0) \times 10^5$ and $4.5 (\pm 0.9) \times 10^5$ cells per flask, and, although there were few cell and cell-debris particles in the medium [$1.2 (\pm 0.7) \times 10^5$ and $0.5 (\pm 0.1) \times 10^5$ counts per flask], LDH release (85 ± 23 and 70 ± 16 units/litre) was almost as much as from confluent cells. This indicates that cell lysis had occurred, and this could account for the release of bFGF. There was no significant release of IGF-1 from sub-confluent cell monolayers.

Bioactivity of BREC-conditioned medium

Medium from serum-free cumulative-release flasks sampled on days 1–6 produced increased [³H]thymidine uptake into BREC as compared with medium sampled at zero time (Table 1). bFGF (5 pM) also gave a significant stimulation of [³H]thymidine uptake.

Identity of bFGF in BREC-conditioned medium

To confirm the identity of the bFGF measured in the radioimmunoassay, the heparin-Sepharose elution profile of BREC-conditioned medium was compared with that of rbFGF. The rbFGF was eluted at 1.9 M-NaCl, reaching a peak at 2.2 M. Recovery as a percentage of rbFGF applied to the column was 34.3 %. BREC-conditioned medium from serum-free cumulative-release flasks was eluted as three peaks at 0.15 M-, 0.8 M- and 2.3 M-NaCl, the final peak corresponding to the elution position of rbFGF (Fig. 8).

DISCUSSION

Retinal endothelial-cell proliferation is a requirement for new vessel growth as seen in proliferative diabetic retinopathy. The stimulus to this proliferation has not been determined, although the development of large areas of ischaemic retina usually precedes this event. We have shown that in monolayer culture BREC release large quantities of bFGF, which is a potent mitogen for these cells. The maximum release of bFGF from these monolayers into serum-free medium is 105.2 pM/day and into serum-containing medium is 61.3 pM/day; this compares with an ED_{50} for the stimulation of BREC proliferation by rbFGF of 2.8 pM (R. A. Brooks, unpublished work) and by purified bFGF of 0.8 pM [11] and 5.5 pM [24]. The amount of bFGF released by BREC monolayers is therefore in excess of that required to bring about retinal endothelial-cell proliferation. We have also shown that serum-free DMEM + 0.01 % CHAPS conditioned by BREC monolayers is mitogenic for BREC, and this ability to stimulate BREC proliferation is similar to that shown by rbFGF (5 pM).

The lack of a classic signal-peptide sequence on the mRNA transcript coding for bFGF, which would allow secretion, has led to speculation as to the source of bFGF measured in endothelial-cell-conditioned culture medium. Several authors have suggested that bFGF is mainly stored in the extracellular matrix, where it is bound to heparan sulphate proteoglycans [15,16,18,21] and can be released by heparinitase and heparin [15,22]. Others have shown release of bFGF in viable confluent cultures of bovine corneal endothelial cells [30] and BREC [24] by an unknown mechanism. Rifkin & Moscatelli [31] have pointed out that, even in viable cultures, there is a small amount of cell death, and this could be responsible for the measurable bFGF. Endothelial cells from bovine aorta, adrenal capillaries

and retinal vessels have been shown to contain extractable bFGF [17,24]. Using bovine aortic endothelial cells, Gajdusek & Carbon [23] demonstrated the presence of large quantities of bFGF, which can be released by cell lysis and then bind to extracellular matrix, where it could be released by heparin and the enzyme heparinidase.

We have now shown the release of bFGF from bovine retinal endothelial cells into both serum-free and serum-containing medium and related this to parameters of cell damage and lysis. In serum-free medium, bFGF increases along with a decrease in cell number in the monolayer. Its release is also associated with increased cells and cell debris in the culture medium and with release of the enzyme LDH, which provides a marker of cell lysis [32]. The quantity of bFGF released was variable and was dependent on the batch of cells used. The quantity of cells and debris in the medium increased more rapidly than the decrease in monolayer cell number. This could be due to either cell proliferation in the monolayer or degradation of released cells, and, although the former cannot be ruled out, the appearance of small particles in the conditioned medium indicates that cell break-up did occur. Additionally, the serum-free medium in these flasks would not readily support the proliferation of retinal endothelial cells even in the presence of released growth factors. In serum-containing medium, bFGF release can also be linked to parameters of cell damage and lysis. Although there is an initial increase in monolayer cell number and a less marked linear increase in cell debris in the medium, owing to less cell break-up, LDH release was similar to that seen in serum-free medium, indicating that cell lysis did occur. The fact that LDH concentration increases linearly to day 6 in both serum-free and serum-containing medium, whereas bFGF release forms a plateau after day 4, is probably due to progressive degradation of bFGF by proteases released from damaged cells, leaving it unable to bind to the antibody in the radioimmunoassay.

In order to investigate more rigorously the relationship between cell damage, lysis and bFGF release, cell growth conditions were chosen to produce minimum (sub-confluent cells) and maximum (lipopolysaccharide-treated cells) cell damage. Endotoxin-mediated cell damage has been used previously to investigate the release of growth-promoting activity from bovine aortic endothelial cells [23]. Release of bFGF in both serum-free and serum-containing medium was greatest when cells were treated with lipopolysaccharide, which also produced the largest decrease in monolayer cell number and increase in cell debris and LDH concentration in the medium. A lower but significant release of bFGF was also seen when serum-free or serum-containing DMEM was conditioned by sub-confluent BREC monolayers. Although there were few cell-debris particles released into the medium, indicating little cell break-up, LDH was released in quantities approaching those seen from confluent monolayers, showing that cell lysis had occurred. This would provide an explanation for bFGF release. Insufficient extracellular matrix was produced by these cells, under the experimental conditions used, to allow extraction and measurement of bFGF. The absence of this means of bFGF sequestration may have increased the measurable concentration of this growth factor in BREC-conditioned medium.

The identity of bFGF released from the monolayer was shown by heparin-Sepharose affinity chromatography. The concentration of NaCl at which both rbFGF and bFGF in BREC-conditioned medium are eluted from the column is higher than that observed by other investigators [17,33]. However, these values have been shown to be very dependent on the batch of heparin-Sepharose used [34]. Two other peaks of bFGF immunoreactivity were observed in the elution profile of BREC-conditioned medium, at 0.15 M- and 0.8 M-NaCl. The first of

these could correspond to the weakly bound high-molecular-mass fraction observed by Courty *et al.* [6] in retinal extract and the second to acidic FGF. If this were due to cross-reactivity of acidic FGF in the radioimmunoassay, then it would imply very high concentrations of acidic FGF in BREC-conditioned medium. Baird *et al.* [35] have reported that bFGF can be co-eluted with a low-salt fraction from a heparin-Sepharose column, and this is a more likely explanation.

At the end of each experiment, the flasks to which serum-free DMEM + 0.01 % CHAPS had been added daily showed more disruption to the BREC monolayer than in the flasks used for measuring serum-free cumulative growth-factor release, but the number of cells remaining in the monolayer was similar. The cumulative cell and debris count in the serum-free medium and the LDH levels were the same in both sets of flasks, as was IGF-1 release until levels reached a plateau. This plateau could be due to a cessation of IGF-1 release in these flasks or to degradation of IGF-1 by proteases released from the lysed cells.

IGF-1 may contribute to the retinal endothelial-cell proliferation seen in proliferative diabetic retinopathy. It has been shown by King *et al.* [36] to be mitogenic for retinal endothelial cells in culture, and it can interact with bFGF in stimulating the progression of cells through the cell cycle [37]. Hyer *et al.* [38] showed a transient rise in the serum level of IGF-1 in diabetics at the time of new vessel growth, which returned to normal levels after successful laser treatment, suggesting a role for IGF-1 *in vivo*. We have shown that IGF-1 is released from BREC monolayers into serum-free medium; however, there was no release into serum-containing medium or when the cells were damaged by the addition of lipopolysaccharide. These results suggest that IGF-1 is not released from damaged BREC. The fact that IGF-1 can be conventionally secreted from cells [39] suggest this as a likely explanation for its release into serum-free medium. In serum-containing medium, there may be negative feedback on release owing to the presence of IGF-1 in the serum.

Both bFGF and IGF-1 have been reported to be elevated in the vitreous of patients with proliferative diabetic retinopathy [40,41]. Our results show that both of these growth factors can be released from BREC, but that their mechanism of release is different. IGF-1 is likely to be secreted, whereas bFGF is released after cell damage and lysis, although this does not preclude additional release of bFGF from viable cells by an as yet unknown mechanism. Retinal capillary damage is a feature of advanced diabetic retinopathy, and further endothelial-cell damage could occur in the ischaemic retina before neovascularization. Although not wishing to exclude the contribution of growth factors released from other retinal cells, our results are further evidence for the involvement of bFGF in this sight-threatening condition.

We thank Dr. A. Baird for donation of the bFGF antiserum, and Dr. L. Underwood and Dr. J. J. van Wyck through the National Hormone and Pituitary Program of the National Institute of Diabetes, Digestive and Kidney Diseases for donation of the IGF-1 antiserum. This work was supported by the Medical Research Council and by a group grant from the British Diabetic Association.

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Received 12 September 1990/11 January 1991; accepted 30 January 1991