Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management

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Abstract

Aim—Many growth factors are implicated in proliferative diabetic retinopathy (PDR). It was decided to test the hypothesis that no one factor is predominant but that a regular profile of levels of different growth factors might be operating, and that the profile might differ according to whether or not insulin therapy was part of the patient's glycaemic management. The levels of several growth factors in vitrectomy samples were therefore determined from diabetic patients with tractional, non-haemorrhagic sequelae of PDR and these levels were correlated with (a) each other (growth factor profile), (b) neovascular activity, and (c) the method of glycaemic management (insulin treated (IT) or non-insulin treated (NIT)).

Methods—72 samples of vitreous were obtained from either diabetic patients with PDR (n = 51) or non-diabetic (control) patients (n = 21). Levels of bFGF, IGF-I, EGF, and insulin were determined by radioimmunoassay; levels of TGF- β 2 by ELISA; and levels of IGF-I binding protein by western ligand blotting. The data were analysed using appropriate statistics.

Results—There was no regular growth factor profile. bFGF levels were significantly greater in vitreous from NIT patients compared with IT patients and controls. The highest levels of bFGF were found in NIT patients with actively vascularised membranes. TGF- β 2 levels were significantly greater in vitreous from IT patients compared with NIT patients and controls. The highest levels of TGF- β 2 were found in IT patients with actively vascularised membranes. IGF-I levels were significantly greater in diabetics (irrespective of insulin treatment) than non-diabetics and the highest levels of IGF-I were found in IT patients with actively vascularised membranes. A 34 kDa IGFBP was the predominant IGFBP identified in vitreous and was found to be elevated in diabetics patients.

Conclusion—In PDR there is a correlation between intravitreal growth factor levels and both disease state (whether active or fibrotic) and method of glycaemic management.

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Proliferative diabetic retinopathy (PDR) is characterised by preretinal neovascularisation and fibrosis, ultimately leading to vitreous haemorrhage and tractional retinal detachment.¹ There is now considerable evidence that a variety of growth factors are involved in initiating and perpetuating the neovascular process.²⁻⁴

Studies to date have clearly demonstrated that the levels of many potent angiogenic factors are elevated in vitreous from patients with PDR when compared with vitreous from patients without vasoproliferative retinopathies. Those factors whose intravitreal levels have been shown to be increased in PDR include: basic fibroblast growth factor (bFGF),^{5,6} insulin-like growth factor-I (IGF-I),^{7,8} and its binding proteins (IGFBPs),^{8,9} interleukins,^{10,11} vascular endothelial growth factor (VEGF),^{12,13} and endothelial cell stimulating angiogenesis factor (ESAF).¹⁴ However, previous studies have often failed to correlate intravitreal growth factor levels with: (a) activity of neovascularisation or (b) method of glycaemic management. Furthermore, most studies have only measured a single growth factor in the vitreous samples thus ignoring any potential interrelation between the levels of different growth factors within the vitreous in PDR.

Therefore, this study has attempted to overcome these limitations in our knowledge by measuring the intravitreal levels of several growth factors known to be angiogenic in vivo^{15,16}—that is, bFGF, transforming growth factor β (TGF- β), IGF-I, insulin and epidermal growth factor (EGF), and by undertaking a detailed analysis on the data obtained. We wished to test the hypothesis that no one factor is predominant in PDR but that a regular profile of levels of different growth factors might be operating, and that the profile might differ according to whether or not insulin therapy was part of the patient's glycaemic management.

Materials and methods

COLLECTION OF VITREOUS SAMPLES

Vitreous samples were obtained from Moorfields Eye Hospital, London and the Royal Eye Hospital, Manchester at the time of vitreoretinal surgery in eyes with PDR (51 eyes) or, in the case of non-diabetic patients (controls), either idiopathic macular hole (13 eyes) or rhegmatogenous retinal detachment without

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(or with only a minimal degree (grade 3 or less) of) associated proliferative vitreoretinopathy (PVR) (eight eyes); in PVR preretinal proliferations are primarily non-vascularised. There was no clinically evident intragel, retrohyaloid, or subretinal haemorrhage in any of the eyes studied.

The diabetic patients had either tractional retinal detachment or combined traction-rhegmatogenous detachment involving the macula. All diabetic eyes had previously undergone a variable degree of peripheral laser photocoagulation, though the exact extent of this treatment could not be readily quantified. Of the diabetic patients 33 had been injecting insulin as part of their glycaemic management (IT) for at least 6 months and 18 were not receiving insulin treatment (NIT) but did use oral hyperglycaemic drugs. The mean age of the IT patients was 53.8 (SD 12.1) years and that of NIT patients 54.8 (13.6) years. Neovascularisation was considered to be 'active' if there were perfused preretinal new capillaries (26 IT, 11 NIT) and 'fibrotic' if the vasoproliferation consisted only of large (that is, remodelled) vessels within the membranes at surgery.

Undiluted vitreous samples (0.5–2.0 ml) were obtained at the start of the vitrectomy operation as previously described.¹⁷ All samples were frozen within 15 minutes after removal and sent immediately to the laboratory (on dry ice) where they were stored at –70°C until required for laboratory analysis.

PREPARATION OF VITREOUS SAMPLES

Before analysis the vitreous samples were thawed and liquefied by aspiration through a 27 gauge needle. The liquefied samples were divided into aliquots containing appropriate volumes for subsequent assays and immediately refrozen at –70°C. Aliquots for individual assays were thawed and used in the growth factor assays. Each assay was undertaken on all samples at the same time to avoid interassay variation. The number of factors assayed in individual samples depended on the initial volume of that sample—that is, there was insufficient material to measure all the growth factors in some samples. Owing to the limited volume of certain samples a minimum of duplicate tests were undertaken for each vitreous sample in each assay. Saline was substituted for vitreous in negative controls.

RADIOIMMUNOASSAYS FOR bFGF, EGF, IGF-I, AND INSULIN

The radioimmunoassays (RIAs) for bFGF, IGF-I, insulin, and EGF were undertaken as previously described.^{18,19} The threshold of detection for each assay was; bFGF 50 pg/ml, IGF-I 100 pg/ml, insulin 1 µU/ml, and EGF 50 pg/ml.

RIA for bFGF

Bovine bFGF (R & D Systems, British Biotechnology) was diluted in RIA buffer (PBS plus 0.1% Tween 20, 0.1% BSA) to provide a standard curve ranging from 0 to 100 ng/ml. The RIA reaction mixture comprised: 50 µl

¹²⁵I-bFGF (with a specific activity of 970 Ci/mmol, Amersham) at 8000–12 000 cpm, 50 µl unlabelled bFGF, and 50 µl polyclonal antibody to bovine bFGF (R & D Systems, British Biotechnology) at a dilution of 1:10 000 (as determined by an antibody titre curve). The RIA mixture was incubated in LP3 tubes (Luckham) overnight at 4°C. After incubation, 50 µl of rabbit IgG coated with cellulose (Saccel, International Diagnostics) was added and the mixture incubated for 1 hour at 37°C. Thereafter, 1 ml of RIA buffer was added and the tubes centrifuged at 2000 *g* for 15 minutes. The supernatant was discarded and the pellet was assayed on a gamma counter (Packard). For analysis of vitreous samples, 50 µl of test sample replaced the unlabelled bFGF in the RIA reaction mixture.

RIA for IGF-I

Before the assay of vitreous samples for IGF-1, IGFBPs were removed from the vitreous samples by acid-ethanol extraction.¹⁹ Briefly, vitreous samples (100 µl) were incubated for 30 minutes at room temperature with 400 µl of an extraction mixture (87.5% ethanol/12.5% 2 M HCl, v/v). Extracted vitreous samples were then centrifuged at 1850 *g* for 30 minutes. A 250 µl aliquot of the supernatant was then transferred into fresh tubes and neutralised with 0.855 M TRIS (pH 11) at a ratio of 5:2. IGF-1 within the vitreous extracts was assayed against human IGF-1 (International Reference Preparation coded 87/518; National Institute of Biological Standards and Control, Pottery Bar, Herts), serially diluted over the range 0.1–125 ng/ml in RIA buffer (PBS plus 0.1% (v/v) Tween-20, and 0.1% (w/v) bovine serum albumin) and extracted in an identical fashion to the vitreous samples. The RIA reaction mixture comprised 50 µl ¹²⁵I-IGF-1, 50 µl unlabelled IGF-1 (in the case of standard/test samples) and 50 µl polyclonal rabbit anti-human IGF-1 antiserum (donated by Dr Helen Beere, Manchester University¹⁹) at a dilution of 1:37 500. Reagent additions were carried out on ice and the tubes were incubated overnight at 4°C. After incubation, 100 µl of anti-rabbit IgG coated with cellulose (IDS) was added to each reaction tube except those for total counts. The mixtures were incubated for 1 hour at 37°C. Thereafter, 0.5 ml of RIA buffer was added and the tubes were centrifuged at 1850 *g* for 15 minutes. The supernatant was discarded, and the pellet was assayed on a gamma counter.

Insulin

Human insulin (Sigma) was diluted in RIA buffer to provide a standard curve ranging from 0–250 µU/ml. The RIA reaction mixture comprised: 50 µl ¹²⁵I-insulin (Amersham, 650 cpm per tube), 50 µl unlabelled insulin, and 50 µl polyclonal antibody to insulin (Amersham) at a dilution of 1:4500 (as determined by an antibody titre curve). The RIA mixture was incubated in LP3 tubes (Luckham) overnight at 4°C. After incubation the separation of the bound/free form of the labelled antigen was carried out by addition of 0.2 ml cold PBSA

containing 3% (w/v) charcoal (BDH), and 20% (v/v) horse serum (Gibco) to each tube. After incubation at 4°C for 10 minutes the samples were centrifuged at 2400 *g* for 20 minutes and the unbound tracer in the supernatant counted in a gamma counter. For analysis of vitreous samples, 50 µl of test sample replaced the unlabelled insulin in the RIA reaction mixture.

EGF

Human EGF (Boehringer Mannheim) was radio-labelled with ^{125}I using the chloramine T method. ^{125}I -EGF was diluted in RIA buffer (PBS plus 0.1% Tween 20, 0.1% BSA) to provide a standard curve ranging from 0 to 100 ng/ml. The RIA reaction mixture comprised: 50 µl ^{125}I -EGF (16 000–20 000 cpm), 50 µl unlabelled EGF, and 50 µl monoclonal anti-human antibody to bovine EGF (R & D Systems, British Biotechnology) at a dilution of 1:10 000 (as determined by an antibody titre curve). The RIA mixture was incubated in LP3 tubes (Luckham) overnight at 4°C. After incubation, 50 µl of rabbit IgG coated with cellulose (SacCel, International Diagnostics) was added and the mixture incubated for 1 hour at 37°C. Thereafter, 1 ml of RIA buffer was added and the tubes centrifuged at 2000 *g* for 15 minutes. The supernatant was discarded and the pellet was assayed on a gamma counter (Packard). For analysis of vitreous samples, 50 µl of test sample replaced the unlabelled EGF in the RIA reaction mixture.

ELISA FOR TGF-β2

Vitreous was heat activated at 85°C for 5 minutes in order to activate all the latent TGF-β. Measurements for total TGF-β2 in vitreous were obtained following the manufacturer's instructions using a TGF-β2 Quantikine ELISA kit (R & D systems, British Biotechnology). In brief, 100 µl of standard TGF-β2 or appropriate vitreous samples were pipetted into each well of a 96 well plate precoated with a monoclonal antibody specific for TGF-β2 and allowed to incubate for 2 hours at room temperature. After incubation the plate was washed four times with wash buffer, and a peroxidase linked anti-TGF-β2 antibody added. After further incubation for 2 hours at room temperature, the plate was washed four times with wash buffer. The plate was blot dried, and the substrate solution (hydrogen peroxide/trimethylbenzidine) was added. The colour was allowed to develop for 20 minutes, after which 50 µl stop reagent (2 N sulphuric acid) was added. The absorbance of the wells was obtained at 450 nm and 570 nm, within 30 minutes of adding the stop reagent. Values for TGF-β2 in the vitreous were derived from the standard curve. The threshold of detection for each assay was 31 pg/ml.

WESTERN LIGAND BLOTTING FOR IGFBPs

IGFBPs were identified as previously described.^{19,20} To assess relative levels and determine molecular weights of the vitreous IGFBPs 10 µl samples were subjected to non-reducing SDS-PAGE (12.5% gel) under 100 V

(continuous voltage) and then transferred to nitrocellulose filters (0.45 µm pore size). To evaluate both equal and complete protein transfer membranes were routinely stained for 10 minutes with Ponceau S solution and destained with distilled water. In brief, the nitrocellulose membranes were sequentially blocked for 30 minutes in TRIS buffered saline (TBS; 10 mM TRIS/HCl and 0.15 M NaCl, pH 7.4) containing 3% (v/v) Nonidet P-40 (Sigma); 2 hours in TBS containing 1% (w/v) BSA and finally 10 minutes in TBS containing 0.1% (v/v) Tween-20. Binding of ^{125}I -IGF-II (1×10^6 cpm/5 ml) to the blot was conducted for 3 hours in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. Thereafter, the nitrocellulose membrane was washed twice in TBS plus 0.1% (v/v) Tween-20 and then three times in TBS alone. The vitreous IGFBPs visualised by autoradiography after 3 to 7 days. Comparative western ligand blots were also carried out using ^{125}I -IGF-1 as radioligand. Owing to the high level of signal in some samples the relative signals were assessed subjectively as high (3), medium (2), low (1) or non-detectable (0). Both human plasma and a range of molecular weight markers were run in parallel to confirm the molecular weight of each band.

STATISTICS

Parametric (ANOVA) and non-parametric (Mann-Whitney U) tests²¹ were undertaken on

Figure 1 Scatter plots of bFGF levels in vitreous samples from diabetic (with proliferative diabetic retinopathy) and non-diabetic patients. (A) All diabetic samples (DM) versus non-diabetic samples (MH = macular hole, O = rhegmatogenous retinal detachment). (B) Diabetic samples subdivided according to glycaemic management and activity of neovascularisation (A = active, F = fibrotic). Horizontal bar = mean of each subgroup. **Significantly different ($p < 0.05$) from the other groups.

Figure 2 Scatter plots of TGF-β levels in vitreous samples from diabetic (with proliferative diabetic retinopathy) and non-diabetic patients. (A) All diabetic samples (DM) versus non-diabetic samples (MH = macular hole, O = rhegmatogenous retinal detachment). (B) Diabetic samples subdivided according to glycaemic management and activity of neovascularisation (A = active, F = fibrotic). Horizontal bar = mean of each subgroup. **Significantly different ($p < 0.05$) from the other subgroups.

the data using the Simfit data analysis program.²² All data were examined using both statistical methods and levels of significance are only quoted if comparisons were significant in both tests.

Results

bFGF, TGF-β2, and IGF-I were detected in all vitreous samples tested. IGFBPs were detected in over 90% while EGF was only detectable in 40% of the total ($n = 72$) samples tested.

bFGF

bFGF levels ranged between 0.3 and 6.3 ng/ml (Fig 1). There was no significant difference ($p > 0.07$) between the mean levels of bFGF in diabetic vitreous compared with vitreous from the macular hole or rhegmatogenous retinal detachment patients (non-diabetic control groups) (Fig 1A). The variation in bFGF levels was greater in the NIT subgroup than in the IT subgroup and the mean bFGF level was significantly greater ($p < 0.01$) in vitreous from NIT patients than that from either IT patients or non-diabetics. Further subdivision of patients into those with active neovascular membranes and those with fibrotic membranes demonstrated that the highest mean level of bFGF (2.7 ng/ml) was seen in vitreous from eyes with active neovascular membranes in the NIT subgroup ($p < 0.01$) (Fig 1B). The mean bFGF level in eyes with fibrotic membranes

Figure 3 Scatter plots of IGF-I levels in vitreous samples from diabetic (with proliferative diabetic retinopathy) and non-diabetic patients. (A) All diabetic samples (DM) versus non-diabetic samples (MH = macular hole, O = rhegmatogenous retinal detachment). (B) Diabetic samples subdivided according to glycaemic management and activity of neovascularisation (A = active, F = fibrotic). Horizontal bar = mean of each subgroup. **Significantly different ($p < 0.05$) from the other subgroups.

was similar to levels observed in vitreous from either IT or control patients.

TGF-β2

TGF-β2 levels ranged between 0.9 and 2.9 ng/ml (Fig 2). There was no significant difference ($p > 0.1$) between the mean levels of TGF-β2 in diabetic vitreous compared with vitreous from non-diabetic controls (Fig 2A). The mean vitreous level of TGF-β2 was significantly greater ($p < 0.01$) in IT patients than in the other subgroups. Levels in the macular hole group were very variable. The highest mean level of TGF-β2 (2.4 ng/ml) was seen in vitreous from IT patients with active neovascular membranes (Fig 2B). The mean TGF-β2 level in the fibrotic subgroup was similar to levels observed in either NIT or control patients.

IGF-I, IGFBPs, AND INSULIN

IGF-I levels ranged between 0.7 and 17.0 ng/ml (Fig 3). There was a significant difference ($p < 0.05$) between the mean level of IGF-I in diabetic vitreous compared with vitreous from control patients (Fig 3A). The mean level of IGF-I was significantly greater ($p < 0.01$) in IT patients than in either the NIT or control groups. The highest mean level of IGF-I (6.8 ng/ml) was seen in vitreous from IT patients with active membranes ($p < 0.01$) (Fig 3B). The mean IGF-I level in the fibrotic IT

Figure 4 Western ligand blot of insulin-like growth factor binding proteins present in vitreous samples from patients with diabetic retinopathy (D) and non-diabetic patients with macular hole (MH). The data presented are representative of all the samples analysed. C = human plasma run as a control. The estimated Mr (kDa) of each form was derived by comparison with defined Mr standards.

subgroup was intermediate between that in the IT active subgroup and either NIT or non-diabetic patients.

The majority (75%) of the vitreous samples contained a ~34 kDa IGFBP and 30% of the samples studied contained a ~30 kDa IGFBP. Where both were present the ~34 kDa IGFBP was the predominant isoform. Band intensities were highest in diabetic patient compared with non-diabetic patients (Fig 4).

Mean insulin levels did not vary significantly ($p > 0.1$) between patient groups, but there was a greater distribution of insulin levels in the IT group (Fig 5).

EGF

EGF levels were either very low or below the detection limit of the assay (50 pg/ml). No correlation could be found with glycaemic management or activity of neovascularisation.

PROFILES AND ADDITIONAL ANALYSIS

Further analysis of the raw data using repeated measures analysis of variance have demonstrated the following: (i) there was no correlation between growth factor levels and either the sex or age of the patient; (ii) there was a positive correlation between increased levels of IGF-I and TGF- β in IT patients and an inverse correlation between these two growth factors and the increased levels of bFGF in NIT patients. However, analysis of growth factor levels within individual IT patient samples

Figure 5 Scatter plots of insulin levels in vitreous samples from diabetic (with proliferative diabetic retinopathy) and non-diabetic patients. Diabetic samples subdivided according to glycaemic management and activity of neovascularisation (A = active neovascularisation, F = fibrotic). MH = macular hole, O = rhegmatogenous retinal detachment. Horizontal bar = mean of each group.

failed to show any correlation between IGF-I and TGF- β ; (iii) there was a weak (but significant) correlation ($p = 0.04$) between high insulin levels, high IGF-I levels, and high 34 kDa IGFBP band intensity in the vitreous from IT patients.

Discussion

Vitreous bFGF levels have previously been determined in two studies and, while our results are comparable with one report,⁵ the levels are considerably lower than those observed by Sivalingham *et al*⁶ who reported bFGF levels of greater than 30 ng/ml in some PDR patients compared with our maximal value of 6.2 ng/ml. This discrepancy may reflect (a) the exclusion of patients with clinically recognisable vitreous haemorrhage from our study, (b) different methods of preparation of the vitreous samples, and/or (c) the use of different assay methods. To our knowledge TGF- β 2 levels have not been determined previously in vitreous from PDR patients. However, values obtained in our study do fall within the range of those previously reported for non-diabetic human eyes with uveitis²³ or PVR.²⁴ The IGF-I levels measured are within the range of those previously reported by others.^{7,8,25} The observation of both a ~34 kDa and a ~30 kDa IGFBP in the vitreous, with the ~34 kDa isoform predominating, is in agreement with previous reports.^{8,9,25} There have been no reports of significant levels of EGF in the vitreous despite evidence for EGF immunolocalisation in PDR preretinal membranes.²⁶

The results presented in this study demonstrate that most of the statistically significant differences between mean levels of vitreous growth factors depended not only on division of patients into groups (that is, diabetic/non-diabetic patients), but also on further subdivision into subgroups within the diabetic group (that is, IT/NIT patients and active/fibrotic membranes). Thus IGF-I was the only growth factor which demonstrated significantly higher levels in the vitreous of PDR patients when all diabetic patients (irrespective of disease type and neovascular activity) were compared with non-diabetic patients. This difference in growth factor levels between diabetics and non-diabetics cannot be due to retinal detachment/panretinal photocoagulation (diabetic patients had retinal detachments while the majority of non-diabetics did not and photocoagulation was only performed in eyes with PDR) since subdivision of the diabetic patients on the basis of glycaemic control and neovascularisation revealed that only a specific subgroup demonstrated increased levels compared with non-diabetic patients. When the diabetic group was subdivided into IT and NIT patients, bFGF, TGF- β 2, and IGF-I demonstrated significantly higher levels in specific patient subgroups, bFGF being significantly higher in the NIT subgroup and IGF-I and TGF- β 2 significantly higher in the IT subgroup. The fact that growth factor profiles varied between the two groups suggests that insulin administration may affect the PDR process.

Further subdivision demonstrated that these increased vitreal growth factor levels were especially associated with active neovascularisation; diabetic patients with quiescent neovascular activity and non-diabetic patients exhibited significantly lower levels of these growth factors. Earlier reports also have demonstrated that vitreal growth factor levels are highest in diabetic patients with active neovascularisation. Sivalingham *et al.*⁶ reported that increased bFGF levels had a positive correlation with active proliferative retinopathy and Aiello *et al.*¹² obtained a similar result for VEGF. Although we did not measure VEGF levels in this study we would anticipate that these would be variably raised in both groups. This increase in vitreous growth factor levels may result from (i) excess secretion/release of growth factors from the ischaemic retina, thus 'driving' neovascularisation^{18 25 27 28}; (ii) overproduction of growth factors by cells in active neovascular membranes—there is clear evidence at both the protein and gene level that a variety of angiogenic factors are produced locally by cells within preretinal membranes as well as in the retina^{28 29}; (iii) a contribution from systemic sources³⁰; or (iv) decreased degradation of growth factors—the presence of IGFBPs and soluble FGF receptors in the vitreous, for example, may protect these growth factors from proteolysis.^{31 32} It is also unclear what relation, if any, elevated levels of intravitreal growth factors have on the development and progression of preretinal neovascularisation; the potential action of any individual growth factor depends on the levels of other growth factors and the presence of their respective receptors.^{33 34} In addition, some factors may be present in latent form—for example, the majority of TGF- β 2 in vitreous from eyes with PVR is present in the latent form.²⁴

The lack of correlation between the levels of different growth factors within individual patients with PDR infers that (a) the elevation of different growth factors within the vitreous can be associated with a clinically indistinguishable neovascular response, or (b) there is no regularly concerted effect or cascade of those growth factors assayed, or (c) the levels of intravitreal growth factors do not reflect their autocrine/paracrine role in preretinal neovascularisation. While we have demonstrated a correlation between mean levels of intravitreal growth factors and both disease type and activity of neovascularisation in PDR, further studies are required at the molecular level to elucidate the precise role of these and other growth factors in vasoproliferation.

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