Angiopoietin concentrations in diabetic retinopathy

J I Patel, P G Hykin, Z J Gregor, M Boulton, I A Cree

Background/aim: Angiopoietin 1 and 2 interact with vascular endothelial growth factor (VEGF) to promote angiogenesis in animal and in vitro models. Although VEGF concentrations are elevated, there is little information regarding angiopoietin concentration in the vitreous of patients with diabetic retinopathy.

Methods: Angiopoietin concentrations were measured by luminescence immunoassay in vitreous samples from 17 patients with non-proliferative diabetic retinopathy (NPDR) and clinically significant diabetic macular oedema (CSMO), 10 patients with proliferative diabetic retinopathy (PDR), and five patients with macular hole (controls) obtained at pars plana vitrectomy.

Results: Angiopoietin 1 concentrations were low in patients with macular hole (median 17 pg/ml) while in NPDR with CSMO they were 2002 pg/ml (range 289–5820 pg/ml) and in PDR 186 pg/ml (range 26–2292 pg/ml). Angiopoietin 2 concentrations in NPDR with CSMO were a median of 4000 pg/ml (range 1341–14329 pg/ml). For both macular hole and PDR patients angiopoietin 2 was below the limit of detection.

Conclusions: Angiopoietin 2 concentration was twice that of angiopoietin 1 in NPDR with CSMO. Angiopoietin 2 is the natural antagonist of angiopoietin 1 which is thought to act as an anti-permeability agent. The predominance of angiopoietin 2 may allow VEGF induced retinal vascular permeability in patients with CSMO. The relatively low concentration of both angiopoietin 1 and 2 in patients with proliferative diabetic retinopathy may reflect the established nature of the neovascularisation in cases proceeding to vitrectomy.
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Quantitative assessment using the computer software (Zeiss-analysed quantitatively using the retinal mapping software. taken at each time point, and assessed qualitatively and diameter radial scans centred on the point of fixation were times with 100 mm (BSA) for 1 hour at room temperature. Then either 30 washed six times with PBS. Thereafter, either 30 m phosphate buffered saline, pH 7.4). The wells were then washed, and 30 m of a tertiary antibody of anti-rabbit goat antibody for angiopoietin 2) was added to each well depending on which cytokine was tested (Regeneron Pharmaceuticals Inc, USA). Both secondary antibodies were used at 1:10 000 in PBS-0.5% BSA. The wells were then washed with PBS. Thereafter, either 30 ml of NTL-1 (secondary antibody for angiopoietin 1) or NTL-2 (secondary antibody for angiopoietin 2) was added to each well depending on which cytokine was tested (Regeneron Pharmaceuticals Inc, USA). Both secondary antibodies were used at 1:10 000 in PBS-0.5% BSA. The wells were then washed, and 30 ml of a tertiary antibody of anti-rabbit goat immunoglobulin added at 1:10 000 for 1 hour at room temperature. Then the wells were washed with PBS followed by addition of 30 ml of LumIGLO reagent and hydrogen peroxide (New England Biolabs, USA) for 5 minutes and the plate read in a luminometer. The angiopoietin 1 standard curve was from to 5 pg/ml–100 ng/ml. All of the results of the samples fell within this range. Similarly, for angiopoietin 2, the standard curve ranged from 1 ng/ml to 100 ng/ml. The lower sensitivity of the angiopoietin 2 assay is explained by the lower affinity of angiopoietin 2 for Tie 2 in comparison with angiopoietin 1. Vitreous samples were tested in triplicate and the aqueous samples tested in duplicate.

RESULTS

Mann-Whitney test and non-parametric (Spearman) correlation tests were used to analyse the data.

The average duration of diabetes was 14 years (5–22 years) with Hba1C of 9% (9–12%) in the diabetic patients whose average age was 58 (40–72 years). All had type 2 diabetes. Those patients with non-proliferative retinopathy also demonstrated clinically significant macular oedema which had been present for a median of 15 months (12–18 months) before pars plana vitrectomy. They had received on average three macular laser treatments (range 1–6). Those with proliferative disease had received extensive panretinal photocoagulation and underwent PPV for delamination of membranes and further endolaser.

The median vitreous angiopoietin 1 concentration for five patients with full thickness macular hole (FTMH) used as a control population was 150 pg/ml (range 3–227 pg/ml) compared to 1933 pg/ml (range 289–5820 pg/ml) in 17 patients with non-proliferative diabetic retinopathy (NPDR) and 186 pg/ml (range 26–2,292 pg/ml) for 10 patients with treated proliferative diabetic retinopathy (PDR) (see fig 1A). The angiopoietin 1 concentration in NPDR was higher than in FTMH and PDR (p<0.001). The angiopoietin 1 concentration (Ang 1 and Ang 2) in non-proliferative diabetic retinopathy with clinically significant macular oedema. Angiopoietin 1 concentration was approximately half that of angiopoietin 2.

Figure 1 (A) Vitreous angiopoietin 1 concentration in full thickness macular hole (FTMH) (n = 5), non-proliferative diabetic retinopathy with clinically significant macular oedema (NPDR-CSMO) (n = 17), and treated proliferative diabetic retinopathy (PDR) (n = 10). The angiopoietin 1 concentration was greater in the NPDR than in FTMH and PDR (p<0.001). (B) Vitreous angiopoietin concentration (Ang 1 and Ang 2) in non-proliferative diabetic retinopathy with clinically significant macular oedema. Angiopoietin 1 concentration was approximately half that of angiopoietin 2.

an estimation of the foveal thickness and the macular volume, which is derived by an integration process of the average retinal thickness of the six radial scans.

Statistical analysis

A volume of 30 ml recombinant FcTie-2 receptor (Regeneron Pharmaceuticals Inc) was added to wells of a 384 well ELISA plate and incubated overnight at 4°C at a concentration of 3 pg/ml. The wells were then washed six times with 100 ml of phosphate buffer saline (PBS) and then blocked with 100 ml of PBS-0.5% of bovine serum albumin (BSA) for 1 hour at room temperature. Then either 30 ml of either angiopoietin 1 (R and D Systems, Minneapolis MN, USA) or angiopoietin 2 (Regeneron Pharmaceuticals Inc, USA) standards, or test samples were added to the wells for 2 hours at room temperature (samples were diluted 1:4 with phosphate buffered saline, pH 7.4). The wells were then washed six times with PBS. Thereafter, either 30 ml of NTL-1 (secondary antibody for angiopoietin 1) or NTL-2 (secondary antibody for angiopoietin 2) was added to each well depending on which cytokine was tested (Regeneron Pharmaceuticals Inc, USA). Both secondary antibodies were used at 1:10 000 in PBS-0.5% BSA. The wells were then washed, and 30 ml of a tertiary antibody of anti-rabbit goat immunoglobulin added at 1:10 000 for 1 hour at room temperature. Then the wells were washed with PBS followed by addition of 30 ml of LumIGLO reagent and hydrogen peroxide (New England Biolabs, USA) for 5 minutes and the plate read in a luminometer. The angiopoietin 1 standard curve was from to 5 pg/ml–100 ng/ml. All of the results of the samples fell within this range. Similarly, for angiopoietin 2, the standard curve ranged from 1 ng/ml to 100 ng/ml. The lower sensitivity of the angiopoietin 2 assay is explained by the lower affinity of angiopoietin 2 for Tie 2 in comparison with angiopoietin 1. Vitreous samples were tested in triplicate and the aqueous samples tested in duplicate.

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The median angiopoietin 2 concentration in NPDR was 3874 pg/ml (range 1341–9888 pg/ml) while in FTMH and PDR patients, angiopoietin 2 concentration were below the level of sensitivity for the assay. The angiopoietin 1 concentration in NPDR was half that of angiopoietin 2 with the difference significant at p<0.02 (see fig 1B).
Further on comparing the foveal thickness to the angiopoietin 1 concentration in patients with NPDR, all of whom had persistent CSMO, there was a non-significant trend towards correlation (p<0.08 and R = 0.44) (see fig 2). No such trend was seen on comparing angiopoietin 2 with these macular structural indices.

In two patients there was sufficient postoperative aqueous available (initially drawn as part of a separate study) to evaluate postoperative aqueous angiopoietin concentration over a 6 week period. This concentration varied according to whether there was a coincidental increase or decrease in the foveal thickness after surgery. In one patient there was a fall in the foveal thickness, with the angiopoietin 1 concentration increasing from a baseline of 845 pg/ml to 3839 pg/ml at 6 weeks as the foveal thickness changed from 216 µm to 260 µm over the same period, but at 3 months the thickness had decreased to 174 µm (fig 3). The angiopoietin 2 level remained relatively static (3223 to 3591 pg/ml over the period of observation) (fig 3). However, in the second patient, the foveal thickness increased and the angiopoietin 1 concentration decreased over the 6 week period (2752 to 1828 pg/ml) while angiopoietin 2 showed a reciprocal increase from 3069 to 6079 pg/ml over the same period (see fig 4).

DISCUSSION
Diabetic macular oedema is the leading cause of blindness among the diabetic population. Its pathogenesis is multifactorial and incompletely understood, but chronic hyperglycaemia has been shown to disrupt the blood-retinal barrier allowing for the development of oedema. Cytokines and growth factors are considered important in the process of damage to the blood-retinal barrier, in particular vascular endothelial growth factor (VEGF). VEGF is a vasopermeability inducing agent with known disruptive effects on retinal endothelial tight junctions. VEGF has been shown to work in concert with the angiopoietins which interact with the Tie 2 receptor. To date only concentrations of VEGF in the ocular fluids of diabetic patients are known. This is the first report of the concentration of angiopoietins in the vitreous of diabetic patients.

In PDR, the vitreous angiopoietin 1 level was similar to those of macular hole (FTMH) patients. The angiopoietin 2 concentration was below the sensitivity of the assay for both FTMH and PDR. This finding reflects the role of angiopoietin 2 in facilitating the process of angiogenesis at sites of active remodelling. Therefore, if there is no active angiogenesis (as in FTMH), the angiopoietin 2 level would not be elevated. Our results in PDR vitreous samples appear to contradict the role of angiopoietin 2 as a facilitator of angiogenesis, but may simply reflect the fact that in long standing and treated PDR, there is little active vessel replication. In established PDR, at the time of vitrectomy, new vessels are mature with limited active angiogenesis and therefore low concentrations of the angiopoietins would be expected. Furthermore in such a clinical picture of minimal vascular turnover, VEGF levels would also be at basal levels and, correspondingly, a decrease stimulus for angiopoietin 2 production. In FTMH no active neovascular processes are known to occur, explaining the minimal concentration of angiopoietin 1. In fact, FTMH patients represent the normal non-neovascular vitreoretinal environment where angiopoietin 1 is at the basal level required to promote and maintain endothelial integrity. In contrast, angiopoietin 1 promotes stabilisation of immature neovascularisation by promoting the recruitment of supporting cells. In treated PDR, where neovascularisation would be regressing, then angiopoietin 1 may be expected to contribute to this process as facilitator for the recruitment of supporting cells—for example, fibroblasts. There, in both FTMH and treated PDR similar levels of angiopoietin 1 may be expected.
In contrast, patients with NPDR had angiopoietin 1 vitreous concentrations that were approximately half those of angiopoietin 2. In our series, patients had at least moderate and five cases had severe NPDR. From clinical observation there is established retinal ischaemia and capillary closure in such cases leading to the formation of IRMA (intraretinal microvascular abnormalities—precursors of new vessels) as a result of increased VEGF production. This represents the point in the natural history of diabetic retinopathy where early neovascularisation occurs with the development of angiogenic sites and endothelial remodelling. Such dynamic changes within the retina are probably reflected by the increased angiopoietin 2 concentration combined with a relative change in the angiopoietin 1 to angiopoietin 2 ratio allowing angiopoietin 2 to predominate at the Tie 2 receptor. The affinities of these two angiopoietins are similar for the Tie 2 receptor but angiopoietin 2 competitively inhibits angiopoietin 1 at the receptor. Our results confirm that in NPDR, angiopoietin 2 concentration is double that of angiopoietin 1 concentration. Therefore the predominance of angiopoietin 2 at the Tie 2 receptor would promote increased vascular permeability in combination with elevated VEGF in NPDR thus facilitating breakdown of the blood-retinal barrier.

Interestingly, our findings in two patients who had intraoperative and two postoperative aqueous samples concur with the role of angiopoietin 2 as a pro-permeability agent. In the one patient, in whom the foveal thickness had deceased at 3 months after surgery, the angiopoietin 1 concentration had progressively increased during the initial 6 week period following surgery. We postulate that in this case, the anti-permeability effect of angiopoietin 1 acted to reduce the amount of macula oedema as measured by OCT. In the second patient in which the postoperative macular oedema increased up to 3 months, there was a concomitant reduction in angiopoietin 1 aqueous concentration leading to increased permeability and macular oedema mediated by angiopoietin 2 (probably with VEGF). It seems that the morphological effects of the early changes in angiopoietin 1 after surgery may be borne out some weeks later. If this model of angiopoietin 1 function is correct, then an increase in angiopoietin 1 in this situation would precipitate a cascade of intracellular events which would eventually lead to the “tightening” of the blood-retinal barrier. However, a larger study correlating macular volume, as measured by OCT, and postoperative aqueous angiopoietin concentrations would be required to confirm these findings.

This is the first report to quantify the concentration of the angiopoietins 1 and 2 in the ocular fluids of diabetic patients and controls. It shows that both angiopoietin 1 and 2 are elevated in moderate and severe NPDR at a time when intraretinal neovascularisation is initiated. It is presumed that angiopoietin 2 predominates in this context to promote neovascularisation and possibly increased vascular permeability together with a decrease in angiopoietin 1 to precipitate macular oedema.

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