Vitreous and plasma changes of endothelin-1, adrenomedullin and vascular endothelium growth factor in patients with proliferative diabetic retinopathy

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Abstract. – OBJECTIVE: To assess vitreous and plasma changes of vascular endothelial growth factor A (VEGF-A), adrenomedullin (ADM) and endothelin-1 (ET-1) in proliferative diabetic retinopathy (PDR).

PATIENTS AND METHODS: 9 patients with PDR in type 2 diabetes (T2DM) and 11 age-matched non-diabetic patients were enrolled. The levels of VEGF-A, ADM and ET-1 were measured using an enzyme (ELISA) and a radioimmunoassay (RIA) both in vitreous and plasma samples.

RESULTS: Vitreous ADM and VEGF-A levels were significantly higher in PDR patients (p=0.04 and p=0.02), whereas no differences were found in ET-1 levels (p=0.29). Plasma ADM levels were significantly higher in the PDR group (p<0.01), whereas no significant differences were found in the plasma ET-1 and VEGF-A levels (p=0.30 and p=0.37). The ADM vitreous/plasma ratio was significantly reduced in PDR group.

CONCLUSIONS: The role of ET-1 in advanced PDR is still controversial; it has been supposed a role limited to induce hypoxic state and promote angiogenesis in the early phases. Once the neo-angiogenic process starts, other mediators are mainly involved as VEGF and ADM. Our findings suggest that ADM is an important marker of advanced PDR as well as VEGF. Conversely, ET-1 is not significantly involved in the advanced stage of PDR.

Key Words: Neoangiogenic factors, Diabetes, Microvascular complications, Proliferative diabetic retinopathy, Vasactive peptides.
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University “Sapienza”, Rome, Italy. The patients were divided into two groups. In the first group, we included 9 T2DM patients with PDR (age range: 43-84 years). The control group consisted of 11 age-matched non-diabetic patients (age range: 47-78 years), with no evidence of diabetic retinopathy, vitreous hemorrhage and proliferative vitreoretinopathy (PVR).

All subjects gave written informed consent before enrollment. Institutional Board approval (IRB) was obtained from Ethics Committee of our Department. The research was conducted in conformity to the Declaration of Helsinki.

Patients who had T2DM and advanced PDR, as defined by the Early Treatment Diabetic Retinopathy Study (ETDRS) were included in the study group. The criteria to perform vitrectomy were best-corrected visual acuity less than 20/40, diabetic vitreous hemorrhage at least 2 months of duration with or without tractional retinal detachment or epiretinal membranes.

Exclusion criteria were prior treatment with steroid or non-steroid anti-inflammatory drugs (FANS), history of acute or chronic infections, fever, cancer or organ failure, thrombotic events, other ophthalmic disorders, including intraocular or vitreoretinal surgery within 6 months, and history of intravitreal injection therapies.

The control group consisted of patients who had undergone vitrectomy for other conditions, such as epiretinal membrane (n=5), retinal detachment (n=4) and macular hole (n=2). These patients were free from any systemic diseases.

At the beginning of standard three-port vitrectomy, 0.3 ml of undiluted vitreous fluid was aspirated via pars plana with a vitreous cutter into a syringe, before opening the infusion line. The vitreous fluid samples were collected into sterile tubes and quickly frozen at -80°C. Blood samples were collected into EDTA tubes before start vitrectomy and were subjected to centrifugation for 10 minutes at 3000 g. Then plasma was immediately frozen and stored at -80°C.

The determination of the ET-1, ADM and VEGF-A levels in the plasma and the vitreous fluid was assessed according to the manufacturer’s instructions, using specific kits, as previously described7-9.

ET-1 was determined by specific radioimmunoassay (RIA) using rabbit anti-endothelin antibody (Peninsula Laboratories, Belmont, CA, USA). Inter- and intra-assay variabilities of ET-1 measurements were 13% and 9%, respectively.

ADM was measured by using commercially available RIA (Phoenix Pharmaceuticals, Mountain View, CA, USA). The intra- and inter-assay coefficients of variation were 5.1% and 12%, respectively. VEGF-A was determined by using commercially available enzyme-linked immunosorbent assay (ELISA) (Cusabio Biotech, Carlsbad, CA, USA).

Statistical Analysis

Quantitative data were reported as mean ± standard deviation (SD) and distribution normality verified through Shapiro-Wilk normality test. The significance of differences between groups was assessed using unpaired t-test or U-Mann Whitney test, depending on the types of distribution. Chi-squared test was used to compare categorical variables. Pearson or Spearman’s rank correlation test (r) was used to assess the relationship between two variables. Statistical significance was set at p<0.05. All calculations were carried out using SPSS software (ver.19; SPSS, Inc., Chicago, IL, USA).

Results

Baseline characteristics of the study groups are shown in Table I. The vitreous fluid analysis revealed that ADM and VEGF-A levels were significantly higher in the PDR eyes than controls (+0.30 ng/ml, 95%CI: -0.20,0.80 and +10.50 pg/ml, 95% CI: -2.99, 24.01, respectively). Instead, the levels of ET-1 were not different between groups (0.003 ng/ml, 95% CI: -0.25, 0.03) (Table II). Figure 1 shows the levels of ET-1, ADM and VEGF-A in the vitreous fluid in both groups.

The blood plasma concentration of ADM was significantly higher in the PDR eyes (+7.26 ng/ml, 95% CI: -0.12, 14.64) than control group, whereas ET-1 and VEGF-A did not change significantly between the groups (0.003 ng/ml, 95% CI: -0.25, 0.03) (Table II). Figure 2 reported the blood plasma concentrations of the studied factors.

A positive correlation was found between vitreous and plasma concentrations of ADM (r=0.70, p<0.03) as well as VEGF-A (r=0.77, p<0.01) in the PDR group solely.

The vitreous fluid/plasma ratios for ET-1 and VEGF-A were comparable between groups, whereas the ADM vitreous fluid/plasma ratio was lowest in the PDR group (Table II).
No statistically significant correlations were found between vitreous or plasma factors and age, disease duration or glycemia. Instead, a positive correlation between serum creatinine and plasma- tic ET-1 levels was detected ($r=0.80$, $p<0.01$).

### Discussion

Diabetic retinopathy is a common microvascular complication, involving more than 60% of patients with T2DM after 20 years. The most...
serious and vision-threatening stage is proliferative diabetic retinopathy\textsuperscript{10}.

In T2DM both metabolic and vasoactive factors contribute to the development of diabetic microvascular complications\textsuperscript{11} and endothelial dysfunction is an early feature of the disease\textsuperscript{12}.

The vascular endothelium can maintain vascular tone, performing also an important hemostatic function. The endothelial dysfunction plays an important role in the pathogenesis of diabetic angiopathy\textsuperscript{13}.

An imbalance between some vasoactive substances such as ET-1 (potent vasoconstrictor and pro-inflammatory peptide)\textsuperscript{14} and ADM (potent vasodilator)\textsuperscript{15} represents a prominent feature of the endothelial dysfunction.

Although our results showed that ADM and VEGF-A concentrations were highest in the vitreous samples of the proliferative diabetic eyes, the ET-1 levels did not significant change between groups. Moreover, the plasma analysis demonstrated that only ADM levels were significantly higher in proliferative diabetic eyes than controls.

In our opinion, there are several possible explanations for the behavior of ET-1. Ocular tissues, vascular and extravascular sites in the retina are a rich source for ET-1 expression, and ET-1 itself contributes to abnormal retinal hemodynamics in diabetic retinopathy\textsuperscript{16,17}. The ET-1 expression is associated with several factors, such as clinical course of disease, insulin, sulfonylureas therapy, and serum creatinine levels\textsuperscript{18}. Moreover, panretinal laser photocoagulation can also reduce plasma ET-1 levels\textsuperscript{19}.

Indeed, vitreous and/or plasma modifications of the ET-1 are still controversial in proliferative diabetic retinopathy. Adamiec-Mroczek et al\textsuperscript{20} reported no significant differences in plasmatic ET-1 concentration between PDR patients and control group, but a significant increase in the vitreous fluid. However, Ogata et al\textsuperscript{21} reported lower ET-1 levels in the vitreous fluid of PDR patients compared with controls.

Our observations suggest that ET-1 levels in plasma and vitreous are strongly influenced by various factors, resulting in high variability of its expression. In fact, a strong positive correlation is observed between ET-1 levels and ADM and VEGF-A concentrations.

### Table II. Vitreous and blood serum concentrations of ET-1, ADM and VEGF-A among study groups and their ratios.

<table>
<thead>
<tr>
<th></th>
<th>PDR group (N=9)</th>
<th>Control group (N=11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitreous concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1 (ng/ml)</td>
<td>0.10</td>
<td>0.09</td>
<td>0.29*</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.08, 0.12</td>
<td>0.08, 0.10</td>
<td></td>
</tr>
<tr>
<td>ADM (ng/ml)</td>
<td>0.47</td>
<td>0.17</td>
<td>0.04*</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.001, 0.77</td>
<td>0.11, 0.23</td>
<td></td>
</tr>
<tr>
<td>VEGF-A (pg/ml)</td>
<td>29.51</td>
<td>19.03</td>
<td>0.02*</td>
</tr>
<tr>
<td>95%CI</td>
<td>16.75, 40.78</td>
<td>14.21, 23.86</td>
<td></td>
</tr>
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</table>

| **Blood plasma concentrations** |                |                      |         |
| ET-1 (ng/ml)            | 0.38           | 0.43                 | 0.30*   |
| 95%CI                   | 0.29, 0.64     | 0.35, 0.50           |         |
| ADM (ng/ml)             | 10.79          | 3.53                 | <0.01*  |
| 95%CI                   | 2.72, 14.92    | 1.68, 5.38           |         |
| VEGF-A (pg/ml)          | 162.15         | 144.69               | 0.37*   |
| 95%CI                   | 124.06, 194.62 | 60.13, 229.24        |         |

| **Vitreous/serum ratio** |                |                      |         |
| ET-1                   | 0.25           | 0.23                 | 0.34*   |
| 95%CI                  | 0.19, 0.32     | 0.20, 0.27           |         |
| ADM                    | 0.04           | 0.07                 | 0.05*   |
| 95%CI                  | 0.02, 0.06     | 0.05, 0.11           |         |
| VEGF-A                 | 0.17           | 0.16                 | 0.42*   |
| 95%CI                  | 0.12, 0.22     | 0.13, 0.20           |         |

PDR, proliferative diabetic retinopathy; 95% CI 95% confidence interval; *unpaired student t-test; $^b$U Mann-Whitney test;
was found between serum creatinine and plasma ET-1 levels, in agreement with previous studies reporting also an increased kidney glomerular filtration rate or mesangial expansion.

In the current study, the VEGF-A was elevated in the vitreous fluid of PDR patients, as previously reported. Otherwise, the plasma levels of VEGF-A were higher than controls but not significantly, even if directly related to its vitreous concentration. The VEGF is a well-known factor in the development of proliferative diabetic retinopathy. VEGF acts as proangiogenetic factor, involved in both vascular proliferation and increased vascular permeability.

Interestingly, vitreous and plasma levels of ADM were highly elevated in the PDR eyes and directly correlated each other. ADM is a potent vasorelaxant peptide found not only in normal adrenal medulla but also in various tissues organs including vascular smooth muscle cells, endothelial cells, and human retinal cells. In detail, during inflammatory process, various types of cells in the eye may produce ADM, including RPE cells, vascular endothelial and smooth muscle cells and fibroblasts.

ADM production in the eye is stimulated by hypoxic state, which is a typical pathological feature of diabetic microangiopathy leading to angiogenesis. ADM seems to be implicated in the advanced PDR in which fibrovascular membranes are formed. Not surprisingly, the same cells forming the fibrovascular membranes are also involved in the ADM production.

Conclusions

In summary, ET-1 may be considered as an early mediator that acts, as demonstrated previously, inducing hypoxic state and promoting angiogene-
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sis, but it is not remarkable in advanced PDR, in which seems to be involved mainly other mediators especially VEGF and ADM.

To support these hypotheses, Masuzawa et al in a diabetic rat model reported that treatment with an ET receptor antagonist, in the early stage of the diseases, might be useful in preventing the progression of diabetic retinopathy.

This is the first study that simultaneously measures concentrations of ET-1, ADM, and VEGF-A, the peptides involved in the PDR pathogenesis. Our results suggest that ET-1 may be a role as target molecule in the early stages of diabetic retinopathy, whereas ADM seems to be involved in the advanced stages as well as VEGF. Also, the high levels of ADM detected in both vitreous and plasma may represent an important marker of proliferative diseases. These findings are important to clarify the pathogenic mechanisms and to assess the potential target molecules at different stages of diabetic retinopathy.

Further studies with a large number of patients are encouraged both to confirm and extend these findings and especially to evaluate the potential role of ADM as a new target molecule.

Conflict of interest
The authors declare no conflicts of interest.

References


