Plasma endothelin-1 concentrations in patients with retinal vein occlusions

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Abstract

***Aims*—To investigate whether plasma levels of endothelin-1 (ET-1), a potent vasoconstricting peptide that is crucial in regulating retinal blood flow, were elevated in patients with retinal vein occlusion (RVO).**

***Methods*—ET-1 plasma concentrations were determined by radioimmunoassays in a double blind fashion in a group of 18 selected patients with RVO, in 20 healthy age matched non-smoking, normoglycaemic, normotensive control subjects, and in 15 patients with uncomplicated essential hypertension in the same age range.**

***Results*—Patients with RVO had significantly increased ET-1 plasma levels (14.22 (SD 4.6) pg/ml) compared with both normal subjects (7.90 (1.6) pg/ml; $p < 0.05$) and hypertensive patients (8.50 (2.9) pg/ml; $p < 0.05$). The highest concentrations of circulating ET-1 were found in patients with RVO of the ischaemic type (16.97 (3.5) pg/ml; $p < 0.01$; $n = 7$). Systemic hypertension alone did not account for the observed increase in plasma ET-1 concentrations.**

***Conclusions*—These findings raise the possibility that the increased circulating ET-1 levels in patients with RVO may be a marker of the occlusive event, thereby suggesting that ET-1 homeostasis may be relevant to RVO pathogenesis and retinal ischaemic manifestations.**

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Endothelin-1 (ET-1) is a vasoactive peptide produced and released by endothelial cells.[1] Its actions on both arteries and veins are mediated by at least two main types of receptors, ET_A and ET_B.[2 3] In the human eye, ET-1-like immunoreactivity has been documented in association with retinal blood vessels,[4] and ET receptors have been found on both retinal vessels and pericytes.[5 6] Vasoconstriction induced by ET-1 on retinal arterial vessels is primarily ET_A mediated[7–9] and is produced by pericytes,[6–10] which are contractile cells that have a regulatory role on retinal blood flow[11–13] and on the growth and function of endothelial cells.[10 14 15] In addition, ET-1 has been shown to promote retinal pericyte proliferation in a dose dependent manner.[10] The combination of the local paracrine effects of ET-1, along with release of nitric oxide and other vasoactive factors,[16 17] is likely to play a relevant physiological role in the regulation of retinal blood flow,[4–10 16 17] especially because of the absence of autonomic innervation on retinal vessels.[18] Recent experimental evidence suggests that ET-1 might also contribute to ocular pathological manifestations, promoting retinal capillary non-perfusion and ischaemia.[19–23]

The pathogenesis of retinal vein occlusions (RVOs) is still incompletely understood.[24] Clinically, RVOs are characterised by a blockage of venous outflow with marked stasis and intraretinal haemorrhagic manifestations (non-ischaemic type, NIT). In many instances RVOs can be further complicated by capillary non-perfusion (ischaemic type, IT). The combination of the aforementioned factors can promote a chain of events that leads to intraocular neovascular complications.

Why some but not all RVOs are complicated by ischaemic events, and why not all IT RVOs progress to intraocular neovascularisation remains to be clarified. Vascular endothelial growth factor (VEGF) has been implicated in determining intraocular neovascular manifestations both in animal models and in human disease.[25 26] However, elevated VEGF expression has also been observed both in retinal vessels[27] and glial cells[28] of diabetic retinas before neovascular proliferations, as well as in mouse retinas in which the neovascular response had been inhibited by somatostatin analogues.[29] VEGF is therefore unlikely to act alone in determining retinal neovascular growth. Because of the demonstrated potential of ET-1 to induce retinal ischaemia,[19–23] and since ET-1 has been readily measurable in the plasma for nearly a decade, our study was designed to determine, in a well characterised group of patients, whether RVO is associated with increased plasma ET-1 concentrations, and whether correlations existed between retinal status and ET-1 levels.

Subjects and methods

STUDY POPULATION

Thirty two consecutive patients with uniocular RVO have been examined. Patients with systemic conditions known to be associated to increased ET-1 levels (such as, congestive heart failure,[30] myocardial infarction,[31] or diabetes[32 33]) were excluded from this study, as were patients with other intercurrent retinal diseases. Also, not eligible for this investigation were patients with RVO who had already undergone retinal laser photocoagulation at the time of enrolment. Based on these inclusion criteria a group of 18 patients (group A, 11 men; seven women; mean age 63.3 (SD 8.4) years) with a well established diagnosis of uniocular RVO were enrolled in this study

(Table 1). All patients were examined upon self referral or following referral from the ophthalmic emergency unit. Time between diagnosis and examination was variable, ranging between 1 day for patients referred by the emergency unit up to several months for self referred patients. For some subjects it was not possible to define precisely when the occlusion had occurred.

Patients were diagnosed as having NIT or IT RVO (groups A1 and A2, respectively) based on findings on first examination, which also corresponded with the time when blood samples were drawn. The presence of inner retinal ischaemia was ascertained with fluorescein angiography and also with electroretinography.[24 34] Branch occlusions (BRVOs) were diagnosed according to the criteria used by the Eye Disease Case-Control Study Group.[35] Central occlusions (CRVOs) were classified according to a combination of the criteria indicated by Hayreh,[36] and, more recently, by the Central Vein Occlusion Study (CVOS) group.[37] The laboratory personnel was maintained masked to the clinical diagnosis matching each blood sample by letter coding, and so were the grading clinicians to subsequent ET-1 level determinations.

At the time of RVO occurrence, 11 of the 18 patients had a pre-existing history of systemic hypertension, and in seven history was positive for elevated intraocular pressure (IOP) (see Table 1). One patient (No 13) had a narrow anterior chamber angle bilaterally upon examination, and another (No 14) had a history of borderline IOP (18–20 mm Hg range). Consistent with the results reported in large RVO patient populations by the Eye Disease Case-Control Study Group,[35 38] the majority of cases in the IT RVO group had systemic hypertension. None of the other subjects included in this study had other relevant systemic conditions. Laboratory tests revealed hypercholesterolaemia in two of the hypertensive patients (Nos 9 and 15), and one case of increased platelet aggregation (case 1). Only one patient (No 17) was a smoker at the time of the investigation, and he refrained from smoking on the morning of blood sample collection.

Two age matched control groups were included in this study. Measurements from 20 healthy non-smoking, normoglycaemic, normotensive subjects (group B, 12 men; eight women; mean age 60.3 (6.0) years) were used as normal controls. In view of the high incidence of systemic hypertension in RVO patients, findings were also compared with data from a group of 15 patients (group C, eight men; seven women; mean age 61.0 (5.5) years) with uncomplicated essential hypertension, selected from a larger hypertensive patient population currently under investigation for distinct purposes (Letizia *et al*, study in progress).

All samples were drawn between 8:30 and 9:30, in a quiet room, following a minimum of 30 minutes of rest. Five ml of blood were collected in EDTA (1 mg/ml) and aprotinin (500 kIU/ml) containing tubes and placed on ice. Samples were immediately shipped to the laboratory, identified only by the initials of the subjects and date of sampling, leaving the laboratory masked to the subtype diagnosis of each patient. Plasma was separated by centrifugation at 3000 *g* at 4°C for 10 minutes, and immediately stored at −70°C until assayed. Informed consent was obtained from all subjects after full explanation of the nature of the study. This investigation was approved by the bioethics committee of the University of Rome La Sapienza.

ENDOTHELIN-1 DETERMINATION

Plasma ET-1 was determined by specific radioimmunoassay (RIA) according to a recently described technique.[33] In brief, on the day of assay ET-1 was extracted from samples with C18 columns (Sep-column) after acidification with 1 ml of 0.1% trifluoroacetic acid (pH 3), and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. The extracts were evaporated under nitrogen and then assayed with a specific RIA (RIK-6901, Peninsula Laboratories, Belmont, CA, USA). Cross reactivity with ET-2 and ET-3 was 7%, and 17% with human big endothelin. All assays were performed in duplicate. Interassay and intra-assay variabilities were 13% and 9%, respectively. Concentrations of ET-1 were expressed in pg/ml. Results of the assays for each patient remained masked to the ophthalmologists until data processing was completed.

STATISTICAL ANALYSES

Analyses were performed by a statistician provided with a list of the patients' ET-1 values and the letter coded grouping but masked to the clinical diagnosis of patients and the identity of control subjects. Upon completion of analyses, masking was broken, and assay results were matched to clinical diagnoses. Statistical measures were performed on an IBM personal computer (Atlanta, GA, USA) using SAS statistical software (SAS Institute Inc, Cary, NC, USA). The one way ANOVA test with Duncan's post hoc multiple range test was used for intergroup comparisons. Correlations were investigated by linear regression analysis and calculation of the correlation coefficient *R*. Statistical significance limits were fixed at p values <0.05.

Results

A diagnosis of NIT RVO was established in 11 patients (group A1). Of these, six had a BRVO. The other seven subjects fulfilled criteria to be assigned to the IT RVO group (group A2). Three of them (patients 15, 16, and 17, identified by two asterisks in Table 1) had already developed iris neovascularisation at the time of examination. Three of the patients in the IT RVO group had a branch occlusion involving one retinal quadrant, associated with extensive areas of capillary non-perfusion on fluorescein angiography.

Table 1 summarises both the clinical features and the laboratory findings for each patient in the two RVO subgroups considered. ET-1 levels in the RVO patient group as a whole were

Table 1 Clinical and laboratory findings

Patient No	*Occlusion type*	*Age (years)/sex*	*ET-1 levels (pg/ml)*	*Systemic hypertension (+/−)*	*Ocular hypertension (+/−)*
Group A, RVO patients (n = 18):			14.22 (4.6)*†		
Group A1: NIT RVO (n = 11)			12.46 (4.3)*†		
1	BRVO	57/M	5.6	−	+
2	BRVO	64/F	10.3	+	−
3	BRVO	65/M	18.3	−	+
4	BRVO	66/F	8.7	+	−
5	BRVO	66/M	12.6	−	−
6	BRVO	73/M	13.5	−	−
7	CRVO	41/M	9.0	−	−
8	CRVO	52/M	14.1	−	+
9	CRVO	63/F	14.4	+	−
10	CRVO	72/F	10.2	+	+
11	CRVO	74/F	20.4	+	+
Group A2: IT RVO (n = 7)			16.97 (3.5)*† ‡		
12	BRVO	62/M	18.4	+	−
13	BRVO	67/M	13.7	−	−(††)
14	BRVO	68/M	14.2	+	−(‡‡)
15	CRVO**	54/F	18.3	+	+
16	CRVO**	58/M	16.2	+	−
17	CRVO**	68/M	24.2	+	+
18	CRVO	70/F	13.8	+	−
Group B, Normal subjects (n=20)			7.90 (1.6)*		
Group C, Uncomplicated hypertensives (n=15)			8.50 (2.9)*		

NIT RVO = non-ischaemic type retinal vein occlusions; IT RVO = ischaemic type retinal vein occlusions; BRVO = branch retinal vein occlusions; CRVO = central retinal vein occlusions.
*Mean (SD).
†p Value <0.05 for group A and A1 *v* groups B and C, respectively, and for group A2 *v* group A1.
‡p Value <0.01 for group A2 *v* groups B and C, respectively.
**Patients with neovascular glaucoma at the time of diagnosis and blood sampling.
††Bilateral anterior chamber angle narrowing upon examination.
‡‡History of borderline intraocular pressure (range 18–20 mm Hg).

moderately increased (group A, 14.22 (SD 4.6) pg/ml) compared with both the normal mean (group B, 7.90 (1.6) pg/ml; p <0.05) and data from uncomplicated hypertensives (group C, 8.5 (2.9) pg/ml; p <0.05). There was no significant difference between normal subjects and patients with uncomplicated systemic hypertension (p >0.05).

Interesting differences were found between RVO patients with and without retinal ischaemic manifestations. Patients with NIT RVOs (group A1) had on average ET-1 concentrations of 12.46 (4.3) pg/ml, whereas IT RVO patients (group A2) showed a larger increase in ET-1 plasma levels, averaging 16.97 (3.5) pg/ml (Table 1). Compared with normal controls and uncomplicated hypertensive patients, both subgroups showed significantly increased ET-1 concentrations (p <0.05 for NIT-RVOs, and <0.01 for IT RVOs, respectively). In addition, ET-1 plasma levels were significantly higher in the IT RVO than in the NIT RVO subgroup (p <0.05).

To determine if during the first days following the occlusion ET-1 levels were particularly elevated, we plotted ET-1 concentrations as a function of the time elapsed between the occlusive event and blood sample collection. A wide scatter of the data was observed (data not shown), with no detectable correlation between these two variables (R = 0.041; p = 0.88). In addition, we plotted ET-1 levels as a function of the age of the RVO patients (data not shown). Also in this case no significant correlation could be observed (R = 0.289; p = 0.245), thus excluding that ET-1 increases may have reflected age related factors.

We also subdivided the RVO patients listed in Table 1 based on the presence or absence of systemic hypertension and elevated IOP, respectively, to evaluate whether these variables could account for some of the observed differences in the RVO patients. Although systemic hypertensive patients (n = 11; mean age 65.8 (6.1) years) with RVO showed slightly higher mean ET-1 concentrations (15.37 (4.7) pg/ml) than normotensive RVO subjects (12.40 (4.1) pg/ml; n = 7; mean age 60.7 (10.6) years), this difference was not statistically significant (p >0.05). Both subgroups, however, had significantly higher ET-1 concentrations than those found in uncomplicated hypertensive patients (p <0.05). Similarly, no statistically significant difference was observed when comparing patients with pre-existing IOP greater than 20 mm Hg (n=7; mean age 63.9 (8.9) years) with those without ocular hypertension (n=9; mean age 63.1 (9.1) years), although the former had slightly higher ET-1 plasma concentrations (15.9 (6.4) *v* 13.0 (3.2) pg/ml). Patients 13 and 14, in whom there was only indirect evidence that ocular hypertension may have been a factor in determining the RVO, were not included in this analysis. None the less, their ET-1 plasma levels were not different from the general mean for RVO patients or from the mean of the ocular hypertensive patients.

ET-1 concentrations in the subgroups of patients with and without systemic hypertension and with or without elevated IOP were significantly higher (p < 0.05) compared with both group B (normotensive controls) and group C (uncomplicated systemic hypertensives), respectively.

Discussion

Findings from our investigation indicate that patients with RVO have increased circulating levels of ET-1. To the best of our knowledge, this investigation is the first to demonstrate that elevation of plasma ET-1 concentrations can also occur in association with disorders

limited to small vessels, such as those in the retina, and not only for systemic conditions such as congestive heart failure,[30] acute myocardial infarction,[31] and non-insulin dependent diabetes mellitus,[32 33] that have all been found to be associated with increased ET-1 plasma levels.

Systemic hypertension, which was present in 11 of the 18 RVO patients, was not a factor in determining the observed increase in ET-1 concentrations because (a) the control group of uncomplicated hypertensive patients had ET-1 concentrations superimposable on normal values (8.50 (2.9) *v* 7.90 (1.6) pg/ml; $p > 0.05$); (b) RVO patients showed, on average, ET-1 plasma levels (group A, 14.22 (4.6) pg/ml) significantly higher than those of normal subjects and uncomplicated hypertensives, respectively ($p < 0.05$ in both cases); and (c) ET-1 levels in normotensive RVO patients (12.40 (4.1) pg/ml) were significantly higher than in patients with uncomplicated hypertension ($p < 0.05$). These findings confirm Lerman and others' observations that ET-1 levels do not increase in systemic hypertension unless in the presence of advanced atherosclerotic organ damage.[39]

Presence of elevated IOP before the RVO, observed in seven patients, did not contribute significantly to the observed ET-1 increases, and no correlation was observed between age and the ET-1 levels in the RVO group, thereby also excluding that ET-1 concentrations may have varied as a function of age. This observation is consistent with both our previous experience (unpublished observations) and previous studies,[30 39] that clearly indicated that ET-1 plasma levels do not vary significantly with age.

The small size of the RVO subgroups identified on clinical grounds precludes any certain conclusion to be drawn about the differences in plasma ET-1 levels observed between them. However, some considerations of potential diagnostic and prognostic relevance are possible. Compared with both normal subjects and uncomplicated hypertensives, NIT RVO patients had significantly elevated ET-1 concentrations ($p < 0.05$). This is consistent with the notion that NIT RVO entails the presence of endothelial damage with venous stasis and ongoing enhanced thrombin release, all conditions that have been extensively documented to stimulate ET-1 production.[40–43] Another well known ET-1 release enhancing factor is hypoxia.[44–47] Of note, when hypoxia coexisted with venous stasis and thrombogenesis as in IT RVOs, ET-1 concentrations were significantly higher compared with normals ($p < 0.01$), with uncomplicated hypertensives ($p < 0.01$), and with NIT RVOs ($p < 0.05$), respectively. These findings raise the intriguing possibility that hypoxia, venous stasis, and thrombogenesis may have had an additive effect, resulting in an enhanced ET-1 production, and perhaps accounting for the differences observed between the clinically distinct subgroups.

To minimise the possibility that elevated ET-1 concentrations may had been present in some cases before the occlusive event, RVO patients with conditions known to be associated with elevated ET-1 plasma levels were excluded at time of enrolment (see exclusion criteria in the Methods section). Only two of the selected RVO patients had hypercholesterolaemia, and no other relevant systemic conditions were present in the study group. Therefore, it seems unlikely that the observed elevated ET-1 levels were a mere result of preexisting advanced systemic atherosclerosis or generalised ischaemia.

Elevated ET-1 levels are a well known marker of vascular endothelial dysfunction.[30–33 39] It is possible that, at least in some patients, increased basal ET-1 circulating levels could directly promote or favour vascular retinal occlusions, or otherwise reflect a baseline condition predisposing to RVO. However, findings from our study better fit a scenario in which the ET-1 plasma increase may represent a marker of the occlusion, possibly as a "spillover" phenomenon from a locally activated system.[41] This possibility is supported by several lines of evidence. Firstly, the normal plasma concentrations of ET-1 are very low (nearly 8 pg/ml according to our methods—that is, 8×10^{-12} g/ml, equal to 2.4×10^{-8} g in 3000 ml of circulating plasma), so that also relatively small vascular accidents can determine major concentration shifts. In addition, elevated expression of ET-1 receptor genes has been observed only at the sites of hypoxia and not in the remainder of the systemic vascular bed, despite increased ET-1 plasma levels.[47] Therefore, the presence of peripherally detectable ET-1 increases would not argue against the hypothesis of a local retinal effect, nor would predict a generalised systemic vascular remodelling. Finally, the quality of the vascular accident appears to be a greater determinant of ET-1 plasma concentrations than its size. This concept has been clearly illustrated by Stewart and co-workers,[31] who documented that ET-1 levels in patients with myocardial infarction are not related to the infarction size, but rather to its potential for more severe sequelae.

A potentially important role of ET-1 in retinal vascular disease has been proposed in recent experimental studies. Investigators have demonstrated that retinal capillary nonperfusion and closure of retinal arterioles ensues following intravitreal ET-1 injections,[19–21] and that ET-1 may contribute significantly to retinal vascular abnormalities in diabetic rats,[22 23] thereby suggesting that ET-1 may play a role in retinal ischaemia. In addition, in a recent study on experimental BRVO it has been demonstrated that secondary arteriolar constriction in the territory of the occluded vein is associated with decreased nitric oxide production, but an associated role of ET-1 could not be excluded.[48] Therefore, a local imbalance in the ET-1-nitric oxide system could play a role also in RVO pathogenesis. Our findings support the possibility that ET-1 may be an important factor in RVO pathophysiology, and may explain, at least in part, the efficacy reported in a clinical trial in treating RVOs with defibrotide,[49] a drug that

antagonises ET-1 effects by releasing prostacyclin.[50]

In conclusion, this investigation demonstrated for the first time in patients with RVOs an elevation of ET-1 plasma concentrations, which was more pronounced in patients with occlusions of the ischaemic type. The observed ET-1 increases could not be accounted for by systemic hypertension or other coexisting atherosclerotic or ischaemic conditions. The effects of ET-1 on the ocular microcirculation are certainly protean, and have yet to be completely unravelled, and so are its interactions with other vasoactive factors. To date, the mechanism(s) by which ET-1 could play a role in determining or favouring some of the clinical manifestations of RVOs remain a matter of speculation. Further investigations on larger patient populations and animal models may help clarify this issue, and establish if plasma ET-1 level determinations can be used either as an additional diagnostic variable or as a prognostic criterion in the follow up of patient with RVOs.

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