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Original Article

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Endothelin-1 immunoreactivity in plasma is elevated in HIV-1 infected patients with retinal microangiopathic syndrome

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Abstract. Endothelin-1 is a recently identified cytokine with potent vasoconstrictor activity which is associated with various diseases involving blood vessels. HIV-1 related retinal microangiopathic syndrome is a frequent finding in patients with AIDS or AIDS-related complex, presenting predominantly with retinal cotton-wool spots. We investigated 55 HIV-1 infected patients by ophthalmoscopy and for endothelin-1 immunoreactivity in plasma and an additional 76 HIV-1 infected patients only for endothelin-1 levels. For reference values 13 age-matched healthy subjects were studied. In 18 of 55 patients (33%) investigated ophthalmoscopically we found evidence of microangiopathic syndrome. Overall, the mean endothelin-1 immunoreactivity in plasma of HIV-1 infected patients was significantly elevated as compared to controls (4.28 ± 3.62 versus 2.72 ± 0.67 fmol/ml, $P < 0.0001$). HIV-1 infected patients with retinal microangiopathic syndrome had significantly higher plasma levels of endothelin-1 immunoreactivity (4.59 ± 1.38 fmol/ml) compared to HIV-1 infected patients without microangiopathic syndrome (3.18 ± 1.64 fmol/ml, $P = 0.003$). Correlation analysis revealed that endothelin-1 immunoreactivity in plasma had no significant association with disease progression, CD4 cell count, β_2-microglobulin, neopterin, or age. Endothelin-1 immunoreactivity in plasma was correlated exclusively with retinal microangiopathic syndrome in one or both eyes ($r = 0.45$, $P = 0.0006$) and with the number of cotton-wool spots ($r = 0.50$, $P = 0.0001$). In conlusion, HIV-1 related retinal microangiopathic syndrome is associated with elevated plasma levels of endothelin-1. By virtue of its potent vasoconstrictor activity endothelin-1 may be involved in the pathogenesis of HIV-1 related vascular disease.

Key words: HIV-1 – Endothelins – Endothelin-1 – Cytokine – Retinal microangiopathic syndrome – Vascular disease

Endothelins are a family of potent vasoconstrictor peptides, originally isolated from porcine aortic endothelial cells [23]. They have subsequently been found to be produced by a number of other cell types. Since their identification in monocytes/macrophages and mast cells, they have been considered to be cytokines [1, 3]. To date three distinct forms (endothelin-1, −2, −3) have been discovered, but only endothelin-1 (ET-1) seems to occur in substantial amounts in human plasma. Although data on plasma endothelin concentrations are not unequivocal, elevated levels of ET-1 immunoreactivity (ET-1 IR) have been detected in various diseases involving blood vessels, especially in myocardial infarction [20], vasospastic angina [22], hypertension [15, 21], subarachnoid hemorrhage [4, 16], and diabetes mellitus [13]. These elevated plasma levels together with the known potent and long-lasting vasoconstrictor action may argue for a role of ET-1 in the pathophysiology of a number of vascular conditions.

Vascular involvement seems to be a frequent phenomenon in HIV-1 infection. Microangiopathic changes of retinal vessels similar to those seen in other diseases such as diabetes, leukemia, and malignant hypertension are the most frequent ocular finding in patients with acquired immunodeficiency syndrome (AIDS). This microangiopathic syndrome presents in 40–60% of these patients with cotton-wool spots or other microvascular lesions such as hemorrhages and Roth's spots [12, 17]. Furthermore, clinical and pathological observations

Abbreviations: AIDS = aquired immunodeficiency syndrome; ET-1 = endothelin-1; HIV = human immunodeficiency virus; IR = immunoreactivity; WR = Walter Reed classification

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point to an involvement of cerebral vessels in human immunodeficiency virus type 1 (HIV-1) related neurological disease [6, 14, 18]. Recently we demonstrated a close association of ocular microangiopathic syndrome and cerebral blood flow using hexamethyl-propylene amine oxime single photon emission computed tomography [9]. In addition, we found a close association between HIV-1-related ocular microangiopathy and cognitive deficits, especially impairment of short-term memory [10].

In the present study we investigated ET-1 IR in plasma of HIV-1 infected patients with and without retinal microangiopathic syndrome to elucidate a possible role of endothelins in these vascular disturbances in HIV-1 infection.

Patients and methods

Patients

Fifty-five HIV-1 infected patients were investigated by ophthalmoscopy and for ET-1 IR in plasma after informed consent. To further study the role of endothelin in HIV-1 infection we determined ET-1 IR in an additional 76 HIV-1 infected patients which could not be seen by an ophtalmologist. Blood samples were usually taken with the patient in supine position and after a 10-min rest. Patients with hypertension or diabetes mellitus were excluded from the study. Patients suffering from acute opportunistic infections, especially cytomegalovirus, retinitis were also excluded. Renal function as evaluated by routine laboratory tests was normal in the 131 patients studied. Distribution of these patients to risk groups was as follows: 61.8% of the patients were homosexual, 12.2% intravenous drug users, 9.9% heterosexual, and 2.3% were recipients of blood products. In 13.7% the route of transmission remained unknown. Most of the patients (89.3%) were male. Mean age was 41.2 $\pm$ 10.2 years. Staging was performed according to the Walter Reed (WR) classification system, with 20.7% of the patients being in WR 1 or 2, 25.0% in WR 3 or 4, and 54.3% in WR 5 or 6. Reference values for ET-1 IR in plasma were obtained from 13 healthy volunteers (9 males, 4 females). Mean age of these subjects was 30.0 + 3.3 years.

Ophthalmological investigations

Fundus examination was performed by indirect ophthalmoscopy after dilatation of the pupils. A Nikon 14 dpt lens was used for posterior pole and peripheral retina examination. Additionally, the outer retinal periphery was examined using a Nikon 20 dpt lens, and, if necessary, examination was extended with a Volk 78 dpt lens at the slit lamp. The criterion for the diagnosis of ocular microangiopathic syndrome was: cotton-wool spots, hemorrhages, or Roth-spots. Patients were classified into three groups: group I, no microangiopathic changes; group II, one eye showing at least one of the described criteria; and group III, both eyes showing at least one of the described criteria. As an indicator of the severity of the ocular microangiopathic syndrome, the number of cotton-wool spots in both eyes was counted.

Extraction procedures and assays

For ET-1 IR determination blood was drawn into EDTA-containing vacutainer tubes (Greiner, Frickenhausen, Germany) without other additives, placed immediately on ice, and centrifuged at 4°C. Plasma was then stored until analysis at -20°C. Sample analysis was performed blinded to the clinical and ophthalmoscopic findings. Endothelin was extracted and concentrated from plasma as described [19]. In brief, 2.5 ml plasma was applied to the preconditioned column (Sepak C18 Vac 500 mg, Waters, obtained from Millipore, Eschborn, Germany) and washed with 2.5 ml 10% acetic acid and 5 ml ethylacetate. ET-1 was then eluted with 3 ml methanol/0.05 M ammonium bicarbonate (80/20, v/v). The eluate was dried under reduced pressure und reconstituted in radioimmunoassay buffer. Extraction recovery of ET-1 from plasma as determined by the use of a 125 J labeled ET-1 (Amersham, Braunschweig, Germany) was 87–92%, independently of the concentrations tested (1 fmol/ml–100 pmol/ml). The extraction procedure reduced the average protein content of plasma (70 g/l) to 50 mg/l in the eluate and osmolarity from 330 mosm to 10 mosm. Triglycerides and cholesterol were not detectable in the eluate (< 2 mg/dl). Therefore the sample clean-up described resulted in minimal matrix interferences in the eluate compared to other extraction procedures tested [19]. HPLC analysis of plasma samples spiked with pure ET-1 (Serva, Heidelberg, Germany) revealed that ET-1 was stable ($> 95\%$) throughout the extraction process.

Determination of ET-1 IR in the extracted samples was performed using a radioimmunoassay for ET-1 obtained from NEN/Dupont (Dreieich, Germany). Performance of the assay was checked by extracting standard curves prepared with pure ET-1 from different suppliers spiked in human pool plasma or in radioimmunoassay buffer. Detection

limit of the assay was 0.1 fmol/ml. The level of lowest reliable quantification (defined as B < Bo −3 SD) was 0.52 fmol/ml. Determination of ET-1 was linear from 1 to 50 fmol/ml. Intra-assay variability for extracted samples was between 9.3% for 1 fmol/ml added and 8% for 25 fmol/ml added ($n = 6$ for each concentration). Interassay variability was 13.4% and 11.2%, respectively. All determinations of ET-1 IR in plasma were performed in duplicate.

Neopterin in serum was measured by a radioimmunoassay (Henning, Berlin, Germany), and β_2-microglobulin by an enzyme-linked immunosorbent assay (Behring, Marburg, Germany). CD4-positive cells were determined by flow cytometry. All chemicals used were of reagent grade or better. For all analytical procedures regarding ET-1 only polypropylene tubes were used.

Statistical analysis

Statistical calculations were performed using the SPSS software package (SPSS/PC 4.0, Chicago, USA). Tests for statistical significance (two-tailed) were performed with Student's t test, χ^2 test, and analysis of variance. Correlations were calculated by Pearson's or Spearman's correlation coefficient. Data are expressed as mean values ± standard deviation. In patients investigated repeatedly, only the most recent values were taken for statistical analysis.

Results

Of the 55 patients investigated by ophthalmoscopy 18 (33%) had evidence of microangiopathic syndrome. In 6 patients only one eye and in 12 patients both eyes were affected. In 2 patients retinal hemorrhages were observed, but no cotton-wool spots were detectable; in 9 patients 1–3, in 3 patients 4–6, and in 4 patients more than 6 cotton-wool spots were counted. A typical ophthalmoscopic picture in a severly affected patient is shown in Fig. 1.

The mean ET-1 IR in plasma of HIV-1 infected patients was significantly elevated compared to healthy controls (4.28 ± 3.62 versus 2.72 ± 0.67 fmol/ml, $P < 0.0001$). Patients with retinal microangiopathic syndrome had significantly higher plasma levels of ET-1 IR (4.59 ± 1.38 fmol/ml, $n = 18$) compared to patients without microangiopathic syndrome (3.18 ± 1.64 fmol/ml, $n = 37$, $p = 0.003$). Analysis of variance revealed a significant difference in ET-1 IR between patients without microangiopathic syndrome (3.18 ± 1.64 fmol/ml) and patients with monocular (3.69 ± 1.52 fmol/ml, $n = 6$) or with binocular (5.03 ± 1.12 fmol/ml, $n = 12$) microangiopathy ($P = 0.0026$; Fig. 2). In addition, we found a significant correlation (Spearman's correlation coefficient) between ET-1 IR in plasma and microangiopathic syndrome in one or both eyes ($r = 0.45$, $P = 0.0006$) and with the number of cotton-wool spots ($r = 0.50$, $P = 0.0001$).

In contrast, there was no correlation of ET-1 IR in plasma with the risk group, WR stages, CD4

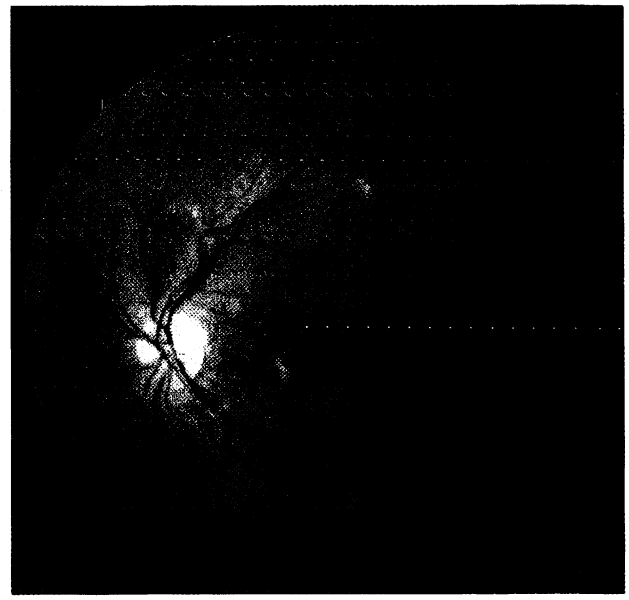

Fig. 1. HIV-1 related microangiopathic syndrome of the retina with 16 cotton-wool spots visible on ophthalmoscopy. These feather-edged fluffy white lesions are typically distributed near the optic disc along the major retinal vessels and usually less than one-quarter optic nerve disc diameter in size

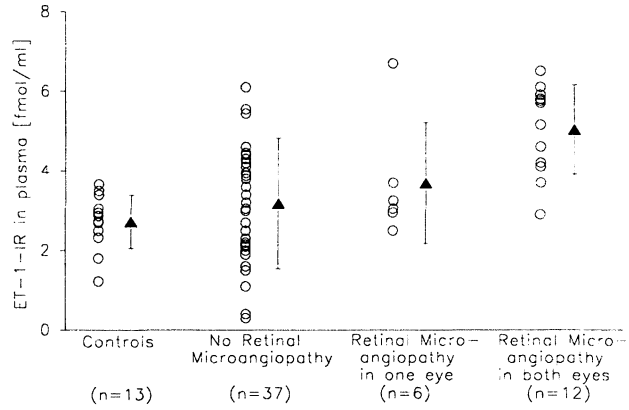

Fig. 2. ET-1 IR in plasma is elevated in HIV-1 infected patients with retinal microangiopathic syndrome. Scattergram of the values and mean ± standard deviations. ET-1 IR is significantly elevated in HIV-1 infected patients compared to controls. Analysis of variance reveals significant differences in ET-1 IR among patients without microangiopathic syndrome or with monocular or with binocular involvement ($P = 0.0026$)

Table 1. Findings in HIV-1 infected patients in advanced disease states (WR 5 or 6) without, with monocular, and with binocular retinal microangiopathic syndrome

	No microangiopathic change ($n=21$)	Microangiopathic syndrome in one eye ($n=6$)	Microangiopathic syndrome in both eyes ($n=12$)
ET-1 IR (fmol/ml)	3.00 ± 1.55	3.69 ± 1.52	5.03 ± 1.12
CD4 (cells/μl)	148 ± 145	144 ± 131	49 ± 66
Hemoglobin (g/dl)	12.1 ± 2.4	11.2 ± 2.8	11.4 ± 1.6
β_2-Microglobulin (mg/l)	4.5 ± 1.0	4.6 ± 1.3	6.4 ± 3.1
Neopterin (nmol/l)	32.1 ± 34.9	31.8 ± 19.2	70.3 ± 101.7

Analysis of variance revealed a significant increase in ET-1 IR ($P=0.0015$) whereas the other parameters were not significantly different between the tree groups

count, β_2-microglobulin, neopterin, hemoglobin level, or zidovudine therapy at the time of the study. This was true for the 55 patients investigated by ophtalmoscopy as well as for the total number of patients (n = 131). Furthermore, there was no correlation between ET-1 IR and age or sex either in the healthy or in the HIV-1 infected subjects.

Correlation analysis revealed a significant (P = 0.027) association of microangiopathic syndrome and disease progression. It is therefore important to note that all patients with microangiopathic syndrome were in advanced disease states, i.e., WR 5 or 6. In contrast, only 21 (56.8%) of the 37 patients without microangiopathic changes were staged WR 5 or 6. Thus patients with microangiopathic syndrome had significantly lower CD4 counts (85 ± 103 versus 206 ± 153 cells/μl, P = 0.014) compared to patients without microangiopathic syndrome. In addition, there was a trend to higher levels of β_2-microglobulin (5.8 ± 2.5 versus 4.7 ± 4.2 mg/l) and neopterin (58.3 ± 85.6 versus 23.7 ± 28.3 nmol/l) in these patients, although these differences were not statistically significant. However, when comparing patients with microangiopathic syndrome to patients without microangiopathic syndrome but also in WR stages 5 or 6 (n = 21; Table 1) we no longer found significant differences in CD4 counts but still significantly higher ET-1 IR in the former group (4.59 ± 1.38 versus 3.00 ± 1.55 fmol/ml, P = 0.002). Moreover, patients with or without microangiopathic syndrome showed no significant difference with respect to age, hemoglobin level, or zidovudine therapy at the time of the study (Table 1).

We investigated 21 patients several times over a period of 2–4 months by ophthalmoscopy and for ET-1 IR in plasma. Five of these patients had microangiopathic syndrome, whereas 16 had normal ophthalmoscopic findings. Except for one patient who improved with respect to his funduscopic findings, all remained unchanged with regard to their ophthalmological state. In these patients ET-1 levels remained nearly constant over time. Mean ET-1 levels in 11 plasma samples obtained from 5 patients with microangiopathic syndrome were 4.51 ± 1.37 fmol/ml as compared to 3.12 ± 1.42 fmol/ml in 36 plasma samples from 16 patients with normal ophtalmologic findings (P < 0.0001).

Discussion

Our study demonstrates that ET-1 IR in plasma of HIV-1 infected patients exceeds that of healthy subjects. Moreover, in HIV-1 infected patients with microangiopathic syndrome of retinal vessels there is a significantly higher level of ET-1 IR in plasma compared to patients in the same disease stage but without microangiopathic changes.

Microangiopathic syndrome of retinal vessels in HIV-1 infection seems to be a distinct vascular disease [12, 17]. The pathogenetic mechanism underlying retinal microangiopathic syndrome in HIV-1 infection is believed to be related to ischemia, and cotton-wool spots are interpreted as the clinically visible result of ischemic microinfarcts in the superficial layer of the retina [12, 17]. Retinal vessels often reflect vascular disease in other organs. In addition to our previous finding that cerebral blood flow is reduced in HIV-1 infected patients with retinal microangiopathy [9], there is a report on a possible connection between proteinuria and retinal microangiopathic syndrome in patients with AIDS-related complex [7].

ET-1 apparently acts in a paracrine/autocrine way rather than systemically [2, 11]. Nevertheless, high local levels of this peptide appear to be reflected by increased levels in the peripheral circulation. ET-1 exerts its vasoconstrictor activity predominantly from the the adventitial side of intact vessels.

It is therefore of interest that, among other cell types, ET-1 is produced by human monocytes/macrophages [1]. Monocytes/macrophages are target cells of HIV-1, and infection leads to an enhanced penetration of these cells into perivascular tissue and increased production of cytokines. Monocyte derived cytokines, for example, tumor necrosis factor-α or interleukin-6, have been shown to be mediators of HIV-1 related tissue damage. Recently we demonstrated enhanced transcription of the ET-1 gene in monocytes isolated from peripheral blood of HIV-1 seropositive individuals, which cannot be observed in healthy controls [5]. However, no data are yet available on ET-1 plasma levels in HIV-1 infected patients.

In the present study we found significantly elevated levels of circulating ET-1 IR in HIV-1 infected individuals compared to healthy controls. The source of plasma ET-1 IR in HIV-1 infection remains to be elucidated. Endothelial cells is one possible source of circulating ET-1. In addition, monocytes have been shown to produce high levels of ET-1 upon stimulation in vitro [1]. However, ET-1 IR in plasma did not significantly correlate with β_2-microglobulin or neopterin, two other substances produced and released by monocytes upon stimulation.

As in this study, other investigators have shown that microangiopathic syndrome develops predominantly in advanced stages of HIV-1 infection [7, 8]. Interestingly, however, there was a distinct difference in ET-1 IR levels between patients in advanced disease states with and without microangiopathic syndrome. This suggests that ET-1 elevation is related to vascular disease rather than to advanced disease state in general.

Some of our patients were investigated several times within a few months. Patients with microangiopathic syndrome had constantly higher ET-1 IR levels than patients with normal ophthalmological findings, suggesting a reliable association between plasma ET-1 IR and microangiopathic syndrome.

In conclusion, the results of our study argue for an involvement of ET-1 in the pathophysiology of HIV-1 related microangiopathic syndrome. By virtue of its strong vasoconstrictor action it may contribute to ischemic damage in this condition. The availability of endothelin antagonists for clinical use in the near future may help to elucidate the functional significance of ET-1 in HIV-1 related retinal microangiopathic syndrome and may open therapeutic avenues.

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