Endothelin-1 release from cultured endothelial cells induced by sera from patients with systemic lupus erythematosus

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Abstract

Objectives—To clarify the pathophysiological role of endothelin-1 (ET-1) in the vascular injury associated with systemic lupus erythematosus (SLE) by investigating the effect of sera from patients with SLE on ET-1 release from cultured human umbilical vein endothelial cells.

Methods—Confluent monolayers of cultured human umbilical vein endothelial cells were incubated with serum samples (diluted 1:10) from 25 patients with SLE and 16 normal controls for two hours at 37°C and ET-1 concentration in the culture supernatant was measured by enzyme immunoassay.

Results—The mean release of ET-1 from endothelial cells in the presence of serum from SLE patients was greater than in the presence of serum from normal controls (p < 0.005). ET-1 release from endothelial cells significantly correlated with the titre of IgM anti-endothelial cell antibodies (IgM-AECA) and immune complex concentration in sera from SLE patients (p < 0.05 and p < 0.01, respectively). After gel chromatography of the serum from an SLE patient, those fractions containing IgM-AECA or immune complex were shown to stimulate ET-1 release from endothelial cells. Heat aggregated IgG also stimulated ET-1 release from endothelial cells in a concentration dependent manner.

Conclusions—IgM-AECA and immune complexes may stimulate ET-1 release from endothelial cells and ET-1 may play an important role in the initiation and development of vascular injury, such as pulmonary hypertension and lupus nephritis, in SLE.

Endothelin-1 (ET-1), a 21 amino acid polypeptide with two intramolecular disulphide bonds, is produced by endothelial cells and induces sustained vasoconstriction in vitro and a long lasting increase in blood pressure in vivo.1 In addition, ET-1 stimulates the proliferation and contraction of vascular smooth muscle cells and mesangial cells.2,3 Such actions may have an important pathophysiological role in the initiation and development of vascular injury such as lupus nephritis and pulmonary hypertension in patients with systemic lupus erythematosus (SLE). Indeed, high plasma concentrations of ET-1 have been demonstrated in patients with SLE and were shown to be significantly correlated with a history of nephritis.4 A significant increase in plasma ET-1 concentration has also been observed in patients with Raynaud's phenomenon, including patients with SLE, immediately after immersion of their hands in cold water.5 Moreover, the concentration of ET-1 in arterial plasma is greater than that in venous plasma in patients with primary pulmonary hypertension, and it has been suggested that pulmonary production of ET-1 may contribute to increased pulmonary vascular resistance.6 Pulmonary hypertension in SLE usually resembles that seen in the primary idiopathic type of pulmonary hypertension, with a clear lung field and no evidence of pulmonary thromboembolism.7 To clarify the pathophysiological role of ET-1 in the vascular injury associated with SLE, we have investigated the effects of sera from SLE patients on the release of ET-1 from cultured human umbilical vein endothelial cells.

Patients and methods

PATIENTS AND SERUM SAMPLES

Serum samples were collected from 25 patients with SLE (24 females, one male; mean age 32.1 (SD 10.8) years, range 16–61 years), all of whom fulfilled the American College of Rheumatology revised criteria for the diagnosis of SLE,8 and from 16 healthy matched control subjects (15 females, one male; mean age 32.8 (11.5) years, range 15–60 years). Serum samples were stored at −20°C until required for use.

CELLS

Endothelial cells were prepared from fresh human umbilical veins and cultured as previously described.9 Cells were passaged serially by brief exposure to 0.25% trypsin (Difco, Detroit, MI) and 0.04% EDTA (Sigma, St Louis, MO). Only cells from the second or third passage were used. The cells were positive for von Willebrand factor.

Different preparations of the cells were exposed to the following three test samples to evaluate the effect of the samples on release of ET-1: whole sera from SLE and control subjects; fractions of SLE and control sera; and monomeric and heat aggregated IgG. The various cell preparations were also assayed for...
release of ET-1 in the presence of phosphate buffered saline (PBS) alone.

ET-1 RELEASE FROM ENDOTHELIAL CELLS
Cells (1.5 × 10^5/well) were seeded in 48 well flat bottom tissue culture plates (Costar Corporation, Cambridge, MA) that had been coated with 5% gelatin (Sigma). The cells formed a confluent monolayer after two to three days in culture, after which the medium was removed and the cells washed twice with medium 199 (Nissui, Tokyo, Japan).

Test samples were prepared in three different volumes for use in separate studies: 250 μl samples of serum fractions; 500 μl samples of whole serum diluted 1:10 in PBS; and 1 ml samples of monomeric or heat aggregated IgG diluted serially in PBS. The samples were added to each well, and the cells incubated for two hours at 37°C. The supernatant in each well was then collected and stored at −20°C until required for assay of ET-1. After removal of the supernatant, the cell monolayer remained confluent. All samples were tested in triplicate, and the results were averaged.

The concentration of ET-1 was measured in cell supernatants by the enzyme immunoassay developed by Suzuki et al. The assay, which uses two different antibodies for capture and detection of ET-1, has a lower limit of detection of 0.4 pg/ml of ET-1, and does not cross react with big ET-1 or ET-3.

FRACTIONATION OF SERA
Serum from one normal control that did not significantly stimulate ET-1 release from endothelial cells and serum from one SLE patient that did significantly stimulate ET-1 release from endothelial cells were fractionated in an attempt to identify the components responsible for stimulation of ET-1 release. The serum samples (1 ml) were applied to a Sephacryl S-200 (Pharmacia, Woerden, The Netherlands) column (1.5 × 120 cm) that had been equilibrated in 0.01 mol/l PBS (pH 7.2). The fractions (2 ml) were subsequently eluted with PBS and assayed for protein, IgG, IgA and IgM concentrations, IgG- and IgM-AECA titre, immune complex concentration, and ability to stimulate ET-1 release from endothelial cells. The IgG, IgA and IgM concentrations in each fraction were measured by radial immunodiffusion.

PREPARATION OF HEAT AGGREGATED IgG
Heat aggregated IgG was prepared before each experiment by heating monomeric IgG isolated from normal human serum (Sigma) at 63°C for 20 minutes, followed by centrifugation at 3000 g for 15 minutes to remove insoluble aggregates.

ENZYME IMMUNOASSAY FOR ANTI-ENDOTHELIAL CELL ANTIBODIES
IgG and IgM anti-endothelial cell antibody (IgG- and IgM-AECA) titres of the cultured human umbilical vein endothelial cells were measured by enzyme immunoassay as previously described.

ENZYME IMMUNOASSAY FOR IgG ANTI-CARDIOLIPIN ANTIBODIES
IgG anti-cardiolipin antibodies (IgG-ACA) in the sera of SLE patients were measured with an enzyme immunoassay kit (Yamasa, Tokyo, Japan) in the presence or absence of the anticardiolipin cofactor (β2-glycoprotein I). The kit uses high titre IgG-ACA positive serum as standard.

MEASUREMENT OF ANTI-DNA ANTIBODY TITRES
Anti-DNA antibody titres were measured using the Farr assay. This method used 125I-labelled recombinant double stranded DNA (Amersham International plc, Buckinghamshire, England), which is free from contamination with single stranded DNA. The assay was performed according to the manufacturer’s instructions.

STATISTICAL ANALYSIS
Statistical comparisons between groups were made with the Mann-Whitney U test. Correlation of ET-1 release from endothelial cells with serum AECA titre, IgG-ACA titre, immune complex concentration or anti-DNA antibody titres was assessed by Spearman’s rank correlation.

Results
EFFECT ON ET-1 RELEASE OF PBS ALONE
The endothelial cell preparation used in the evaluation of the effects of monomeric and heat aggregated IgG on ET-1 was found to release 20-0 pg/1×10^5 cells when incubated in the presence of PBS alone. The two used in the evaluation of both whole and fractionated sera from SLE patients and normal controls each released 34-0 pg/1×10^5 cells in the presence of PBS alone.

EFFECT OF SERUM FROM NORMAL CONTROLS AND SLE PATIENTS
ET-1 release from endothelial cells in the presence of serum from SLE patients (88-6 (17-9) pg/1×10^5 cells) was significantly greater (p<0.005) than that in the presence of serum from normal controls (72-6 (7-2) pg/1×10^5 cells) (fig 1). The release of ET-1 in the presence of serum from 15 of the 25 patients with SLE was greater than the mean +2SD for normal controls (86-9 pg/1×10^5 cells).
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Figure 1  Release of endothelin-1 (ET-1) from endothelial cells in the presence of serum from normal controls and SLE patients. Bars represent mean ± SD. Horizontal dashed line represents the mean ± 2SD release in the presence of serum from normal controls (86.9 pg/1.5×10⁶ cells).

Figure 2  Correlation between release of endothelin-1 (ET-1) from endothelial cells in the presence of serum from SLE patients and serum IgM-AECA titre (r = 0.472, n = 24, p < 0.05).

Figure 3  Correlation between release of endothelin-1 (ET-1) from endothelial cells in the presence of serum from SLE patients and serum immune complex concentration (r = 0.606, n = 23, p < 0.01).

**Correlation between ET-1 release from endothelial cells and serum AECA titre, IgG-AECA titre, anti-DNA antibody titre or immune complex concentration**

There was no significant correlation between the extent of ET-1 release from endothelial cells in the presence of serum from SLE patients and IgG-AECA titre (r = 0.035, n = 24), IgG-AECA titre in the presence (r = 0.158, n = 23) or absence (r = 0.200, n = 23) of the antcardiolipin cofactor, or anti-DNA antibody titre (r = 0.397, n = 22). However, ET-1 release was significantly correlated with IgM-AECA titre (r = 0.472, n = 24, p < 0.05) (fig 2), and with immune complex concentration (r = 0.606, n = 23, p < 0.01) (fig 3).

**Effects of fractions of normal control and SLE patient serum on ET-1 release**

The elution profile of IgM-AECA for the normal serum sample showed a peak titre of 0.3 U/ml, which corresponded to the peak of IgM (fig 4). Neither IgG-AECA nor immune complexes were detected. Significant stimulation of ET-1 release from endothelial cells was not observed with any of the fractions of the normal control serum. The elution profile of IgM-AECA for the SLE patient serum sample showed a peak titre of 1.0 U/ml, which corresponded to the peak of IgM (fig 5). Two small peaks of IgG-AECA were detected, one each side of the IgG peak. Immune complex concentration increased in the first half of the IgG peak. Two large peaks of increased ET-1 release from endothelial cells corresponded to the IgM-AECA peak and the first half of the immune complex peak.

**Effects of monomeric IgG and heat aggregated IgG on ET-1 release**

Heat aggregated IgG, but not monomeric IgG, stimulated ET-1 release from endothelial cells in a concentration dependent manner (fig 6).

**Discussion**

Serum from normal controls stimulated release of ET-1 from cultured endothelial cells. It has been reported that ET-1 production is stimulated by adrenaline, angiotensin II, arginine vasopressin, transforming growth factor β, thrombin, and interleukin-1. Our results suggest that serum from normal control subjects may contain such stimulating factors, or others which remain unknown, which induce a release of ET-1 from cultured endothelial cells which is greater than that caused by PBS. Furthermore, serum from patients with SLE stimulated a significantly greater release of ET-1 from cultured endothelial cells compared with the normal controls.

ACA are frequently present in patients with SLE and are associated with clinical episodes
of thromboembolic manifestations in some of these patients.\textsuperscript{18} Indeed, it has been suggested that these antibodies may bind to phospholipids on the surface of endothelial cells and cause the thromboembolic episodes.\textsuperscript{19} However, the extent of ET-1 release from cultured endothelial cells induced by serum from SLE patients was not correlated with IgG-ACA titre. It has been reported that anti-DNA antibodies bind to the endothelial cells through DNA,\textsuperscript{20} and we investigated whether anti-DNA antibodies bind to the endothelial cells and stimulate them to release ET-1 from endothelial cells. However, the amount of ET-1 release from cultured endothelial cells induced by serum from SLE patients was not correlated with anti-DNA antibody titre but, in contrast, it correlated significantly with the IgM-AECA titre and immune complex concentration in serum from those patients. Furthermore, those fractions of serum from an SLE patient which contained IgM-AECA or immune complexes were found to stimulate ET-1 release. Heat aggregated IgG also stimulated ET-1 release from endothelial cells, in a concentration dependent manner. These results suggest that IgM-AECA or immune complexes may stimulate ET-1 release from endothelial cells in SLE patients. However, the fractions containing IgM-AECA from a normal control subject did not stimulate ET-1 release. It has been reported that AECA in sera of patients with SLE react with a wide spectrum of molecules with different molecular weights,\textsuperscript{21} so this difference of ET-1 release by IgM-AECA between an SLE patient and a normal control may be due to different endothelial antigens recognised by IgM-AECA.

We also compared the mean extent of ET-1 release from cultured endothelial cells in the presence of sera from patients with and without lupus nephritis, but detected no significant difference between these groups of patients (data not shown). Deposits of IgG, IgM, and complement component C3 along glomerular capillary loops, and glomerular immune complex deposits have been demonstrated in patients with lupus nephritis.\textsuperscript{22} We have previously shown that the patients with this condition have significantly greater serum IgM-AECA titres than those without lupus nephritis.\textsuperscript{13} In addition, increased plasma concentrations of ET-1 have been demonstrated in patients with SLE and were shown to correlate significantly with a history of nephritis.\textsuperscript{7} These studies suggest that immune complexes or IgM-AECA may bind to the endothelial cells of glomerular capillary loops and stimulate ET-1 release, resulting in the development of lupus nephritis.

Only two of the SLE patients we examined had pulmonary hypertension. The stimulation of ET-1 release from cultured endothelial cells by serum from these two patients was significantly greater than the upper limit of the normal range (data not shown). We have previously shown that SLE patients with pulmonary hypertension have a markedly increased serum IgM-AECA titre relative to those without pulmonary hypertension.\textsuperscript{15} Quismorio et al\textsuperscript{23} detected IgG deposits in the
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walls of pulmonary arteries in SLE patients with pulmonary hypertension and suggested that these immune complexes may be important in the pathogenesis of this condition in SLE patients. Together with the observation that the concentration of ET-1 in arterial plasma is greater than that in venous plasma in patients with primary pulmonary hypertension,3 these studies suggest that IgM-AECA and immune complexes may stimulate ET-1 release from pulmonary endothelial cells and that the released ET-1 may play a pathophysiological role in the initiation and development of pulmonary hypertension in SLE.

Evidence for the expression of the Fcγ receptor by non-placental endothelial cells is controversial and may be related to technique. However, heat aggregated IgG has been shown to bind to human umbilical vein endothelial cells, possibly through Fcγ receptor.24 On the basis of their observation of a sinusoidal pattern of staining, Micklem et al reported that anti-Fcγ receptor II monoclonal antibodies immunostained hepatic endothelium.25 These findings suggest that immune complexes or heat aggregated IgG may stimulate ET-1 release from endothelial cells, possibly through Fcγ receptor mediated binding. Although the endothelial antigens recognised by IgM-AECA have not been characterised, IgM-AECA may also stimulate ET-1 release from endothelial cells as a result of interaction with these antigens. Further studies are required to investigate these hypotheses and other factors stimulating the release of ET-1.

This study was supported by a grant from the Ministry of Health and Welfare of Japan.