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Endogenously produced nitric oxide inhibits endothelial cell growth as demonstrated using novel antisense cell lines

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1 Proliferation of endothelial cells is a vital component of vascular repair and angiogenesis. The endothelial cell mediator, nitric oxide (NO) has been reported both to inhibit and to promote endothelial cell proliferation. In this study we have generated cell lines which constitutively express antisense RNA to a region of inducible nitric oxide synthase (iNOS) from a murine endothelial cell line, sEnd-1.

2 In response to stimulation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) these antisense cells had no detectable RNA for endogenous iNOS, barely detectable iNOS protein and produced 82% less NO than did the control transfected line.

3 Stimulation of the control transfected line caused significant NO production and inhibition of cell growth whereas for the antisense line, producing little NO in response to stimulation, proliferation remained the same as for unstimulated cells. No differences in cell death were observed between unstimulated and LPS/IFN-γ stimulated cells.

4 The data presented in this study directly demonstrate that NO derived endogenously from iNOS inhibits proliferation of endothelial cells. This approach overcomes problems in other studies where NO donors or non-isoform specific inhibitors of NO synthase have been used.
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Abbreviations: cNOS, constitutive nitric oxide synthase; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; L-NMMA, NG-monomethyl-L-arginine; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase

Introduction

The vascular endothelium, which lines the luminal surface of all blood vessels, produces a variety of substances that modulate vascular tone, coagulation, cell adhesion and growth. The integrity of the vascular layer is maintained by controlled migration and proliferation of both endothelial and vascular smooth muscle cells.

Endothelial cell proliferation is particularly important in angiogenesis and vascular repair following injury to the vessel wall. The process of angiogenesis, new vessel generation from a pre-existing microvascular network, involves the controlled proliferation of endothelial cells. Endothelial cells normally have a low rate of proliferation whereas in angiogenesis-dependent processes, such as reproduction and tumour metastasis, endothelial cells actively proliferate. When the endothelial layer is damaged, repair of the vessel is achieved by migration and proliferation of adjacent endothelial cells. If the vessel is not repaired then the subendothelial layer remains exposed and platelet adhesion and thrombus formation can occur, events that have an important role in the development of atherosclerosis (Cooke & Dzau, 1997). Endothelial cells are influenced by many endogenous factors, some of which stimulate whereas others inhibit angiogenesis.

Endothelial cells produce the endogenous vasodilator nitric oxide (NO) which is a potent regulator of vascular homeostasis. NO is continuously produced by a constitutive isoform of NO synthase (cNOS) in endothelial cells. Cytokines cause the expression of the inducible isoform of the enzyme (iNOS) in endothelial cells, inflammatory cells and vascular smooth muscle cells (Farrell & Blake, 1996). The amount of NO produced by cells in which iNOS has been induced can be several orders of magnitude greater than in cells producing NO *via* cNOS.

NO decreases the proliferation of a number of cell types including lymphocytes, smooth muscle cells, macrophages and lung and skin fibroblasts (Dubey & Overbeck, 1994; Kosonen *et al*., 1997). Studies of the effects of NO on endothelial cell growth have given conflicting results. An anti-proliferative effect of NO is suggested by Yang *et al*. (1994), Sarkar *et al*. (1995), RayChaudhury *et al*. (1996) and Lopez-Farre *et al*. (1997), whereas in contrast a proliferative effect has been reported by Ziche *et al*. (1994) and Morbidelli *et al*. (1996). These differing results may reflect problems in interpreting data from the approaches taken – namely using NO donors and NOS inhibitors, which can lead to non-specific effects.

It was the aim of this study to address this controversy using a novel approach. Using the murine endothelial cell line, sEnd-1 (Williams *et al*., 1989), which expresses both cNOS and iNOS and produces substantial levels of NO on cytokine stimulation (Walter *et al*., 1994), we have generated endothelial cell lines containing antisense RNA to iNOS. This permanent inhibition of the inducible production of NO, an approach which overcomes the problems of other methods of manipulating NO, allowed us to study the involvement of endogenously produced NO in endothelial cell proliferation.

Methods

Cell culture

Murine endothelial cells (sEnd-1, Williams *et al*., 1989) were cultured in Dulbecco's modified Eagle's medium containing

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2 mM L-glutamine, 0.375% (w v^{-1}) sodium bicarbonate, 100 u ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 10% (v v^{-1}) FCS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were routinely passaged every 4 days using trypsin/EDTA.

Preparation of plasmids containing antisense and control sense sequences to inducible nitric oxide synthase

A DNA fragment corresponding to bases 2476–2969 of macrophage iNOS mRNA (Lyons *et al*., 1992) was produced according to methods described in Cartwright *et al*. (1997). The 493 bp product was purified and subcloned into the plasmid vector pBluescriptII (Stratagene Ltd., Cambridge, U.K.) at the *Eco*RV site. The identity of the insert was confirmed by double stranded dideoxy sequencing. The resultant plasmid was digested with *Hinc*II and *Bam*HI linkers were ligated to make the *Hinc*II site into a *Bam*HI site. The linearized plasmid was then digested with *Bam*HI to isolate the iNOS DNA plus a 45 bp part of pBluescriptII (total, 538 bp). This fragment was purified and subcloned into the vector pSG5 (Stratagene), at its *Bam*HI site, which allows the expression of the inserted DNA under the control of the early SV40 promoter. Restriction enzyme digests were carried out to determine the orientation of the insert (antisense or sense direction with respect to the SV40 promoter/enhancer). CsCl gradients were used to make large-scale preparations of plasmids of interest.

Transfection of sEnd-1 cells

Murine endothelial cells (sEnd-1) were transfected using a modification of the method described in Dong *et al*. (1993). Medium was replaced with normal culture medium containing two plasmids – the pSG5 vector with iNOS insert and pbabepuro (a gift from Prof F. Farzaneh, King's College School of Medicine & Dentistry, London), in a molar ratio of 10 : 1, at a final concentration of 10 μg DNA per 3 ml per 9 cm plate and 10 μg ml^{-1} of poly-L-ornithine. Positive transfectants were selected by resistance to puromycin encoded by pbabepuro. The pSG5 vectors containing iNOS inserts in either the sense or antisense orientation were used in separate transfections. The sense insert was the same length as the antisense and thus would not generate a full-length coding region. Mock transfections, without DNA were also carried out. Plates were incubated for 6 h at 37°C with gentle mixing every 1.5 h. The medium was aspirated and replaced with 30% (v v^{-1}) DMSO in culture medium for 4 min at room temperature. The plates were then washed rapidly with medium and 10 ml of fresh culture medium added. After 3 days, the medium was replaced with culture medium containing 2 μg ml^{-1} puromycin for the isolation of stable transformants. After a further 3 days, medium was replaced with fresh selection medium and this was repeated every 3 days until puromycin resistant colonies appeared. The concentration of puromycin used was previously determined as the lowest concentration that caused death of non-transfected sEnd-1 cells within 5 days. Single colonies were isolated and expanded in culture. Cells were screened by Northern analysis using a cDNA probe for iNOS and positive cell lines were characterized further.

Total RNA isolation and analysis

Total RNA was isolated from confluent 175 cm^3 flasks of sEnd-1 cells or transfected lines (approximately 2×10^7 cells) using RNAzol B (Biogenesis Ltd., Bournemouth, U.K.) according to the manufacturer's instructions. Samples of RNA (approximately 10 μg) were subjected to electrophoresis in 1.5% agarose-formaldehyde gels. The RNA was transferred to a Hybond-N+ membrane (Amersham Life Sciences, Aylesbury, Bucks, U.K.) according to the manufacturer's instructions. Transfer was by diffusion overnight with 20 × SSPE as buffer (3.6 M sodium chloride, 0.2 M sodium phosphate pH 7.7, 0.02 M EDTA). The RNA was fixed to the membrane by placing it on a pad of absorbent filter paper soaked in 0.05 M sodium hydroxide. The cDNA probe for iNOS (Bogle *et al*., 1994) was labelled with α-[^{32}P]-dCTP (3000 Ci mmol^{-1}) using a multiprime DNA labelling system (Amersham) according to the manufacturer's instructions. Prehybridization solution (5 × SSPE, 50% (v v^{-1}) deionized formamide, 5 × Denhardt's solution (0.1% (w v^{-1}) Ficoll, 0.1% (w v^{-1}) polyvinylpyrrolidone, 0.1% (w v^{-1}) bovine serum albumin), 0.1% (w v^{-1}) SDS) supplemented by 20 μg ml^{-1} denatured herring sperm DNA was incubated with the membranes for 1 h at 42°C. For hybridization, membranes were incubated for 12 h at 42°C in the above buffer with the addition of denatured ^{32}P-labelled iNOS probe. The hybridization solution was then removed and the filters were washed once with 50 ml of 2 × SSPE, 0.1% (w v^{-1}) SDS for 15 min at room temperature, twice with 50 ml of 2 × SSPE, 0.1% (w v^{-1}) SDS for 30 min at 65°C and once with 50 ml of 1 × SSPE, 0.1% (w v^{-1}) SDS for 30 min at 65°C. The membranes were air dried, wrapped in Saran-wrap and subjected to autoradiography with an intensifying screen at −70°C.

Stimulation of nitric oxide production by transfected sEnd-1 cells

Transfected sEnd-1 cell lines were seeded in 24-well tissue culture plates at a density of 3.5×10^5 cells ml^{-1}, 500 μl per well and incubated at 37°C overnight. Medium was replaced with medium containing lipopolysaccharide (LPS, from *E. coli* serotype 055:B5, Sigma) and murine interferon-γ (IFN-γ, Sigma) at the stated concentrations in a volume of 500 μl and incubated for 24–72 h. NO production was assessed by measuring the amount of nitrite accumulated over the 24–72 h using the Greiss reaction. The remainder of the tissue culture medium was removed; the cells washed with 200 μl PBS and then solubilized in 200 μl 1 M sodium hydroxide. The plates were left for 30 min at room temperature and then stored frozen until assayed for protein (Bradford, 1976, Bio-Rad, Hemel Hempstead, Herts, U.K.).

Measurement of nitric oxide produced by transfected sEnd-1 cells (Greiss reaction)

Nitrite and nitrate are stable products formed from NO upon release from cells (Hibbs *et al*., 1988). The production of NO by transfected sEnd-1 cell lines was determined by measuring the accumulation of nitrite in the tissue culture medium (Greiss reaction) according to Di Rosa *et al*. (1990). Standards of sodium nitrite in the range 0–100 μM were prepared in sEnd-1 culture medium. Aliquots of 75 μl of either standards or supernatants to be assayed were added to 96-well microtitre plates followed by 25 μl of Greiss reagent (1% (w v^{-1}) sulphanilamide, 0.1% (w v^{-1}) napthyl-ethylenediamine, 2.5% (v v^{-1}) phosphoric acid). The plates were left for 10 min at room temperature and then the absorbance at 540 nm was determined using a Titertek Multiskan microtitre plate reader.

Western blot analysis

Endothelial cells (sEnd-1) or transfected lines were either unstimulated or stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24 h. To detect iNOS protein expression, confluent 9 cm plates of sEnd-1 cells or transfected lines (approximately 2.5×10^6 cells) were washed in PBS and harvested at 4°C in 0.9 ml RIPA buffer (1 × PBS, 1% (v v^{-1}) NP40, 0.5% (w v^{-1}) sodium deoxycholate, 0.1% (w v^{-1}) SDS, 0.1 mg ml^{-1} PMSF, 30 μl ml^{-1} aprotinin, 1 mM sodium orthovanadate). Cells were scraped and the lysate passed through a 21-gauge needle to shear the DNA. PMSF was added (10 μl of a 10 mg ml^{-1} solution) and incubated for 1 h on ice. The lysate was centrifuged at 15,000 × *g* for 20 min at 4°C and the amount of solubilized protein determined (Bradford, 1976). A constant amount of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis on a 7% polyacrylamide gel and transferred to a nitrocellulose membrane. Following incubation in blocking buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20, 5% (w v^{-1}) milk powder) overnight at 4°C, the membrane was incubated with rabbit polyclonal anti-mouse iNOS (M-19, Santa Cruz Biotechnology, U.S.A.) at 0.1 μg ml^{-1} in the blocking buffer for 45 min. After washing, the membrane was incubated with anti-rabbit IgG peroxidase (A6154, Sigma) at a 1 in 500 dilution in the blocking buffer for 30 min. Detection of membrane bound antibodies was carried out according to the manufacturer's instructions using a chemiluminescence kit (Boehringer Mannheim, Germany).

Endothelial cell growth studies

Transfected sEnd-1 lines were seeded at a density of 5×10^4 cells ml^{-1} in 24-well plates, 500 μl per well and incubated for 7 h to allow them to adhere. Cells were stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24–72 h and their growth monitored by determination of both protein content and cell number.

Protein The culture medium was removed after the stated times for the determination of nitrite by the Greiss reaction and the cells were washed twice with PBS and 200 μl 1 M NaOH added. The plates were incubated overnight at 4°C and then stored frozen until assayed for protein (Bradford, 1976).

Cell number After 72 h the cells were washed twice with PBS and detached using trypsin/EDTA. Cells were centrifuged at 150 × *g* for 10 min and resuspended in 200 μl culture medium. Cell number was determined using a haemocytometer following staining with erythrosin B.

Time-lapse digital image microscopy (TLDIM) determination of cell death

TLDIM was carried out to determine whether apoptosis was induced by incubation of the cells with LPS and IFN-γ. Transfected sEnd-1 lines were seeded at a density of 5×10^4 cells ml^{-1} in 6-well plates, 2 ml per well and incubated for 7 h to allow them to adhere. Cells were stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) and incubated on an Olympus IX70 microscope in an environmental cabinet at 37°C, 5% CO_2 in air. Using a computer driven motorized stage, images were taken every 30 min from a number of areas, which were revisited over 72 h. Forty cells were randomly chosen in a field of view and analysed by scoring cells as apoptotic by morphological appearance. Apoptosis is characterized by a number of distinct morphological changes, such as cell shrinkage, plasma membrane blebbing and membrane blistering, usually occurring in a progressive manner. Apoptotic cells appear phase bright while necrotic cells appear phase dark. Experimenters blinded to the treatment used carried out all analyses of apoptosis.

Statistical analysis

Results were analysed using the non-parametric Mann-Whitney U-test. Differences between populations/samples were considered to be significant if the P value was less than 0.05.

Results

Screening of cell lines by Northern blot analysis

Production of iNOS RNA by cell lines selected for puromycin resistance was analysed by Northern blotting using a ^{32}P-labelled double-stranded DNA probe corresponding to the 493 bp murine iNOS insert. An analysis of the cell lines termed 'A1' (derived with the antisense construct) and 'S7' (derived with the control sense construct) is shown in Figure 1; a band at approximately 1.2 kb, corresponding to the size of the exogenous iNOS RNA fragment plus β-globin from pSG5, was observed in both cell lines. After stimulation with LPS/IFN-γ, S7 and untransfected sEnd-1 cells contained an additional band corresponding to endogenous iNOS RNA at 3.6 kb. In contrast, stimulation of the antisense line A1 produced no detectable endogenous RNA for iNOS.

Amount of NO synthase in transfected sEnd-1 cells

Western blot analysis of transfected sEnd-1 cells (Figure 2) showed that on stimulation with LPS/IFN-γ the sense transfected line, S7, had high levels of iNOS present whereas the antisense transfected line, A1, had barely detectable iNOS even with protein loading as high as 200 μg. The band for

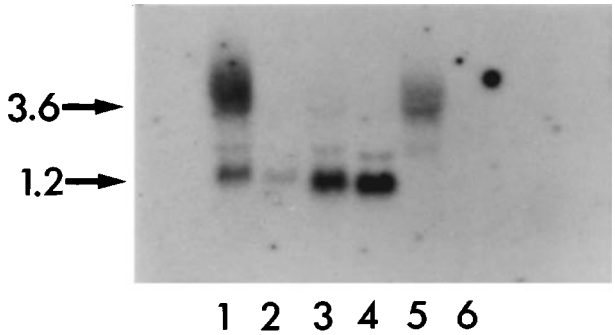

Figure 1 Northern blot analysis of iNOS expression in cell lines transfected with pSG5/iNOS. RNA was electrophoresed through a 1.5% agarose gel, transferred to Hybond N+ membranes and hybridized with ^{32}P-labelled double-stranded probe to murine iNOS (includes the region used for sense/antisense). Cells were either unstimulated or stimulated for 4 h with LPS/IFN-γ (1 μg ml^{-1} LPS, 50 u ml^{-1} IFN-γ, 25 ml^{-1} 175 cm^3 flask 2×10^7 cells^{-1}). The bands of antisense or sense sequence plus β-globin from pSG5 are approximately 1.2 kb. The band for endogenous iNOS mRNA (in the stimulated sEnd-1 and S7 but not visible in A1) is approximately 3.6 kb. Lane 1: stimulated S7; lane 2: unstimulated S7; lane 3: stimulated A1; lane 4: unstimulated A1; lane 5: stimulated untransfected sEnd-1; lane 6: unstimulated untransfected sEnd-1.

iNOS appeared in the same position as the positive control cell extracts from LPS-stimulated J774.2 cells.

Nitric oxide production by transfected sEnd-1 cells

NO production by transfected sEnd-1 cells was measured by detecting nitrite in the tissue culture medium (Greiss reaction, Figure 3). Both the sense transfected line S7 and the antisense line A1 had a basal level of nitrite detectable from unstimulated cells (sEnd-1 cells have cNOS as well as iNOS). On LPS/IFN-γ stimulation of S7 cells, the level of nitrite increased by over 3 fold ($P<0.0001$, $n=24$). LPS/IFN-γ stimulation of A1 cells only increased the level of nitrite slightly (1.4 fold, $P=0.0112$, $n=24$). The antisense transfected line produced 82% less NO (after subtracting basal) in response to LPS/IFN-γ than did the sense line.

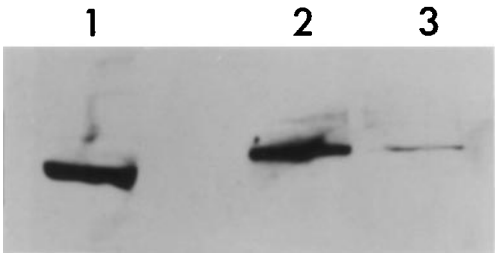

Figure 2 Western blot analysis of iNOS expression in cell lines transfected with pSG5/iNOS. The control sense transfected line S7 and antisense transfected line A1 (2.5×10^6 cells 9 cm^{-1} plate) were stimulated with LPS/IFN-γ (1 μg ml^{-1} LPS, 50 u ml^{-1} IFN-γ) for 24 h. J774.2 cells (2.5×10^6 cells 9 cm^{-1} plate) were stimulated with LPS (10 μg ml^{-1}) for 24 h and were included as a positive control for iNOS production. Cells were harvested, lysed and separated by SDS polyacrylamide electrophoresis (200 μg protein per track) and transferred to a nitrocellulose membrane. Following incubation with rabbit polyclonal anti-mouse iNOS and anti-rabbit IgG peroxidase, bound antibodies were detected by chemiluminescence. Lane 1 shows the positive control of stimulated J774.2 cells. Lane 2 shows stimulated S7 cells. Lane 3 shows stimulated A1 cells.

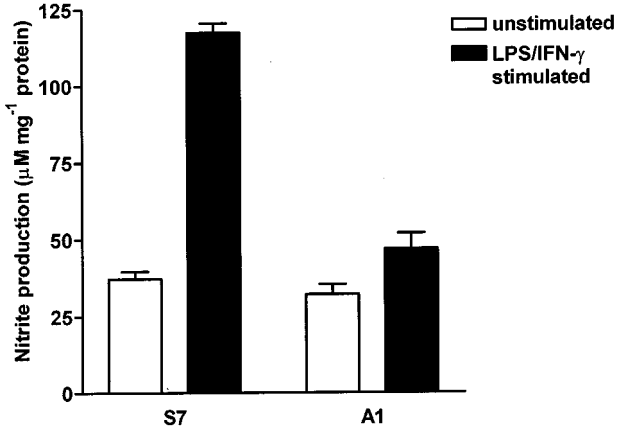

Figure 3 Nitrite accumulation from cell lines transfected with pSG5/iNOS. Transfected lines were seeded at 3.5×10^5 cells ml^{-1} (24-well plate, 500 μl $well^{-1}$) and incubated for 24 h. LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) were added in a volume of 500 μl and the incubation continued for 24 h. Nitrite accumulation was measured by applying the Greiss reaction to 75 μl medium. The remainder of the medium was removed, the cells washed with PBS and lysed with 1 M NaOH. Protein content of each well was measured and the Greiss reaction results adjusted to a standard protein amount. Results shown are mean + s.e.mean of combined results from four separate experiments, each in duplicate, with triplicate measurements of each replicate in the Greiss reaction. $P<0.0001$ for LPS/IFN-γ stimulated S7 cells compared to unstimulated S7 cells. $P=0.0112$ for LPS/IFN-γ stimulated A1 cells compared to unstimulated A1 cells.

Growth studies of transfected sEnd-1 cells

In order to study the effects of endogenous NO production on endothelial cell growth, both protein content and cell number of the sense transfected line S7 and the antisense transfected line A1 were monitored after stimulation with LPS/IFN-γ. The growth of S7 and A1 cells between 24 and 72 h, determined by measuring protein content, is shown in Figure 4 and nitrite accumulation from the same cells is presented in Table 1. At 72 h after stimulation of S7 cells with LPS/IFN-γ, the levels of protein were substantially lower ($P<0.001$, $n=18$) for the stimulated cells, producing high levels of NO, than the unstimulated cells, producing low levels of NO. At 72 h after stimulation of A1 cells with LPS/IFN-γ, the levels of protein were only slightly lower for the stimulated cells than the unstimulated cells ($P=0.03$, $n=18$); no significant increases in nitrite accumulation were seen (Table 1) compared with unstimulated cells. The growth of S7 and A1 cells after 72 h in culture determined by counting cell numbers is shown in Figure 5. Cultures of S7 cells stimulated with LPS/IFN-γ had

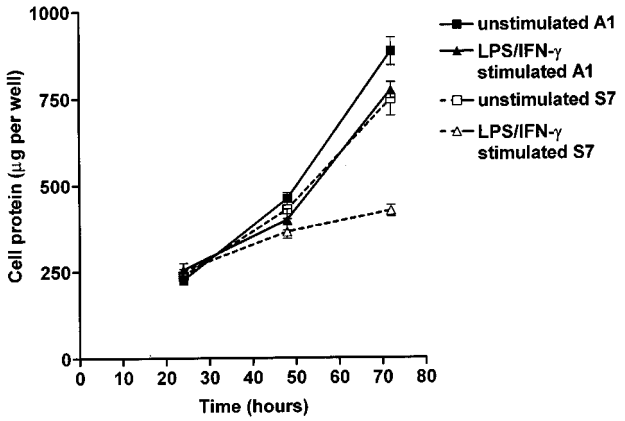

Figure 4 Growth of resting and LPS/IFN-γ stimulated cell lines transfected with pSG5/iNOS determined by protein analysis. Transfected lines were seeded at 5×10^4 cells ml^{-1} (24-well plates, 500 μl $well^{-1}$) and incubated for 7 h to allow them to adhere. Cells were stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24–72 h. The medium was removed after the stated times (and kept for nitrite determination by the Greiss reaction, Table 1) and the cells washed with PBS, lysed with 1 M NaOH and the protein content determined. The results presented are mean ± s.e.mean of combined results from three separate experiments, each in quadruplicate, with duplicate measurements of each replicate in the protein assay.

Table 1 Nitrate accumulation from resting and stimulated transfected sEnd-1 cells used in growth studies (Figure 4)

	Time in culture (h)		
	24	*48*	*72*
A1	0	2.17 ± 0.65	11.76 ± 1.69
Stimulated A1	0	3.56 ± 1.10	7.53 ± 1.17
S7	0.35 ± 0.16	3.80 ± 1.00	9.69 ± 1.44
Stimulated S7	2.62 ± 0.77	13.49 ± 2.42	24.37 ± 2.94

Transfected lines were seeded at 5×10^4 cells m^{-1} (24-well plates, 500 μl $well^{-1}$) and incubated for 7 h to adhere. Cells were stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24–72 h. Nitrite accumulation was measured by the Greiss reaction on 75 μl medium. The results presented are mean ± s.e.mean (μM) of combined results from five separate experiments, each in duplicate, with triplicate measurements of each replicate in the Greiss reaction.

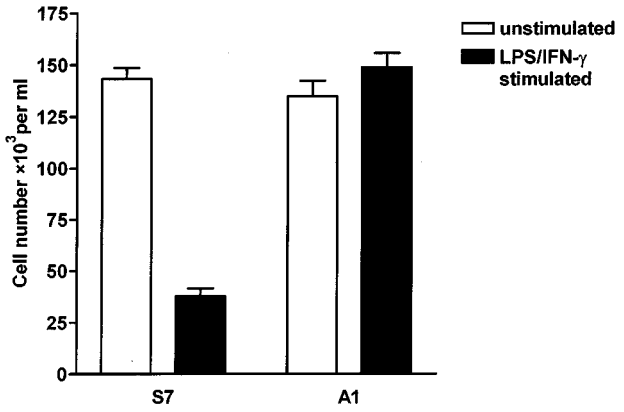

Figure 5 Growth studies of resting and LPS/IFN-γ stimulated cell lines transfected with pSG5/iNOS determined by cell number analysis. Transfected lines were seeded at 5×10^4 cells ml^{-1} (24 well plates, 500 μl well^{-1}) and incubated for 7 h to allow them to adhere. Cells were stimulated with LPS (1 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 72 h and then washed with PBS and detached using trypsin/EDTA. Cells were centrifuged at $150\times g$ for 10 min, resuspended in 200 μl medium and the cell number determined by counting using a haemocytometer. The results presented are mean + s.e.mean of combined results from three separate experiments, each in quadruplicate, with duplicate cell counts of each replicate. $P<0.0001$ for LPS/IFN-γ stimulated S7 cells compared to unstimulated S7 cells.

significantly fewer cells after 72 h than unstimulated cultures ($P<0.0001$, $n=24$). In contrast, cultures of A1 cells stimulated with LPS/IFN-γ had similar cell numbers after 72 h to unstimulated cultures.

Erythrosin B staining of cells prior to cell counting showed no increase in dead or dying cells on LPS/IFN-γ stimulation (mean per cent dead or dying cells $\pm$ s.e.mean was $4.5\pm2.3\%$, $n=3$ for stimulated S7 cells compared to $5.4\pm1.9\%$, $n=3$ for unstimulated S7 cells). The nitrite accumulation shown in Table 1 is lower than in Figure 3 because fewer cells were present.

Time-lapse digital image microscopy determination of cell death

To determine whether the reduced cell number and protein concentration were due to a reduction in cell growth or increased cell death we used TLDIM to monitor morphological changes characteristic of apoptosis or necrosis. Of the forty cells per well picked at random at the start of each experiment less than 2% died after stimulation with LPS/IFNγ. This does not account for the reduction in cell number observed.

Discussion

There have been a number of studies on the involvement of NO in the regulation of endothelial cell proliferation. An increase in endothelial cell growth is seen following treatment with the NO donor SNP and proliferation induced by Substance P, but not basic fibroblast growth factor, is abolished by NOS inhibitors (Ziche *et al*., 1994). Vascular endothelial cell growth factor induced proliferation is also decreased on inhibition of NO (Morbidelli *et al*., 1996). In contrast, Yang *et al*. (1994), Sarkar *et al*. (1995) and RayChaudhury *et al*. (1996), showed that NO donors inhibit proliferation of ECs and Lopez-Farre *et al*. (1997) showed that inhibition of endogenous NO production, using a NOS inhibitor, increased proliferation.

All of these studies have used exogenous NO donors or inhibited NOS in endothelial cells by guanidino-substituted arginine analogues such as L-NMMA. The arginine basis of many of these inhibitors, such as L-NMMA, makes effects on other cell systems possible (e.g. arginine transport; Bogle *et al*. (1995); McDonald *et al*. (1997)) which may be exacerbated by metabolism of the chemical during longer assays (MacAllister *et al*., 1994; Thomae *et al*., 1996). In addition, under certain conditions NO can be produced non-enzymatically from NOS inhibitors (Moroz *et al*., 1998). The conflicting nature of these studies may reflect the fact that the inhibitors of NOS will, to varying extents, inhibit all isoforms of NOS and so in cells such as endothelial cells, which have both cNOS and iNOS, the separate functions of the different isoforms cannot be readily investigated. A NOS inhibitor (1400W) that is more selective for iNOS has been characterized (Garvey *et al*., 1997); however it is not completely selective at higher doses and was not used in the studies of proliferation detailed above.

Antisense technology provides an alternative approach to the chemical inhibition of NOS. This involves binding of antisense sequences to the complementary portion of mRNA preventing translation into protein. We have previously inhibited iNOS in macrophages (Cartwright *et al*., 1997) by transfecting constructs that direct the production of antisense RNA within cells. This approach avoids the problems associated with antisense experiments in which oligonucleotides are added to the culture medium. In the present study this method was applied to endothelial cells in order to permanently inhibit the inducible production of NO and enable an investigation of the involvement of NO in proliferation. The murine endothelial cell line, sEnd-1 (Williams *et al*., 1989), has both cNOS and iNOS and produces substantial levels of NO on cytokine stimulation (Walter *et al*., 1994). The antisense iNOS cell line A1, derived from sEnd-1, produced substantially less NO on LPS/IFN-γ stimulation than the control sense line sEnd-1, S7.

It is likely that under different situations the two isoforms of NOS will have different roles (Noiri *et al*., 1996; Heller *et al*., 1999). The antisense approach allowed us to specifically study endogenous NO from an individual NOS isoform. Parallel pathways, in addition to iNOS induction, will be activated on cytokine stimulation, such as GTP cyclohydrolase, the effects of which would not be determined if NO donors were added. In these studies we have concentrated on investigating the effects on endothelial proliferation of NO produced within endothelial cells themselves in response to induction of iNOS.

Our observations of the antiproliferative effect of NO *via* iNOS has important implications for situations involving angiogenesis and reendothelialization, including tumour growth, inflammation, wound healing and sepsis. These are also situations in which high amounts of NO are produced by the induction of iNOS. A number of inflammatory cytokines would be present that can induce NO production from iNOS in macrophages, smooth muscle cells and the endothelial cells themselves. The NO produced by these cells could inhibit mechanisms by which adjacent endothelial cells migrate and proliferate. Hence, NO may act as an antiangiogenic agent, as a defence mechanism against angiogenesis-dependent diseases such as tumour metastasis and diabetic retinopathy and in pathological situations angiogenesis could be aided by the suppression of NO. Conversely, in atherosclerosis, NO produced from inflammatory cells or cytokine-stimulated smooth muscle or endothelial cells, may inhibit EC proliferation and repair leading to disease progression.

One possible consequence of the induced production of large amounts of NO is local cytotoxicity. NO from activated

macrophages is a cytotoxic molecule (Hibbs *et al*., 1988) and NO generated by smooth muscle cells when activated by inflammatory cytokines has local cytotoxic effects on endothelial cells (Thomae *et al*., 1996). Cytokine pre-treatment may also induce synthesis of other mediators that, along with NO, result in cytotoxicity. In our experiments NO does not alter the cell number by causing cytotoxicity or apoptosis as there was no increase in dead or dying cells. The effect observed is therefore likely to be cytostatic rather than cytotoxic.

There is evidence that NO can influence extracellular matrix degradation, cell proliferation, migration and differentiation, all events required for angiogenesis to proceed (Ziche *et al*., 1994; 1997; Murrell *et al*., 1995; Peunova & Enikolopov, 1995). As with endothelial cell proliferation there is also controversy over the involvement of NO in cell migration. Studies using NO donors, NOS inhibitors and antisense oligonucleotides have shown both induction and inhibition of migration (Ziche *et al*., 1994; Noiri *et al*., 1996; Pipili-Synetos *et al*., 1994; Lau & Ma, 1996; Papapetropoulos *et al*., 1997). Our study provides direct evidence of endogenous NO acting to inhibit endothelial cell proliferation in an autocrine manner. Extension of these studies using the antisense lines will allow further determination of the effects of iNOS (and cNOS, by generation of cNOS antisense lines) on the angiogenic process, determination of antimitogenic signal transduction pathways and endothelial cell migration.

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