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Inducible nitric oxide synthase (iNOS) activity promotes ischaemic skin flap survival

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1 We have examined the role of nitric oxide (NO) in a model of functional angiogenesis in which survival of a skin flap depends entirely on angiogenesis to provide an arterial blood supply to maintain tissue viability.

2 The different effects of nitric oxide synthase (NOS) inhibitors on rat skin flap survival appeared to be explained on the basis of their NOS isoform selectivity. Skin flap survival was decreased by iNOS-selective (inducible NOS) inhibitors, S-methyl-isothiourea, aminoguanidine and amino-ethylthiorea; unaffected by the non-selective inhibitor nitro-imino-L-ornithine; and enhanced by the cNOS (constitutive NOS, that is endothelial NOS (eNOS) and neuronal NOS (nNOS)) inhibitor, nitro-L-arginine methyl ester.

3 Skin flap survival was reduced in mice with targeted disruption of the iNOS gene (iNOS knockout mice), and the administration of nitro-L-arginine methyl ester significantly increased flap survival in iNOS knockout mice ($P<0.05$).

4 iNOS immunoreactivity was identified in mast cells in the angiogenic region. Immunoreactive vascular endothelial growth factor (VEGF) and basic fibroblast growth factor were also localized to mast cells.

5 The combination of interferon-γ and tumour necrosis factor-α induced NO production and increased VEGF levels in mast cells cultured from bone marrow of wild-type, but not iNOS KO mice.

6 The increased tissue survival associated with the capacity for iNOS expression may be related to iNOS-dependent enhancement of VEGF levels and an ensuing angiogenic response. Our results provide both pharmacological and genetic evidence that iNOS activity promotes survival of ischaemic tissue.

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Abbreviations: AET, aminoethylthiourea; AG, aminoguanidine; bFGF, basic fibroblast growth factor; DAB, diaminobenzidine; ELISA, enzyme linked immuno-sorbent assay; Erk, extracellular signal-related kinase; IFNγ, interferon γ; L-NAME, nitro-L-arginine methyl ester; L-NIO, nitro-imino-L-ornithine; LPS, lipopolysaccharide; MC, mast cell; NO, nitric oxide; NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; eNOS, endothelial nitric oxide synthase/nNOS: neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; iNOS KO mice, inducible nitric oxide synthase knockout mice; $ONOO^-$, peroxynitrite; PBS, phosphate buffered saline; RPMI 1640, Roswell Park Memorial Institute Culture Media 1640; SMT, S-methyl-isothiourea; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; WT, wild-type

Introduction

Angiogenesis in the adult occurs physiologically in wound healing and in the menstrual cycle, but may also occur pathologically in conditions such as cancer and chronic inflammation (e.g. rheumatoid arthritis) (Folkman, 1995). There is intense interest in the possibility of therapeutic modulation of angiogenesis to facilitate wound healing and inhibit tumour growth or chronic inflammation (Fan *et al.*, 1995). By contrast, the importance of angiogenesis in reconstructive surgery (transfer of skin grafts, skin flaps and prefabricated tissues) and replantation of limbs or digits has received little attention. Enhancement of the angiogenic process is potentially a major contribution to the safety and speed of these tissue transfers and broadens the scope of re-vascularizations.

Non-developmental angiogenesis involves increases in production and release of angiogenic factors such as vascular endothelial growth factor (VEGF) which directly stimulate endothelial cells to degrade the extracellular matrix, migrate, proliferate and organize endothelial cells to form capillaries (Neufeld *et al.*, 1999). A vast number of pro-inflammatory endogenous mediators including tumour necrosis factor-α (TNF-α) influence angiogenesis by mobilizing inflammatory

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cells such as macrophages and mast cells (Fan *et al*., 1995). In contrast, angiogenic responses to basic fibroblast growth factor (bFGF) and VEGF are not dependent on recruitment of inflammatory cells.

Nitric oxide (NO), a mediator with a complex profile of biological activity was first implicated in angiogenesis by Pipili-Synetos *et al*. (1993) who reported that the nitric oxide synthase (NOS) inhibitor, nitro-L-arginine methyl ester (L-NAME) enhanced vessel growth in the chick chorioallantoic membrane (CAM) model (Pipili-Synetos *et al*., 1993). Additional studies indicated that NO donors reduced tissue angiogenesis and tumour neovascularization (Pipili-Synetos *et al*., 1995; Sakkoula *et al*., 1997). However, opinion on the role of NO in angiogenesis has been polarized by disparate observations in the literature. Ziche *et al*. suggest that NO directly stimulates endothelial cell proliferation (Ziche *et al*., 1993a), that NOS inhibitors inhibit angiogenesis in the rabbit corneal model and that NO donors promote angiogenesis in this model (Ziche *et al*., 1993b). Furthermore, tumour specimens exhibiting high levels of NOS activity induced angiogenesis in the rabbit corneal assay and when NO production was blocked, tumour angiogenesis and growth were suppressed (Gallo *et al*., 1998). Endothelial NOS activity (eNOS) has been implicated in the proliferative effects of VEGF (Ziche *et al*., 1997a) through activation of extracellular signal-related kinase (Erk) (Parenti *et al*., 1998). In addition to acute stimulation of eNOS activity (Ziche *et al*., 1997a) VEGF also increases eNOS expression (Hood *et al*., 1998). Even though bFGF-induced proliferation of endothelial cells occurs independently of NO (Ziche *et al*., 1997a), NO appears to exert its effect through upregulation of bFGF production by endothelial cells which in turn stimulates proliferation and urokinase-type plasminogen activator expression (Ziche *et al*., 1997b). The foregoing observations clearly implicate eNOS activity as an intermediate in the angiogenic cascade between VEGF and bFGF, but the role of iNOS in angiogenesis has not been delineated.

A new model of angiogenesis has been developed to avoid confounding influences, such as developmental angiogenesis in foetal tissue or vessel growth into a normally avascular area, that are associated with other models of angiogenesis (Theile *et al*., 1998). In this new model, the developing vessels are required to provide blood flow to a skin/muscle flap, mimicking the clinical process of angiogenesis in reconstructive surgery. A further advantage of this new model of functional angiogenesis is the use of skin flap survival as a functional measure of the extent of angiogenesis, thereby avoiding the contentious aspects of measurement of the angiogenic response histologically (Jain *et al*., 1997).

We have evaluated the role of iNOS in the angiogenic process subserving skin/muscle flap survival by using a series of NOS inhibitors having different selectivity for inhibition of iNOS or constitutive NOS (eNOS and nNOS) isoforms. In addition, mice with targeted disruption of the iNOS gene (iNOS knockout (MacMicking *et al*., 1995) and their corresponding wild-type control (C57BL/6 strain) have been used to establish the relative importance of iNOS in a more definitive manner than can be achieved using moderately selective pharmacological inhibitors. Our observations provide evidence for an as yet undescribed mechanism by which NO derived from iNOS promotes survival of ischaemic tissue through angiogenesis, possibly by increasing the availability of mast cell VEGF within the angiogenic zone.

Methods

Surgical procedure and drug treatment in rats

These experiments received the prior approval of the St Vincent's Hospital Animal Ethics Committee and conformed to the NHMRC guidelines for care and maintenance of animals. The surgical technique for the creation of the model of angiogenesis has been characterized and described in detail (Theile *et al*., 1998). Briefly, in the first operation adult male Sprague-Dawley rats (200–250 g) were anaesthetized by intra-peritoneal injection of sodium pentobarbitone (30–45 mg kg^{-1}), the right and left inferior epigastric vascular pedicles were isolated and the artery and nerve cauterized to create a gap of 7 mm between the ends of the artery. The vein was left intact to prevent further retraction of the divided arteries. Commencing on the day of operation (day 1), rats received daily i.p. injections of saline (1 ml kg^{-1}), nitro-L-arginine methylester hydrochloride (L-NAME, 30 mg kg^{-1}), which has a limited degree of selectivity for cNOS compared to iNOS (Gross *et al*., 1991; Hickey *et al*., 1997). S-methylisothiourea sulphate (SMT, 3 mg kg^{-1}), aminoethylthiorea bromide (AET, 3 mg kg^{-1}), and aminoguanidine bicarbonate (AG, 30 mg kg^{-1}), all of which are relatively selective as iNOS inhibitors (Hickey *et al*., 1997; Reutten & Thiemermann, 1996; Southan *et al*., 1995) or nitro-iminoethyl-L-ornithine hydrochloride (L-NIO, 30 mg kg^{-1}), which inhibits both iNOS and cNOS to similar degrees (Southan *et al*., 1995). After 7 days, a second operation was performed: on the left side, a standard sized (4×3 cm) island skin flap, sustained by the regenerated epigastric artery, and intact vein, was raised (i.e. dissected away from the underlying tissue) on the abdominal wall. This flap, now solely supplied with blood *via* the epigastric vessels, was sewn back into position. The right epigastric pedicle was harvested for histological analyses. After a further 6 days, per cent flap survival was established by tracing necrotic areas and total flap area and measured by computer-based planimetry.

Surgical procedure in mice

Adult C57BL/6 wild-type (WT) or iNOS KO mice of either sex weighing 20–30 g were anaesthetized using chloral hydrate (40 mg kg^{-1}, i.p.) and underwent two operations as outlined for the rat. However, the gap in the mouse epigastric artery after cauterization (first operation) was 4 mm. After periods of 0, 5, 7, 10, 14 or 21 days, a flap (3×1.5 cm) was raised (second operation). Flap survival was evaluated after a further 6 days.

Measurement of skin flap survival

In mice, the necrotic skin flap area was revealed after intramuscular injection (into the tongue) of fluorescein (400 mg kg^{-1}), since the black skin colour precluded direct visual assessment of necrosis. Fluorescein, identified under

UV illumination, was detected in blood-perfused skin. Necrotic (absence of fluorescein) and surviving flap areas were traced and the percentage survival was determined using the Videopro 32 image analysis system (Faulding Imaging, Clayton, Victoria, Australia).

Assessment of morphological changes

Epigastric pedicles removed from the right side of rats in the second operation were immersion-fixed in buffered formol saline (BFS) for a minimum of 24 h and processed for final embedding in paraffin. Prior to final embedding, the angiogenic zone of the pedicle was transfected and the cross-sectioned surface placed face down in the block to allow 5-μm-thick pedicle cross sections to be cut. These sections were placed on glass slides and stained with haematoxylin and eosin or toluidine blue (1% w v^{-1} in 50% isopropanol) for identification of mast cells. In addition, four epigastric pedicles were removed from two unoperated rats, fixed and processed as described above for comparison with operated (angiogenic) pedicles.

Immunohistochemistry

Sections (5 μm) of the paraffin-embedded pedicles were mounted on gelatin-coated glass slides and stained for bFGF, VEGF, iNOS with an indirect immunohistochemical method. The antibodies used to detect iNOS and VEGF were monoclonal isotypes IgG2a and IgG1 respectively, whilst bFGF was a polyclonal. Antibodies of irrelevant specificity 1gG2a anti-smooth muscle α-actin, 1gG1 anti-EC NOS (endothelial) and collagen II rabbit polyclonal antibody were used as controls. In brief, the sections were dewaxed, rehydrated and washed in distilled water followed by a phosphate buffered saline (PBS, pH 7.4) wash (10 min). Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide (3% in methanol) for 15 min at room temperature. The sections were incubated with diluted sheep serum (1 : 20). The primary antibodies were incubated on the sections overnight at room temperature (rabbit anti-human bFGF, diluted 1 : 200; mouse anti-VEGF, diluted 1 : 640; mouse anti-iNOS, diluted 1 : 25 or antibodies of irrelevant specificity at a dilution similar to their specific antibody match). Negative control slides were prepared by substituting sheep serum for the primary antibody. After 24 h, the slides were washed with PBS and incubated with the secondary antibody (1 : 100 dilution of: sheep anti-rabbit horseradish peroxidase-conjugated antibody (for polyclonal primary antibodies) and with sheep and mouse horseradish peroxidase-conjugated antibody (for monoclonal primary antibodies) for 30 min at room temperature). The peroxidase reaction was developed in PBS (containing 3% hydrogen peroxide), diaminobenzidine (DAB) tetrahydrochloride (0.5 mg ml^{-1}) for 3–5 min. The sections were washed and selected sections were counterstained with Mayer's haematoxylin.

In vitro *culture of mouse-derived mast cells*

Bone marrow cells from the femoral bone of either WT or iNOS KO mice were harvested by lavage and aspiration. The harvested cells were cultured for 4–6 weeks in RPMI 1640 media containing 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine, 10% foetal calf serum and 20% Walter and Eliza Hall Institute-3 D cell conditioned media as described previously (Hartmann *et al*., 1997). After 4 weeks more than 99% of the cells in culture were identified as mast cells (MCs) by metachromatic staining with toluidine blue. Mast cells were stimulated at a concentration of 10^5 cells ml^{-1} for analysis of nitrite in the cell culture media and at 2×10^5 cells ml^{-1} for VEGF analysis in the presence or absence of TNF-α (3 nM) and IFNγ (100 u ml^{-1}) for 24 h. Nitrite concentration was analysed by measuring the absorbance (550 nm) of cell culture supernatants after addition of an equal volume of the Griess reagent as described (Green *et al*., 1982). For VEGF measurements, Triton X-100 (0.01%) was used to lyse the cells and total VEGF was measured by a commercially available ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, U.S.A.).

Materials

Unless otherwise stated all chemicals and drugs were purchased from Sigma chemical company (St Louis, MO, U.S.A.). Rats were purchased from Monash University Animal Services (Melbourne, Australia). Mice WT and iNOS KO (129/S_VE_Vx C57BL/6JF2) were internally bred from original breeding pairs provided by Dr G. Karupiah (John Curtin School of Medical Research, ANU, Canberra, Australia) and originally produced by Dr J. MacMicking (Merck, Rahway, NJ, U.S.A.) and Professor C. Nathan (Cornell University, New York, U.S.A.). Walter and Eliza Hall Institute 3D- cells were obtained from The Walter and Eliza Hall Institute (Parkville, Australia). The following antibodies were used: rabbit anti-human bFGF (Sigma Chemical Company, St Louis, MO, U.S.A.), VEGF monoclonal, (generous gift, Genentech Inc, San Francisco, CA, U.S.A.), mouse anti-iNOS (macrophage inducible, Transduction Laboratories Inc, Lexington, Kentucky, U.S.A.), anti-smooth muscle α-actin (Dako Corporation, Carpinteria, CA, U.S.A.), anti-ECNOS (endothelial) (Transduction Laboratories Inc., Lexington, KY, U.S.A.) and collagen II rabbit polyclonal antibody (Novocastra Laboratories Ltd, U.K.). Sheep anti-rabbit, horseradish peroxidase-conjugated antibodies raised against rabbit primary antibodies; and sheep, anti-mouse, horseradish peroxidase-conjugated immunoglobulin raised against murine monoclonal primary antibodies were from Silenus Laboratories (Hawthorn, Australia). RPMI 1640 media and foetal calf serum were from Commonwealth Serum Laboratories - Biosciences (Parkville, Australia), VEGF ELISA kit was from R & D Systems (Minneapolis, U.S.A.).

Statistical evaluation

Time-course and NOS inhibitor experiments on skin flap survival of rats and mice were analysed by ANOVA followed by Dunnett's test for multiple comparisons after initial arcsine transformation of the data to normalize the ratios of skin flap survival. For *in vitro* experiments using bone marrow-derived mast cells, Student's paired *t*-test was used. Differences were considered to be statistically significant when $P<0.05$.

Results

Effect of NOS inhibitors on rat angiogenic pedicles

In saline-treated rats in which 7 days was allowed for bridging angiogenesis, there was a significant flap survival of $39\pm7\%$ ($n=17$, $P<0.05$ compared with 0% survival when no time was allowed for angiogenesis) (Theile *et al*., 1998). Treatment with the NOS inhibitors had a distinct effect depending on the inhibitor selectivity (Figure 1). L-NAME significantly increased flap survival ($P<0.05$, $n=12$) whereas the non-selective NOS inhibitor L-NIO had no effect ($P>0.05$, $n=6$). The iNOS-selective inhibitor, SMT significantly reduced flap survival ($P<0.05$, $n=10$). Flap survival in rats treated with the iNOS-selective inhibitors, AET ($n=6$) or AG ($n=5$) was not significantly different to that in the SMT- or saline-treated rats. Furthermore, flap survival in rats treated with iNOS-selective inhibitors did not differ significantly from 0% ($P>0.05$) (Figure 1).

Effect of targeted iNOS gene disruption on angiogenesis-dependent skin flap survival in mice

The inhibition of flap survival caused by the three iNOS-selective inhibitors in the rat strongly supported the possibility of a pro-angiogenic role for iNOS, but the selectivity of these compounds for iNOS is less than would be required to draw a definite conclusion. Therefore, we used the iNOS KO mouse (MacMicking *et al*., 1995) to provide a more definite examination of the role of iNOS in functional angiogenesis. The time course of flap survival in wild-type mice (Figure 2) was similar to that observed in rats (Theile *et al*., 1998). In iNOS KO mice, flap survival remained below 30% for 21 days and was significantly less than that in wild-type controls, which showed a progressive increase in survival to approximately 85% by 21 days. An additional experiment was conducted examining daily administration of L-NAME (30 mg kg^{-1} per day, i.p.) in iNOS KO mice on flap survival in which 5 days was allowed for angiogenesis prior to flap elevation. L-NAME treatment increased skin flap survival to $57\pm15\%$, compared with saline treated iNOS KO mice $8\pm4\%$ (Figure 3).

Morphology of the angiogenic zone

In saline-treated (control) rats cross-sections of the reconstituting vascular pedicle revealed that the epigastric vein was surrounded by areas of vascular connective tissue containing microvessels of varying calibre (Figure 4a). In toluidine blue stained sections, large granular metachromatically staining cells, i.e. mast cells, were frequently present adjacent to microvessels (Figure 4b).

Immunoreactive iNOS and growth factor localization in mast cells within the angiogenic pedicle of rat and mouse

Inducible NOS immunoreactivity was more apparent in the operated (Figure 4d) compared with the unoperated pedicle (Figure 4c) and was frequently associated with large, mononuclear granular cells (Figure 4d). These iNOS-positive cells were further identified as mast cells by staining of adjacent sections with toluidine blue (Figure 4e). Macrophages in the rat reconstituting vascular pedicle (identified by their haemosiderin granules) were not immunoreactive for iNOS. The angiogenic pedicles were also subjected to immunohistochemistry for VEGF and bFGF. Both angiogenic factors were localized to mast cells granules (Figure 4g,h). Antibodies of irrelevant specificity, but of identical isotypes to iNOS, VEGF and bFGF did not stain mast cells.

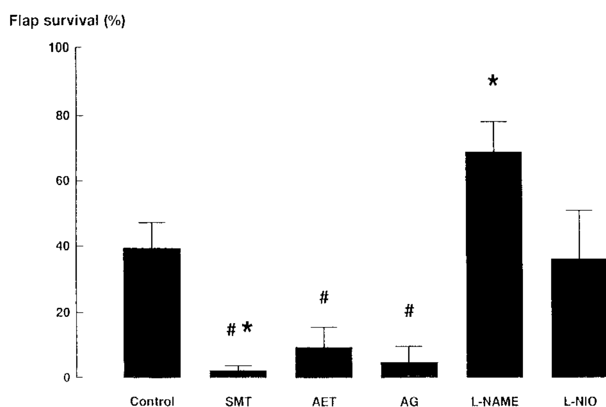

Figure 1 Rat skin flap survival represented as the percentage of the skin flap area that survives 6 days after flap elevation. Rats were treated by daily intraperitoneal injection of saline (1 ml kg^{-1}, control, $n=12$) or one of the following NOS inhibitors: nitro-L-arginine methylester (L-NAME, 30 mg kg^{-1}, $n=12$); S-methylisothiourea (SMT, 3 mg kg^{-1}, $n=10$), aminoethylthiorea (AET, 3 mg kg^{-1}, $n=6$); aminoguanidine (AG, 30 mg kg^{-1}, $n=5$); nitro-iminoethyl-L-ornithine (L-NIO, 30 mg kg^{-1}, $n=6$). *Indicates significant differences ($P<0.05$) between saline-treated controls and drug treatments. # Indicates non-significant differences ($P>0.05$) between drug treatments and 0% survival.

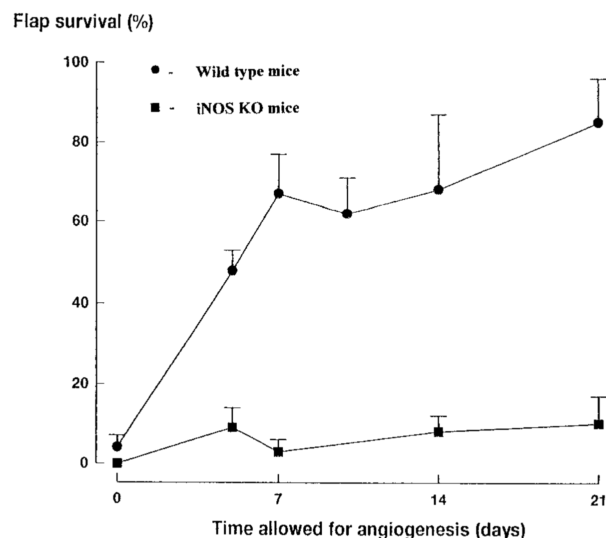

Figure 2 A comparison of the time-course of flap survival in wild-type ($n=6-9$) and iNOS KO mice ($n=6-9$) allowed up to 21 days for angiogenesis to support skin flap survival. The time-dependent increase in skin flap survival in wild-type mice is not observed in iNOS KO mice.

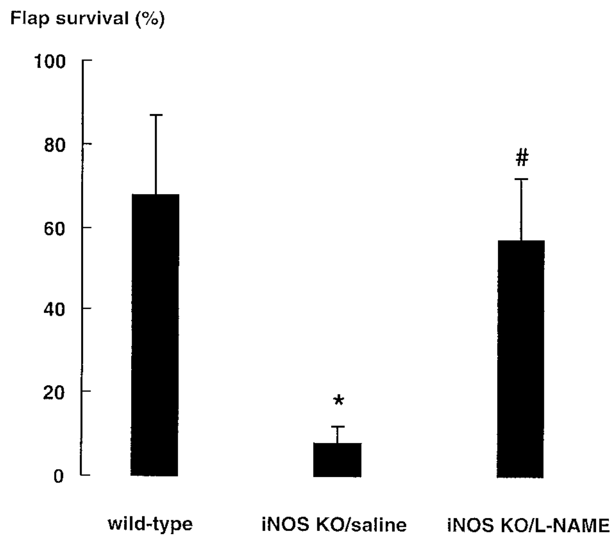

Figure 3 Effects on skin flap survival of L-NAME treatment in iNOS KO mice allowed a 5 day period for angiogenesis. *Indicates significant difference ($P<0.05$) between L-NAME-treated iNOS KO mice and saline-treated iNOS KO mice. # Indicates a lack of difference ($P>0.05$) between L-NAME-treated iNOS KO mice and saline-treated wild-type mice.

Inducible NOS-regulated VEGF expression in mouse bone marrow-derived mast cells

The observed co-expression of VEGF and iNOS in mast cells of the reconstituting vascular pedicle together with the anti-angiogenic effects of iNOS inhibitors raise the possibility that iNOS activity may be linked to expression in VEGF. Thus we examined VEGF levels in mast cells cultured from the bone marrow of wild-type and iNOS KO mice. There were no differences in basal supernatant levels of nitrite between wild-type (4.7 ± 0.7 nmol 10^{6-1} cells) and iNOS KO (4.1 ± 1.2 nmol 10^{6-1} cells) bone marrow-derived mast cells. Moreover, this basal nitrite level was derived largely from the foetal calf serum content of the medium. Incubation of wild-type mast cells with interferon γ (IFNγ, 100 u ml^{-1}) and TNF-α (3 nM) for 24 h induced a significant increase ($P<0.05$, $n=4$) of 1.9 ± 0.3 nmol nitrite 10^{6-1} cells in the cell culture supernatant. The cytokine-induced increase in nitrite concentration was not observed when iNOS KO-derived mast cells were incubated in the same cytokine mixture (non-significant change from the basal level of 0.3 ± 0.2 nmol 10^{6-1} cells, $n=3$). The cytokine treatment significantly increased total cell VEGF ($P<0.05$) in wild-type mast cells, but had no effect on VEGF content of mast cells cultured from iNOS KO mice (Figure 5).

Discussion

In an *in vivo* model which incorporates a pathophysiological type of angiogenesis in the adult (Theile *et al.*, 1998), we have provided pharmacological and genetic evidence that iNOS activity enhances skin flap survival, and by direct inference, angiogenesis. Three lines of evidence implicated iNOS as pro-angiogenic. Firstly, whilst the non-selective NOS inhibitor (L-NIO) had no effect on skin flap survival, the iNOS-selective inhibitors were anti-angiogenic. Secondly, iNOS KO mice, which provide a more definitive experimental paradigm than the relatively selective pharmacological iNOS inhibitors, showed a prolonged delay in angiogenesis compared with wild-type mice. Finally, increased iNOS expression was observed in the operated vascular pedicle of the rats and wild-type mice compared with unoperated control pedicles. In contrast to the anti-angiogenic effects of iNOS inhibition, treatment with L-NAME (selective inhibitor of constitutive NOS isoforms, i.e. eNOS and nNOS) enhanced skin flap survival in rats and iNOS KO mice.

In the INOS KO mice the interpretation of the pro-angiogenic action of L-NAME is restricted to an effect on constitutive NOS, consistent with NO derived from constitutive NOS having anti-angiogenic activity. However, the possibility that L-NAME has unreported actions unrelated to NOS activity cannot be discounted. Further studies using eNOS knockout mice are required to resolve the importance of this NOS isoform.

NO has been implicated in angiogenesis on the basis of observations in different models of angiogenesis, including the chick chorioallantoic membrane (Pipili-Synetos *et al.*, 1993), the rabbit cornea (Ziche *et al.*, 1993b) and different tumour types (Gallo *et al.*, 1998). However, these studies offer conflicting conclusions as to whether NO promotes or inhibits angiogenesis. The proposition that NO has both pro- and anti-angiogenic roles is not difficult to accept when consideration is given to the well documented differences in amount, location and duration of NO production by cNOS compared with iNOS isoforms (Knowles, 1996). The iNOS KO mouse exhibits impaired wound healing compared with wild-type mice (Nathan, 1997), consistent with a role for iNOS in wound healing-associated angiogenesis. This impaired wound healing appears to be one of the few phenotypic changes induced by iNOS KO in these extensively studied mice (Nathan, 1997). The developmental and physiological normality of the iNOS KO mice indicate that the role of iNOS in angiogenesis is likely to be largely restricted to inflammatory or pathophysiological conditions. Our results may help to explain, but do not directly reconcile, the polarized conclusions of previous studies. The restricted role of iNOS in angiogenesis associated with pathophysiological conditions may partly explain the model-dependent roles of NOS isoforms, as these different models are likely to depend to variable degrees on developmental angiogenic mechanisms. The chorioallantoic membrane model, in which inhibition of NOS activity is associated with enhanced angiogenesis, could be considered as having the closest relationship to developmental angiogenesis of any of the models in common use.

Previous studies by Ziche *et al.* (1997a,b) have addressed the importance of NO in angiogenesis, and potential sites of action have been identified. Thus, NO appears to act downstream of VEGF and proximal to bFGF release to induce endothelial cell proliferation (Parenti *et al.*, 1998; Ziche *et al.*, 1997a,b). There are potentially a number of additional roles for NO in the angiogenic process. When rat aortic endothelial cells were cultured in a monolayer, NOS inhibitors had no influence on EC proliferation, but culture in a collagen matrix induced iNOS and inhibitors of iNOS impaired capillary tube formation suggesting a role for NO in the morphogenesis of

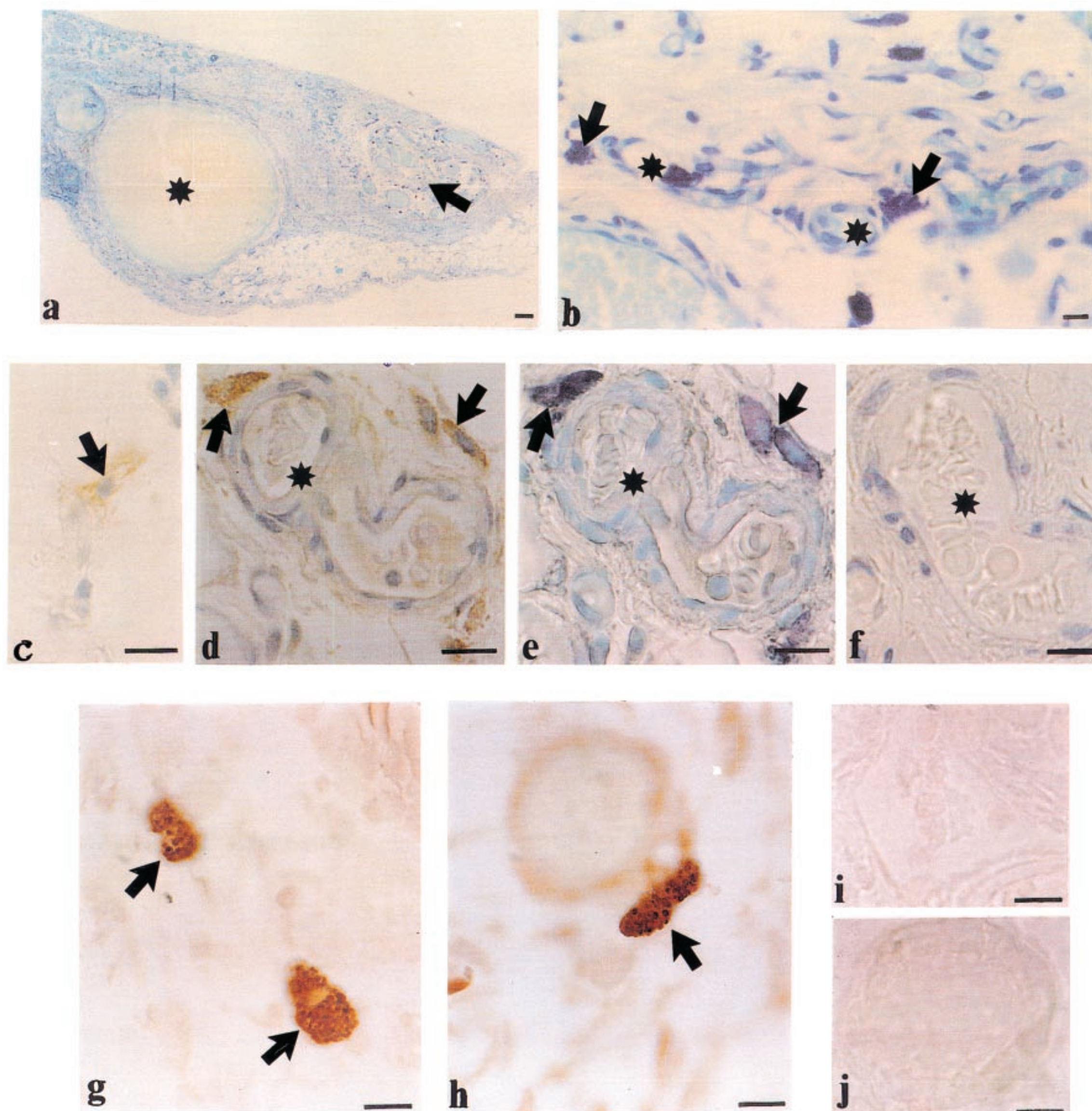

Figure 4 (a,b) Toluidine blue-stained cross-section of the angiogenic zone of an epigastric vascular pedicle obtained 7 days post-cauterization from a saline-treated rat. In (a) note the epigastric vein (asterisk) and surrounding areas of vascularized connective tissue (arrow). (b) A higher power micrograph of the area indicated by the arrow in (a). Large granular purple cells (mast cells, arrows) lie adjacent to microvessels (asterisks). Five-micron-thick paraffin sections. Scale bar in (a) = 100 μm, scale bar in (b) = 10 μm (c) Slight iNOS immunoreactivity in a large granular cell (arrow) in an epigastric pedicle from an unoperated rat. (d–f) The angiogenic zone of an epigastric vascular pedicle obtained 7 days post-cauterization from a saline-treated rat, microvessel indicated by asterisk. (d) Stronger iNOS immunoreactivity in large granular cells (arrows) surrounding an arteriole. (e) The same tissue section as (d) now additionally stained with toluidine blue. Purple (metachromatic) staining indicating mast cells (arrows) is confined to the iNOS immunoreactive cells confirming iNOS occurs in mast cells of the angiogenic zone. (The DAB deposits of iNOS positive granules now appear black). (f) Negative control section (without primary antibody). All sections counterstained with haematoxylin. Scale bars for (c–f) = 10 μm. (g–j) Micrographs of the angiogenic zone of an epigastric vascular pedicle obtained 7 days post-cauterization from a saline-treated rat. (g) Immunoreactive VEGF within granules in mast cells (arrows). (h) Dense bFGF immunoreactive granules in a mast cell (arrow). (i) Negative control (without primary antibody) for (g) and (j) negative control for (h). Scale bars for (g–j) = 10 μm.

neovessels (Papapetropoulos *et al*., 1997). Loss of inhibition of platelet activation by NO with a consequent increase in growth factor release may explain the pro-angiogenic effects of NOS inhibition by L-NMMA in the chorioallantoic membrane model (Pipili-Synetos *et al*., 1993) and the pro-angiogenic action of L-NAME in the current study.

The mast cell is a significant source of bFGF (Qu *et al*., 1995) and all four isoforms of VEGF have recently been identified in the human mast cell tumour line HMC-1, and in isolated human dermal mast cells (Grutzkau *et al*., 1999). Mast cells and macrophages have well-established roles in angiogenesis as sources of angiogenic factors. Immunoreac-

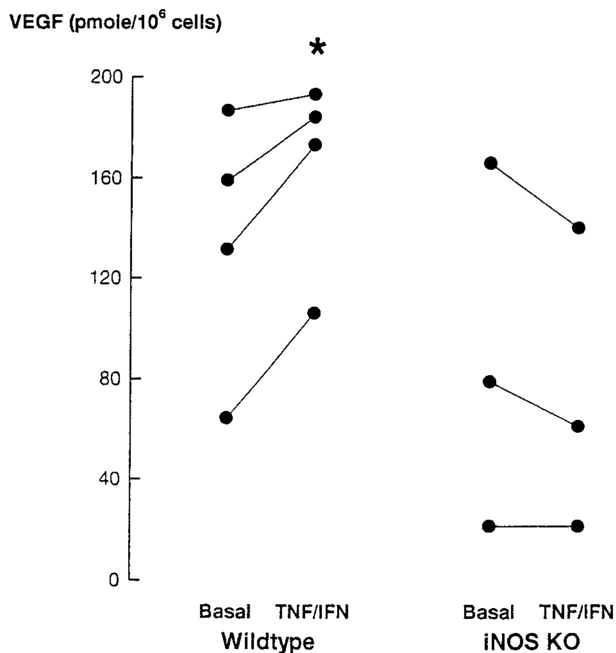

Figure 5 Bone marrow-derived mast cell VEGF content. VEGF was measured in the media of cells incubated in the absence or presence of tumour necrosis factor-α and interferon γ (TNF-α/IFNγ). Cells were obtained from either wild-type or iNOS KO bone marrow. * Indicates a significant ($P<0.05$) increase in levels of VEGF compared with basal levels.

tive iNOS, VEGF and bFGF were co-localized to mast cells in the angiogenic zone of the reconstituting vascular pedicle. In assessing the likelihood that mast cell-derived NO directly influences angiogenesis, it is relevant to consider that the levels of iNOS-derived NO produced by bone marrow-derived mast cells ($\sim$2 nmol 10^6 cells^{-1}) *in vitro* are less than one tenth of those produced by macrophages.

Furthermore, in view of the low tissue density of mast cells, it seems unlikely that mast cell-derived NO is a direct mediator of angiogenesis. We considered the possibility that the influence of mast cell iNOS activity was indirect due to an influence on the release of potent angiogenic factors.

In view of the evidence linking NOS activity and VEGF action (Parenti *et al*., 1998; Ziche *et al*., 1997a,b), and that linking VEGF and NO release (Hood *et al*., 1998; Tuder *et al*., 1995) we examined the influence of iNOS-derived NO on mast cell VEGF levels using mouse bone marrow-derived mast cells. Induction of iNOS was associated with upregulation of levels of VEGF in wild-type, but not KO mice. Furthermore, our previous experiments using the RBL-2H3 mast cell line indicated that lower concentrations of peroxynitrite ($ONOO^-$) enhanced mast cell degranulation (Barker & Stewart, 1998). This latter observation is in contrast to the well established inhibitory effects of NO on mast cell degranulation. Thus NO either directly or by conversion to peroxynitrite, increases both the amount and the release of VEGF from mast cells, thereby facilitating angiogenesis. While further work is required to elucidate the mechanism underlying the iNOS-mediated upregulation of VEGF in mast cells, a similar effect of iNOS activity on VEGF was recently observed in human carcinoma cell lines (Ambs *et al*., 1998). In murine peritoneal macrophages, LPS/IFNγ-induced NOS activity appeared to be responsible for upregulation of VEGF release (Xiong *et al*., 1998). In addition, exogenous NO increased VEGF expression in non-exercised rat skeletal muscle (Benoit *et al*., 1999). NO also appears to have a role in hypoxic induction of VEGF in vascular smooth muscle (Tsurumi *et al*., 1997). Although our data support a role for iNOS in facilitating angiogenesis, impairment of angiogenic responses in eNOS KO mice (Lui *et al*., 1998) suggests that proangiogenic effects of NO are dominant, irrespective of the NOS isoform which produces the NO.

Our observations suggest that VEGF expression and release are upregulated in part by iNOS induction. This mechanism is distinct from, but does not conflict with the model advanced by Ziche and colleagues which suggests that increased endothelial NOS activity in response to VEGF receptor activation leads to endothelial cell proliferation (Parenti *et al*., 1998; Ziche *et al*., 1997b). Given the complexity of the *in vivo* model used in the present study, a combination of these mechanisms could contribute to the effects of NOS inhibitors or iNOS gene knockout on flap survival. Moreover, given the multitude of components of angiogenic processes that are affected by NO, it is not surprising that the overriding influence of NO on angiogenesis is both context and NOS-isoform dependent. Thus, the relationship between NO, VEGF and angiogenic responses is influenced by the context (physiological, injury, hypoxia), amount and location of NO production.

Currently, there is considerable interest in the systemic treatment of chronic inflammatory disease with iNOS inhibitors. Our findings suggest that inhibition of angiogenesis may contribute significantly to the therapeutic effect of iNOS inhibitors in inflammation, but also indicate the possibility that such use of iNOS inhibitors will be accompanied by significantly delayed wound healing (Nathan, 1997). The therapeutic potential of NOS isoform-selective inhibitors for control of angiogenesis in inflammatory conditions and tumour growth warrants further investigation.

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