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Tumour-derived and host-derived nitric oxide differentially regulate breast carcinoma metastasis to the lungs

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To study the role of nitric oxide (NO) in lung metastasis of breast carcinoma, we isolated two cell clones (H and J) from the parental EMT-6 murine breast carcinoma cell line, based on their differential NO production. *In vitro*, EMT-6J cells, but not EMT-6H cells, constitutively expressed inducible NO synthase (NOS II) and secreted high levels of NO. IL-1β increased NO production in both clones, and TNF-α had a synergistic effect on IL-1β-induced NO production, but NO production by EMT-6J cells was always higher than by EMT-6H cells. Proliferation, survival and adhesion to lung-derived endothelial cells of both clones were similar and were not affected by NO. *In vivo*, both clones similarly located in the lungs of syngeneic mice 48 h after injection. However, EMT-6H cells were significantly more tumorigenic than EMT-6J cells as assessed at later time points. Injection of EMT-6J cells and simultaneous treatment of mice with aminoguanidine (AG), a NOS II inhibitor, significantly increased tumour formation. Injection of EMT-6H and EMT-6J cells into NOS II-deficient mice resulted in a significant survival increase as compared with wild-type animals. Simultaneous administration of AG increased the death rate of NOS II-deficient mice injected with EMT-6J cells. These results demonstrate that: (i) NO does not influence the early stages of tumour metastasis to the lungs and (ii) NOS II expression in tumour cells reduces, while NOS II expression in host cells enhances, tumour nodule development. In conclusion, the cellular origin and the local NO production are critical in the metastatic process.

Introduction

The metastatic and growth potential of tumours is dependent on the ability of circulating tumour cells to escape immune responses, to adhere to endothelium, to extravasate and to survive and grow in the target tissue. Nitric oxide (NO) is a potent bioactive molecule produced by nitric oxide synthase (NOS) enzymes. NO has been involved in numerous physiological as well as pathological mechanisms such as vasodilatation and inflammation. Demonstration of NO and of inducible NOS (NOS II) expression in various solid tumours and their metastases as well as in tumour cell lines has suggested that this biological mediator may participate in tumour survival and metastatic process. However, conflicting results have been reported that may reflect a more complex effect of NO than anticipated previously. NO has been shown to decrease the expression of adhesion molecules such as VCAM-1, ICAM-1, E-selectin that mediate interactions between tumour cells and host endothelial cells (1–3). Furthermore, NO could impair tumour cell extravasation and extracellular matrix (ECM) degradation through inhibition of the secretion of matrix metalloproteinases (MMPs) such as MMP-2 (4). Sustained overproduction of NO may also enhance tumour cell susceptibility to apoptosis and death through the expression or activation of cell death effectors such as p53 (5) or caspases and Fas (6). These observations suggest a negative role of NO on tumour progression or metastasis. However, protumorigenic effects of NO have also been reported. NO vasodilatory and angiogenic properties were associated with tumour growth and may participate in tumour-induced immunosuppression by decreasing proliferation and activation of lymphocytes (7). The tumour microenvironment may also influence tumour development as low NO concentrations may favour angiogenesis and tumour growth while higher NO concentrations may be cytostatic (8,9).

Breast tumours mainly metastasize to the lungs and bones (10). In the lungs, mammary tumour cells are retained in capillaries (11) where they adhere to endothelial cells through specific adhesion molecules such as integrins, selectins and N-cadherin. Tumour cells may then invade the lung parenchyma following proteolysis of the ECM through secretion of proteolytic enzymes such as MMP-2 (12). In previous work we had observed high levels of NOS II reactivity in malignant human breast cancer, which negatively correlated with proliferation and tumour grade (13). In order to define in more detail the role of NO in the metastatic process, we developed an experimental model consisting of high and low NO-producing murine breast carcinoma cell clones derived from the parental mammary EMT-6 tumour cell line (14). The *in vitro* proliferation and adhesive properties to murine lung-derived endothelial cells were determined. The implication of tumour-derived and of host-derived NO in the metastatic potential of both clones to the lungs was further investigated in syngeneic NOS II-deficient mice. How the cellular origin of NO may differentially regulate tumour metastasis is discussed.

Abbreviations: AG, aminoguanidine; EMEM, Eagle's minimal essential medium; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS II, inducible nitric oxide synthase.

Materials and methods

Cell selection

The cell clones EMT-6H and EMT-6J, syngeneic to Balb/c mice, were obtained by limiting dilution from the EMT-6 parental breast tumour cell line (kindly provided by Dr M.Lepoivre, CNRS, Orsay, France). They were selected based on NO production: EMT-6J released significant levels of NO while EMT-6H did not. Cells were grown as monolayers at 37°C in Eagle's minimal essential medium (EMEM, Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum (FCS Hyclone, Biovalley, Marne la Vallée, France) and 2 mM L-glutamine (Biowhittaker, Fontenay sous Bois, France), in a humidified atmosphere (5% CO_2 and 95% air). Cultures were tested monthly for *Mycoplasma* contamination with the mycotect kit (Life Technologies, Cergy Pontoise, France) and before each *in vivo* experiment.

Cell culture protocols

For cytokine-stimulated NO production, EMT-6H and EMT-6J cells (5×10^4 cells/well) were grown for 24 h in 96-well plates then treated for another 24 h with the cytokines IL-1β (Sanofi, Labège, France), TNF-α and IFN-γ (Insight Products, UK). When indicated, cells were grown for 24 h with 0.5 mM aminoguanidine (AG) (Sigma-Aldrich, Lyon, France). For proliferation assay, EMT-6H and EMT-6J cells (5×10^3 cells/well) were grown in 96-well plates for 48 h in the presence or not of 0.5 mM AG. Proliferation was assessed after 48 h by incorporation of [^{3}H]thymidine (1 μCi/well, Amersham Pharmacia, Buckinghamshire, UK) for the last 2 h of incubation. The cells were washed twice with PBS and adherent cells were lysed in 1 N NaOH for 2 h at 60°C. Radioactivity was determined by liquid scintillation spectrometry (LKB Rackbeta LKB Wallac, Turku, Finland). Spontaneous cell death was measured as described previously (15). Briefly, 2.5×10^5 EMT-6H or EMT-6J cells were seeded in 6-well plates and incubated overnight at 37°C with 1 μCi [^{3}H]thymidine. The labelled cells were detached and seeded (25×10^3 cells/ml, 200 μl/well) in a 96-well plate for 48 h. Cells were lysed and cell-associated radioactivity determined as above. For the determination of cellular ability to adhere to plastic (15,16), EMT-6H or EMT-6J cells (10^4 cells/well) were seeded in 96-well plates and incubated at 37°C for 48 h. Cells were washed with PBS, fixed in methanol, stained with 1% methylene blue for 30 min, dissolved in 0.1 N HCl and absorbance was measured at 620 nm in a multi-well microtitre plate reader (Anthos, Labtech Instruments, Emerainville, France). Alternatively EMT-6H or EMT-6J cells (5×10^3 cells/well) were seeded in 96-well plates and incubated at 37°C for 48 h. Cells were washed with 0.9% NaCl, stained with neutral red for 2 h, fixed in a 0.4% formalin–1% $CaCl_2$ solution and extracted in 1% acetic acid in 48% ethanol. Absorbance was measured at 540 nm in a multi-well microtitre plate reader. Cellular survival was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (17). Briefly, EMT-6H or EMT-6J cells (5×10^3/well) were seeded in 96-well plates and incubated at 37°C for 48 h. Then 10 μl of 5 mg/ml MTT in PBS were added for 2 h. Formazan crystals were dissolved in 200 μl DMSO and absorbance was measured at 570 nm in a multi-well microtitre plate reader.

Determination of NO production

NO production was determined as nitrite and nitrate accumulation in cell culture media using the Griess microassay (18). Briefly, nitrates were reduced to nitrites using nitrate reductase (Diffchamb, France) and 100 μl of reduced supernatants were added to 100 μl Griess reagent (0.5% sulfanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid). Absorbance was measured at 540 nm in a multi-well microtitre plate reader and nitrite concentration was calculated by comparison with a standard sodium nitrite solution.

NOS II RT–PCR analysis

Total RNA was prepared using the Nucleospin® RNA II kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's protocol. 550 ng RNA were used for NOS II amplification and 125 ng for GAPDH amplification. RT–PCR was performed using the two-enzyme Access RT–PCR system (Promega, Charbonnière, France). First strand cDNA synthesis was carried out at 48°C for 45 min, then the AMV reverse transcriptase was inactivated and the RNA–cDNA hybrid denatured by a 2-min incubation at 94°C. Amplifications were performed using 25 cycles as follows: 94°C/30 s, 63°C/30 s, 68°C/1 min. The sequences of NOS II RNA, located between +1379 and +1877, and GAPDH RNA, located between +256 and +750, were amplified using the following sense and antisense primers: 5′-CCAGTGTCTGGGAGCATCACCCCTG-3′ and 5′-GAACTGAGGGTACATGCTGGAGCC-3′ (NOS II) and 5′-GCCCATCACCATCTTCCAGGAGCG-3′ and 5′-GGGGTAGGAACACGGAAGGCCATGC-3′ (GAPDH). The PCR products migrated as a single band of 498 bp for NOS II and of 504 bp for GADPH.

In vitro adhesion of tumour cell clones to lung-derived endothelial cells

The End p23 murine pulmonary endothelial cell line and the adhesion assay have been described previously (19,20). Briefly, sub-confluent End p23 cells were cultured for 24 h in 48-well microtitre plates and washed twice with PBS immediately before the adhesion assay. EMT-6H and EMT-6J tumour cells were labelled with 0.4 μCi/ml of [^{3}H]thymidine for 16 h and added (10^5 cells/well) to confluent End p23 monolayers in Dulbecco's Modified Eagle's medium (DMEM) without FCS. After 30, 60 or 180 min incubation at 37°C non-adherent cells were removed by washing three times with DMEM. Adherent cells were dissolved overnight in 0.1% SDS/0.1 M NaOH and radioactivity was quantified by liquid scintillation spectrometry. Adhesion (%) was determined as the ratio of the c.p.m. of adherent cells to the c.p.m. of total (adherent and non-adherent) cells × 100.

Maintenance and genotyping of NOS II-deficient mice

C57BL/6×129SvEv mice with a targeted disruption of the NOS II gene (NOS II^{-}/$_{-}$) (21) were obtained from Dr Y.Courtois (INSERM U450, Paris, France) and mated in our animal facility with Balb/c wild type mice ($^{+}$/$_{+}$) (Charles River Laboratories, l'Arbresle, France) for nine generations to produce heterozygous NOS II-deficient mice ($^{+}$/$_{-}$). Heterozygous NOS II-deficient mice were mated to produce NOS II $^{-}$/$_{-}$ mice. Genotyping to confirm the presence or absence of the NOS II gene was performed using PCR of DNA from tail biopsies. Samples were incubated overnight at 54°C in lysis buffer [100 mM Tris, pH 8.5, 5 mM ethylene diamine tetraacetic acid (EDTA), 200 mM NaCl, 0.2% SDS and 100 μl proteinase K (10 mg/ml)]. After centrifugation, the DNA was purified by two cycles of isopropanol precipitation and phenol–chloroform extraction, precipitated and washed in 70% cold ethanol and resuspended in 100 μl of Tris–EDTA (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8). DNA products were amplified by 30 cycles of PCR (94°C/30 s, 60°C/30 s and 72°C/45 s) using *Taq* DNA polymerase (Eurobio, Les Ulis, France). The sequence of the NOS II gene located between −499 and +161 was amplified using the following sense and antisense primers: 5′-GCACAGCCCATCCACTATTCT-3′ and 5′-GAGTGAACAAGACCCAAGCGT-3′. To identify the NOS II +/− and −/− mice, the amplification of the neo resistance gene was performed using the sense and antisense oligonucleotides: 5′-ATCGACAAGACCGGCTTCCATCCGA-3′ and 5′-TCAGCGCAGGGGCGCCCGGTCTTT-3′, which amplify a 475 bp PCR product. The amplified products were electrophoresed in 1% agarose gel containing ethidium bromide.

Tumour nodule induction in lungs

Animal experiments were performed using 8–9-week-old female wild-type mice and NOS II-deficient (NOS II KO) female Balb/c mice. All experimental protocols had been approved by the Ethics Committee of the University of Dijon, France. EMT-6H or EMT-6J cells in exponential growth phase were harvested, dissociated to produce single-cell suspensions, washed and suspended in culture medium. Cell viability was determined by trypan blue exclusion and only cell suspensions with >90% viability were used for animal experimentation. 100 000 cells in 0.1 ml of EMEM were injected into the lateral tail vein of non-anaesthetized mice. The mice were killed after 35 days. Killed and spontaneously dead mice were autopsied and pulmonary nodules were counted. AG-treated mice received intraperitoneal (i.p.) injections of 50 mg/kg AG, an inhibitor of NOS II activity (22), twice a day, throughout the experiment.

^{111}Indium oxine labelling procedure and location of ^{111}In-labelled cells in mice

EMT-6H or EMT-6J cells [2×10^6 cells in 0.5 ml EMEM–HEPES (Biowhittaker, Fontenay sous Bois, France)] were incubated with 85 μCi of ^{111}indium oxine (Mallinckrodt, Les Ulis, France) for 15 min at 37°C as described previously (23). Cells were washed three times with EMEM–HEPES to remove free ^{111}In. Cell-bound ^{111}In radioactivity was measured using a gamma counter (MR 480 Packard, Packard Bioscience, Meriden, CT). Labelled cells (10^5 cells) were injected in the tail vein of mice. Radioactivity location was determined in anaesthetized animals using a single-head Basicam gamma camera (Siemens Medical System, Erlangen, Germany) for image acquisition consisting of 30 images of 1 min each. The images were analysed with the SMV-GE Vision processing system (Sopha Medical Vision-General Electric, Buc, France). Thorax and abdomen were manually defined and the radioactivity in the two regions was calculated based on a summed image. Mice were killed at different time points after injection, and heart, liver and lungs were collected, washed with 0.9% NaCl and their ^{111}In content was determined in an automatic well counter (MR 480 Packard, Packard Bioscience).

Data analysis

Data are presented as mean ± SD. Statistical differences were determined by unpaired Student's *t* test or χ^2 test. Survival rates were calculated using the Kaplan–Meier method, according to tumour cell clone or treatment. Curves

Table I. Spontaneous and cytokine-stimulated NO production by EMT-6H and EMT-6J cells

NO production ($NO_2^- + NO_3^-$) (μM)	EMT-6H cells	EMT-6J cells
Medium	0.29 ± 0.20	4.32 ± 0.85
Medium + AG	0.61 ± 0.10	0.77 ± 0.33[†]
IL-1β	11.84 ± 1.53[*]	29.07 ± 3.05[*]
TNF-α	0.69 ± 0.53	4.11 ± 0.51
IFN-γ	0.05 ± 0.17	4.39 ± 0.53
IL-1β + TNF-α	24.03 ± 0.74[*]	40.49 ± 1.20[*]
IL-1β + IFN-γ	11.72 ± 1.49[*]	29.67 ± 2.80[*]
TNF-α + IFN-γ	0.52 ± 0.31	2.07 ± 0.97
IL-1β + IFN-γ + TNF-α	18.71 ± 3.19[*]	34.01 ± 5.70[*]
IL-1β + IFN-γ + TNF-α + AG	0.56 ± 0.25[#]	0.10 ± 0.53

Cells were grown for 24 h then treated with cytokines for another 24 h. Cytokines were used at the following concentrations, IL-1β, 55 pg/ml; TNF-α, 4.5 ng/ml and IFN-γ, 500 pg/ml. NO production was measured as nitrate + nitrite accumulation in culture media. Results are the means of four independent experiments. The statistical significance of the data was analysed using an unpaired Student's *t* test. NO production by EMT-6J versus EMT-6H cells were always significantly higher ($P < 0.001$) except with TNF-α + IFN-γ.

[*] $P < 0.001$, NO production of stimulated EMT-6H or EMT-6J cells versus unstimulated controls.

[#] $P < 0.001$, NO production of stimulated EMT-6H or EMT-6J cells in the absence versus in the presence of AG.

[†] $P < 0.001$, NO production of unstimulated EMT-6J in the absence versus in the presence of AG.

were compared using the log rank test. Survival rates were computed using the Stata package (Stata 1999, College Station, TX). Differences were considered significant for $P \leq 0.05$.

Results

Two cell clones were isolated by limiting dilution from the parental EMT-6 murine mammary carcinoma cell line and selected from their production of NO (measured as the accumulation of nitrites plus nitrates in the culture supernatant). EMT-6H cells produced NO levels below the detection threshold whereas EMT-6J cells released up to 5 μM $NO_2^- + NO_3^-$/48 h/5 × 10^4 cells (Table I). EMT-6J cells, but not EMT-6H cells, expressed NOS II mRNA (Figure 1) as detected by RT–PCR amplification. IL-1β increased NO production while TNF-α and IFN-γ had no effect (Table I). Synergistic effect was observed with IL-1β plus TNF-α but not with IL-1β plus IFN-γ or TNF-α plus IFN-γ (Table I). In all treatments, NO released in response to cytokines by EMT-6J cells was always higher than that of EMT-6H cells ($P < 0.001$, Table I).

Spontaneous as well as induced NO production was inhibited by 0.5 mM AG (Table I). *In vitro* proliferation, spontaneous cell death, cellular ability to adhere to plastic and cellular metabolic activities were similar for both cell clones and were not affected by AG. These results excluded a direct role of NO on the growth and survival of EMT-6H or EMT-6J tumour cells *in vitro*. *In vitro* proliferation was measured by [^{3}H]thymidine incorporation, EMT-6H cells with or without AG: 266 × 10^3 ± 13 × 10^3 and 234 × 10^3 ± 24 × 10^3 c.p.m., respectively, and EMT-6 J cells with or without AG: 253 × 10^3 ± 32 × 10^3 and 239 × 10^3 ± 14 × 10^3 c.p.m., respectively. Spontaneous cell death was measured by [^{3}H]-thymidine release, EMT-6H cells with or without AG: 100 × 10^3 ± 7 × 10^3 and 96 × 10^3 ± 9 × 10^3 c.p.m., respectively, and EMT-6J cells with or without AG: 112 × 10^3 ± 15 × 10^3 and 134 × 10^3 ± 55 × 10^3 c.p.m., respectively. Cellular ability to adhere to plastic was determined by methylene blue assay, absorbance at 620 nm, EMT-6H cells with or without AG: 1.63 ± 0.21 and 1.46 ± 0.17, respectively, and EMT-6 J cells with or without AG: 1.80 ± 0.09 and 1.77 ± 0.08, respectively. Cellular metabolic activities were assessed by neutral red exclusion and MTT reduction. Neutral red exclusion, absorbance at 540 nm, EMT-6H cells with or without AG: 0.82 ± 0.05 and 0.79 ± 0.04, respectively, and EMT-6J cells with or without AG: 0.82 ± 0.1 and 0.80 ± 0.04, respectively. MTT reduction, absorbance at 570 nm, EMT-6H cells with or without AG: 0.71 ± 0.09 and 0.78 ± 0.06, respectively, and EMT-6J cells with or without AG: 0.65 ± 0.05 and 0.77 ± 0.07, respectively.

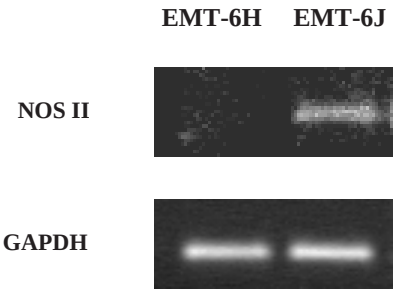

Fig. 1. EMT-6J cells but not EMT-6H cells express NOS II mRNA Cells were grown to sub-confluence then total RNA was prepared for RT–PCR amplification. The results of one representative experiment out of three are shown.

For tumour cell location in organs after i.v. injection, the tumour cells were labelled with ^{111}indium oxine. *In vitro* proliferation and survival of labelled EMT-6H and EMT-6J cells were similar for both clones, as >80% of labelled H or J cells were alive after 48 h culture. 100 000 of ^{111}In-labelled cells were injected in the tail vein of syngeneic mice and tumour cell location was evaluated by whole mice scintigraphy images, within 48 h, both clones located mainly in the thorax. As a control, injection of cell-free ^{111}indium oxine in mice resulted in a homogenous distribution over the whole mice (data not shown). The organs of mice injected with ^{111}In-labelled EMT-6H or EMT-6J cells were excised at different times and radioactivity was quantified. Three independent experiments showed that 48 h after tumour cells injection ~40% of total injected radioactivity was retained in the lungs for both cell clones. The radioactivity ratio (mice injected with EMT-6H to EMT-6J cells) measured in the lungs was stable for 48 h (Figure 2), indicating that short-term tumour cell location in the lungs was independent of clones. Radioactivity was also detected in the liver (results not shown), but as tumour nodules were never observed in this organ, liver-associated radioactivity was attributed to accumulated dead cells.

As *in vivo* experiments showed that both tumour cell clones located in the lungs within 48 h, we evaluated the *in vitro* adhesive properties of EMT-6H and EMT-6J cells on murine lung-derived p23 endothelial cells. Short-term (30 or 60 min) and long-term (3 h) adhesion assays were performed (Figure 3) and showed that both EMT-6H and EMT-6J cells adhered similarly to lung-derived endothelial cells *in vitro*. Taken together these results suggest that tumour-derived NO was not critical in one of the first steps of tumour nodule formation, the initial arrest of breast tumour cells in the pulmonary endothelium.

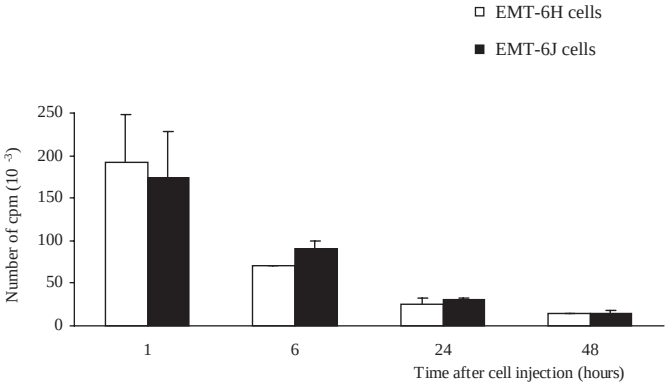

Fig. 2. ^{111}Indium-labelled EMT-6H or EMT-6J tumour cells location in the lungs was independent of clones EMT-6H and EMT-6J cells were labelled with ^{111}indium oxine (^{111}In) and injected in the tail vein of syngeneic mice. The results from one representative experiment out of four are shown. Radioactivity measured in the excised lungs of mice at different times after i.v. injection of 10^5 ^{111}In-labelled EMT-6H (clear bar) or EMT-6J cells (black bar).

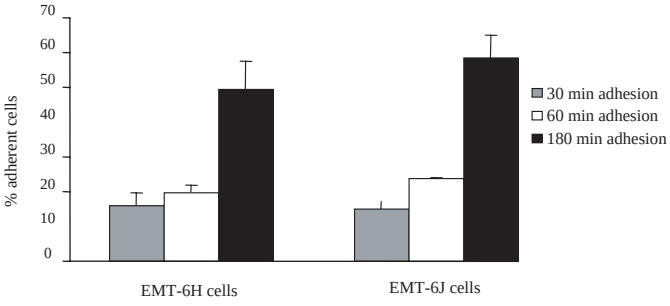

Fig. 3. Adhesion of EMT-6H and EMT-6J cells to murine lung-derived endothelial cells. Adhesion of [^{3}H]thymidine-labelled EMT-6H cells or EMT-6J cells to endothelial cells was measured *in vitro* after either 30 (shaded bar), 60 (clear bar) or 180 min (black bar). Results are expressed in per cent of adherent cells and are the means of triplicate wells ± SD. Three independent experiments were performed. No statistically significant difference was found.

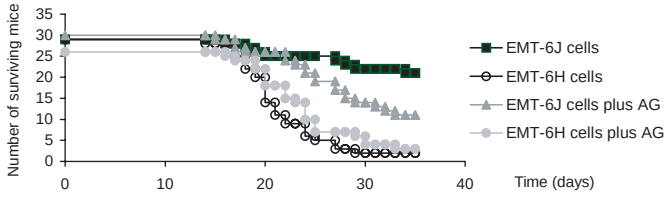

Fig. 4. Survival of mice injected with EMT-6H cells or EMT-6J cells. Mice were injected in the tail vein with 10^5 EMT-6H cells or EMT-6J cells and mice survival was determined. AG was injected i.p. twice a day. Surviving mice were killed 35 days after the injection of tumour cells.

However, long-term survival experiments showed a significant difference between mice injected with EMT-6H or EMT-6J cells. Twenty-seven out of twenty-nine mice (93%) injected with EMT-6H cells died within 35 days, while only eight out of 29 mice (28%) injected with EMT-6J cells died before day 35 ($P < 0.0001$, Figure 4). In contrast, 19 out of 30 mice (63%) injected with EMT-6J cells and receiving AG (50 mg/kg i.p. injected twice a day for 35 days), an inhibitor of NOS II activity, died within 35 days (Figure 4, $P = 0.0071$,

Table II. Counting of tumour nodules in the lungs of mice injected either with EMT-6H or EMT-6J cells

Number of nodules per lung	Number of mice bearing lung tumour nodules/number of mice injected			
	EMT-6H cells	EMT-6J cells*	EMT-6H cells and treated with AG#	EMT-6J cells and treated with AG†
0	0/29	8/29	0/26	5/30
1–10	0/29	9/29	0/26	1/30
11–50	3/29	6/29	2/26	10/30
51–100	3/29	0/29	4/26	2/30
>100	23/29	6/29	20/26	12/30

The number of nodules in the lungs of mice injected in the tail vein with either EMT-6H or EMT-6J cells, and treated or not treated with AG, was counted. The cumulative data are from the autopsies of spontaneously dead mice or mice killed 35 days after tumour cell injection. The statistical significance of the results was analysed using a χ^2 test.
*$P < 0.001$ EMT-6J cells compared with EMT-6H cells.
#n.s. EMT-6H cells + AG compared with EMT-6H cells.
†$P < 0.01$ EMT-6J cells + AG compared with EMT-6J cells.

mice treated with AG compared with untreated mice). On the other hand, the life span of mice injected with EMT-6H cells did not change when mice were treated with AG. EMT-6H cells induced the formation of numerous tumour nodules in the lungs of all the injected mice (Table II). In contrast, the number of mice with pulmonary tumour nodules was lower and the number of nodules per lung was lower ($P < 0.001$) in the EMT-6J group (Table II). AG treatment increased the number of tumour nodule-bearing mice and decreased the number of tumour nodules per lung in mice injected with EMT-6J cells ($P < 0.01$) but had no significant effect in mice injected with EMT-6H cells (Table II). Therefore, there was a relationship between the life span of mice and the number of tumour nodules they bore.

As i.p. injection of AG would result in inhibition of NOS II activity both in tumour cells and in host cells, these experiments did not allow to evaluate the contribution of host-derived NO in the formation of tumour nodules in the lungs. To further investigate the respective roles of tumour cell-derived or host-derived NO, we used NOS II-deficient mice. Twenty-four out of twenty-seven (90%) wild-type mice injected with EMT-6H cells died within 35 days, while 16 out of 27 (60%) NOS II-deficient mice (Figure 5A, $P = 0.0006$) died within the same time. Seven out of twenty-eight (25%) wild-type mice injected with EMT-6J cells, while two out of 30 (7%) NOS II-deficient mice (Figure 5B, $P = 0.06$) died within 35 days. The number of tumour nodules in the lungs of NOS II-deficient mice injected with EMT-6H was lower than in wild-type mice ($P = 0.016$). In mice injected with EMT-6J cells, the number of tumour nodule-bearing mice and the number of tumour nodules per lung were lower in NOS II-deficient mice than in wild-type mice ($P < 0.01$) (Table III). Taken together, these results demonstrated that tumour growth and mice survival was increased in NOS II-deficient mice independently of the cell clone injected.

None of the nine NOS II-deficient mice injected with EMT-6J cells died within 35 days while three out of the nine mice receiving AG died within the same time ($P < 0.05$, Figure 6). The number of tumour nodule-bearing mice and the number of tumour nodules per lung increased in AG-treated mice (data

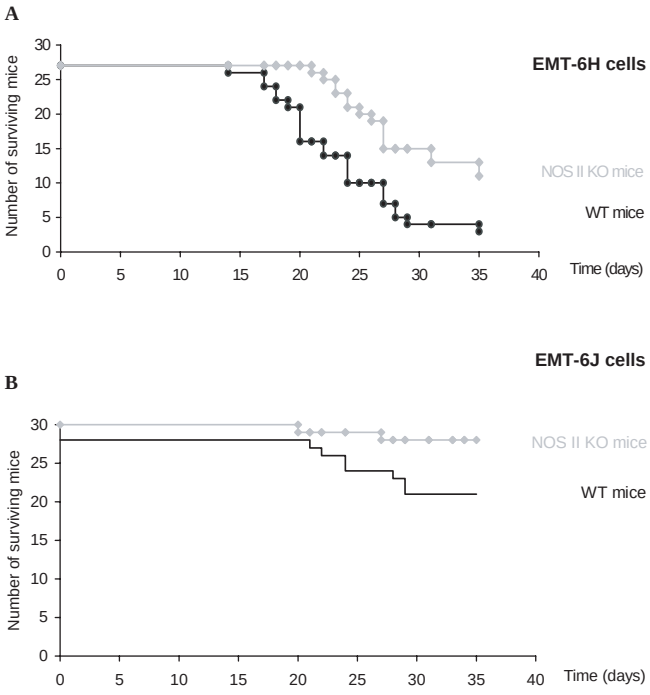

Fig. 5. Survival of wild-type (WT) mice (circles) and NOS II KO mice (diamonds) injected with EMT-6H cells or EMT-6J cells. Mice were injected in the tail vein with 10^5 EMT-6H (**A**) or EMT-6J (**B**) cells and mice survival was determined. Surviving mice were killed 35 days after the injection of tumour cells.

Table III. Counting of tumour nodules in the lungs of wild-type and NOS II-deficient mice injected either with EMT-6H or EMT-6J cells

Number of nodules per lung	Number of mice bearing lung tumour nodules/number of mice injected			
	EMT-6H cells		EMT-6J cells	
	Wild type mice	NOS II-deficient mice*	Wild type mice	NOS II-deficient mice#
0	0/27	0/27	0/28	4/30
1–10	1/27	9/27	16/28	23/30
11–50	1/27	4/27	6/28	3/30
51–100	5/27	2/27	0/28	0/30
>100	20/27	12/27	6/28	0/30

The number of nodules in the lungs of mice injected in the tail vein with either EMT-6H or EMT-6J cells was counted. The cumulative data are from the autopsies of spontaneously dead mice or mice killed 35 days after tumour cell injection. The statistical significance of the results was analysed using a χ^2 test.
*$P = 0.016$ wild-type mice compared with NOS II-deficient mice.
#$P < 0.01$ wild-type mice compared with NOS II-deficient mice.

not presented because of marginal statistical significance). Therefore, NO production by tumour cells prevented tumour progression.

Discussion

It was shown previously that NOS II is heterogenously expressed in human breast cancer (24). NOS II expression has been demonstrated using immunohistochemistry in mammary tumour cells (13,24–26), or in cancer stroma (25,27).

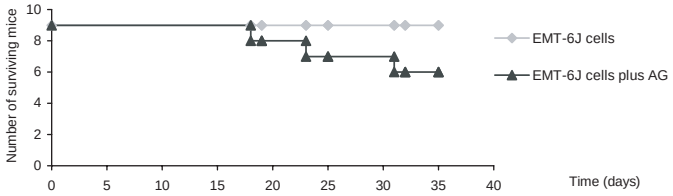

Fig. 6. Survival of NOS II KO mice injected with EMT-6J cells. Mice were injected in the tail vein with 10^5 EMT-6J cells and mice survival was determined. AG was injected i.p. twice a day or not. Surviving mice were killed 35 days after the injection of tumour cells.

Moreover, the evaluation of NOS II activity in human breast cancer gave inconsistent results, being either negatively correlated (13,25), positively correlated (26) or not correlated (24) to tumour grade. The role of NO in breast tumour progression is also controversial: NO may either favour metastasis by promoting cancer cell migration, invasiveness and angiogenesis (28–30,34), or antagonize metastasis by decreasing the expression of adhesion molecules (1–3).

To investigate whether the cellular origin of NO may affect the metastatic potential to the lungs of breast tumour cells we investigated first the role of tumour-derived NO. Toward this goal, we subcloned low and high NO-producing murine breast carcinoma cell lines from the parental EMT-6 cells (14). The results indicated that NO produced by tumour cell was not implicated *in vitro* in tumour cell proliferation, survival or adhesion to murine lung-derived endothelial cells. Thus, both EMT-6H and EMT-6J cells are NO resistant. *In vivo*, tumour cell-derived NO was not involved in the localization of tumour cells in the lungs of syngeneic mice within 48 h following i.v. injection. These data indicated that NO produced from tumour cells was not a determining factor in the initial arrest of tumour cells in the lung capillaries. However, *in vivo*, in the long term, the low NO-producing tumour cells (EMT-6H cells) developed more tumour nodules in the lungs than the high NO-producing tumour cells (EMT-6J cells). The death rate of mice injected with low NO-producing cells was also higher than that of mice injected with high NO-producing cells. These results suggested that NO may be protective at later metastatic stages than the initial tumour cells arrest in the lungs. In long-term *in vivo* experiments, inhibition of NOS II activity by AG (22) in tumour cells and in animals increased the death rate, the number of tumour-bearing mice and the number of nodules in mice injected with EMT-6J cells, but not in mice injected with EMT-6H cells, which rules out a toxic effect of AG. Thus, in NOS II-expressing mice, NO was protective against metastasis. In NOS II-deficient mice, the development in the lungs of tumour nodules was decreased and mice survival increased, whatever the tumour cell clone. These results indicate that NOS II expression by the host cells favours metastasis. Thus, in our model, NO had opposite effects depending on whether it was tumour cell-derived or host-derived. In agreement with our results, it was shown that NOS II disruption in mice enhanced metastasis of NO-sensitive murine ovarian sarcoma cells (31). And NOS II disruption in mice suppressed metastasis of NO-resistant murine melanoma cells, which was dependent on down-regulation of VEGF in melanoma metastases (32).

Thomsen *et al*. (33) have reported that NOS II inhibition by 1400W reduced the size of subcutaneous (s.c.) EMT-6 tumours. Thus, NO functions in the growth of s.c. tumour are different from its role in tumour metastasis to the lungs.

Another explanation of this disagreement is certainly the heterogeneity of the EMT-6 parental cells. Using two mammary tumour cell lines differing in their metastatic phenotype, Orucevic *et al*. (34) showed that NO promoted tumour cell invasiveness. This metastasis-promoting effect of NO released by tumour cell was attributed to NOS III, which produces only low amounts of NO, comparable with the levels secreted by EMT-6H cells. Our present results demonstrated that low levels of tumour-derived NO promoted tumour development. Thus, it appears that not only the cellular origin of NO, but also the level of NO secretion is crucial for its biological effects in cancer.

The mechanisms involved in NO-dependent reduction of the development of tumour nodules in the lungs are not yet elucidated. NO has been shown to decrease leukocyte adhesion to endothelial cells (35–37) through inhibition of the expression of adhesion molecules, either on endothelial cells (1) (such as VCAM-1) or on leukocytes (38) (such as E-selectin or ICAM-1). *In vitro* NO was shown to up-regulate the expression of ICAM-1 on tumour cells (3) and to increase the adhesion of tumour cells to endothelial cells (39). However, *in vivo* only few data are available. Our *in vitro* and short-term *in vivo* experiments did not support a NO-dependent adhesion of breast cancer cells to pulmonary endothelial cells. NO was also shown to inhibit platelet aggregation (40), which promotes metastasis. The regulating role of NO in inflammatory response could also be involved. In support of this possibility, we have shown previously that NO sensitizes colon tumour cells to Fas-mediated cell death *in vitro* (6) implying the involvement of NK or T lymphocytes. However, our studies do not exclude the possibility that phenotypic differences between the EMT-6H and EMT-6J cells other than NO-producing ability, e.g. the degree of MMP expression, may have added to the differences in their metastatic ability or mortality in tumour-bearing hosts.

In conclusion, our results demonstrated: (i) that NOS II expression by breast tumour cells reduces, while NOS II expression by host cells enhances, tumour nodule development in the lungs of syngeneic mice; (ii) that local NO concentration is important in this process; and (iii) that NO is not decisive in the initial arrest of breast tumour cells in lung capillaries, but is critical in later stages of nodule development.

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