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EFFECTS OF NITRIC OXIDE ON THE ADHESION OF HUMAN MELANOCYTES TO EXTRACELLULAR MATRIX COMPONENTS

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SUMMARY

The aim of the present study was to explore whether nitric oxide (NO) interferes with the attachment of human melanocytes to the extracellular matrix (ECM) components. Consequently, the effects have been investigated of the NO-releasing compounds 3-morpholino-sydnonimine (SIN-1) and *S*-nitroso-glutathione (GSNO) on the *in vitro* adhesion of human melanocytic cells to fibronectin. The NO donors induced a concentration-dependent reduction in the adhesion of both $^{51}CrO_4^{2-}$-labelled melanocytes and melanoma cells to fibronectin. Pigmented M14 melanoma cells were more susceptible to the effect of SIN-1 (half-maximal inhibiting effect at about 0·5 mM) than normal human melanocytes and also than the non-pigmented melanoma cells Mel57 (half-maximal inhibiting effects between 0·9 and 2 mM). This effect of SIN-1 also appeared to be related to the melanin content of normal melanocytes, whereas GSNO was significantly less active. Both flow cytometric analysis and immunocytochemical staining showed expression of neuronal NO synthase in all cell lines. The results of this study suggest that aberrant *in vivo* production of NO during infection and inflammation may contribute to loss of melanocytes in, for example, vitiligo, by reducing *de novo* attachment of melanocytes to the ECM. These findings could also be important for understanding the process of metastasis. © **1997 John Wiley & Sons, Ltd.**

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KEY WORDS—**nitric oxide (NO); extracellular matrix (ECM); fibronectin; adhesion; melanocytic cells**

INTRODUCTION

Nitric oxide (NO) is a major endogenous messenger molecule with multiple effects.[1] Under physiological conditions, NO can be interconverted among different redox forms with distinct chemistries,[2] including neutral nitric oxide ($NO^{\cdot}$), nitrosonium ion (NO^+), and nitroxyl radicals (NO^-), which could explain the varied biological responses related to the generation of one or more reactive NO redox species. NO is endogenously synthesized from L-arginine by several isoforms of NO synthase (NOS; EC 1.14.13.39).[3,4] Two of these are constitutively expressed (cNOS) and a third is inducible (iNOS) by variety of stimuli, such as cytokines and lipopolysaccharides. The NOS isoforms are responsible for the endogenous release of small amounts of NO, whereas iNOS generates large quantities of NO over an extended time period. The modulation of iNOS is mainly associated with inflammatory and immune responses.[1,3–5] The presence of *in situ* inflammatory cellular infiltrates in close proximity to normal human melanocytes (NHMs) has been attributed to the disappearance of these melanin-producing cells in vitiligo.[6,7] Indeed, melanocyte stimulation and altered melanin production are generally accompanied by acute and chronic skin inflammation.[8]

In spite of the growing interest in the activation of immune cells such as T lymphocytes and macrophages and NO production, little is known about the pathophysiological functions of NO redox species related to cell–cell and cell–matrix interactions involving NHMs. Adhesive processes are involved in the localization and function of NHMs within the basal layer of the epidermis. The interaction between integrins and extracellular matrix (ECM) components is considered important both for the normal process of repigmentation and for the loss of melanocytes in disease.[9,10] Similarly, interactions between melanoma cells (MCs) and the ECM are also essential to the process of communication with other cells as diverse as monocytes, lymphocytes, fibroblasts, endothelial cells, and parenchymal cells in distant organs, which can influence the outcome of metastasis.[11] Particularly under inflammatory conditions, reactive NO species contribute to the cytotoxic/cytostatic action of inflammatory cells against tumours as well as normal host cells. They might also become damaged in autoimmune diseases when NO is overproduced due to aberrant induction of NOS. Understanding the role of

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NO species in melanocyte functions, particularly with regard to their interaction with the ECM, may therefore be important for some pathological processes in pigmentary disorders.

In the present study, our working hypothesis is that NO, when generated *in vivo* during inflammation, can interfere with the adhesion of melanocytes in the skin and thereby may contribute to depigmentation. In order to test this assumption, we investigated the effects of 3-morpholino-sydnonimine (SIN-1),[12,13] a donor of reactive $NO^{\cdot}$, and *S*-nitroso-glutathione (GSNO),[2,14] as a potential NO^{+} donor, on the adhesion of NHMs and MCs to ECM components. The results of this study indicate that the adhesion to ECM (fibronectin) of melanocytes with different melanin contents can be reduced by NO redox species.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and HEPES (*N*-2-2-hydroxyethyl-1-piperazine-*N*′-2-ethanesulphonic acid) were from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.). Heat-inactivated fetal calf serum (FCS) was purchased from Gibco BRL Life Technologies Inc. (Breda, The Netherlands). Fibronectin, isolated from human plasma; bovine serum albumin (BSA); N^{G}-methyl-L-arginine (NMA); GSNO; and 5-bromo-2′-deoxyuridine (BrdU) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); human interferon-γ (r-hINF-γ) was a gift from Dr P. van der Heide, TNO-Rijswijk, The Netherlands.

The NO donors (SIN-1 and C87-3754),[13] kindly provided by Dr Grewe (Hoechst AG, Frankfurt, Germany), were freshly prepared before measurements.[13] Denitrosylated solutions of SIN-1 as negative controls and GSNO were obtained by incubating 50 mM solutions in DMEM containing 0·25 per cent BSA for 24 h at 37°C. Synthesized standard melanin was a gift from A. Kammeyer (Academic Medical Center, Amsterdam University, The Netherlands).

Culturing cell lines

NHM cultures, established from individual foreskins of different grades of pigmentation, were maintained essentially according to the method described previously by us.[15] The human MC lines were a kind gift from Professor Dr D. Ruiter (Nijmegen University, The Netherlands). The pigmented M14 and the non-pigmented Mel57 were cultured using DMEM containing 10 per cent FCS, essentially according to published methods.[16,17] In these studies, melanocytes were cultured for 24 h after splitting the cells in the appropriate medium. The cells were then maintained in a mitogen-deficient medium, containing phenol red-free DMEM, supplemented with 0·25 per cent BSA, 10 mM HEPES, pH 7·4 (DMEM–BSA) during the investigation period, but not longer than 72 h. The melanocytes were used in passages 3–8 (P_{3-8}) and the different experiments were performed only with cells from the same passage number.

Cell adhesion assays

In a pilot experiment, we examined the *in vitro* baseline adhesion of cultured NHMs and MCs to polystyrene plates coated separately with different ECM components. It was found that under the present experimental conditions for evaluating the effects of short-lived NO donors, the NHMs adhered respectively to fibronectin, laminin, collagen IV, and hyaluronic acid 9·7, 3·9, 3·3, and 1·6 times more effectively than to uncoated plastic. For subsequent experiments, fibronectin was chosen to study the inhibitory effects of NO on melanocyte adhesion by an adhesion assay described by Danen *et al.*[18,19] Briefly, $Na_2{}^{51}CrO_4{}^{2-}$-labelled NHMs and MCs (2×10^5 cells per well) in DMEM–BSA were seeded immediately after addition of NO donors and were allowed to adhere for 75 min at 37°C in 5 per cent CO_2 atmosphere. The non-adherent cells were washed off, the attached cells were lysed, and the radioactivity was measured. As negative controls, DMEM–BSA without SIN-1 and also denitrosylated SIN-1 were added.

FACS analysis of nitric oxide synthase

For analysis of NOS expression, the melanocytes were grown as a monolayer in DMEM–BSA medium for 12 and 24 h in the absence (cNOS) and presence of 500 U/ml INF-γ (iNOS). After washing with phosphate-buffered saline (PBS), harvested cells were fixed in acetone. Approximately 4×10^5 cells were preincubated for 10 min with PBS containing 0·4 per cent FCS and 0·01 per cent sodium azide (PFA), supplemented with 10 per cent pooled normal human AB serum (PFAN) (CLB, Amsterdam, The Netherlands) followed by incubation with mouse anti-brain (1:40), anti-macrophage (1:40), and anti-endothelial NOS (1:20) monoclonal antibodies (MAbs) (Transduction Laboratories, Lexington, KY, U.S.A.) or preimmune IgG for 45 min at 4°C. After washing with PFA, cells were incubated with biotinylated $F(ab)_2$ rabbit anti-mouse antiserum (DAKO a/s, Glostrup, Denmark; dilution 1:50) for 30 min at 4°C. Phycoerythrin-labelled streptavidin (PE-Strept; DAKO; dilution 1:25) was added after a subsequent washing procedure and the cells were incubated for 30 min at 4°C. Fluorescence of 5000 cells per sample of the stained melanocytes was then measured with a FACScan analyser (Becton Dickinson Immunocytometry Systems, U.S.A.). Negative controls were similarly stained by omitting the primary antibodies to determine background fluorescence.

FACS analysis of cell proliferation

The effects of SIN-1 on cell proliferation were measured using only Mel57 and M14 but not with NHMs because the latter were found to have an extremely slow doubling time.[20] The MCs were maintained for 12 h in DMEM–BSA. SIN-1 (0·1 or 1 mM) was added every 12 h, starting with the medium change.

Proliferation rates of the MCs were compared by BrdU incorporation assay essentially according to Bakker *et al.*[21] Cells (5×10^5) were incubated with 10 μM BrdU for an additional 6 and 12 h after the second addition of SIN-1, respectively. In a pilot kinetics study, these MCs only showed measurable BrdU incorporation between 4 and 12 h. For this reason, the time period of 6 and 12 h was used. Subsequently, cells were harvested by scraping and pretreated in 95 per cent formamide in buffer containing 0·15 M NaCl and 0·05 M sodium citrate, pH 7·0, for 45 min at 70°C prior to incubation with mouse anti-BrdU MAb (DAKO; dilution 1:40) for 45 min at 4°C. Then the samples were stained with fluorescein-conjugated rabbit anti-mouse IgG (RAM-FITC) (DAKO; dilution 1:100) for 45 min at 4°C and analysed by the FACScan.

FACS analysis of integrin expression

The effects of SIN-1 on the expression of integrins in melanocytes were determined by indirect immunofluorescence staining, employing rabbit anti-mouse antiserum (DAKO) and followed by PE-Strept as described above. All anti-integrin subunit MAbs were a kind gift from Professor Dr C. Figdor (Nijmegen, The Netherlands) and Dr A. Sonnenberg (Amsterdam, The Netherlands), unless stated otherwise. The following anti-integrin subunit MAbs were used: 10G11 (anti-α_2), J143 (anti-α_3), SAM1 (anti-α_5), GoH3 (anti-α_6), NKI-M_7 (anti-α_v), 4B4 (anti-β_1), and C17 (anti-β_3). 3E1 (anti-β_4) was used as a negative control, and melanocyte-specific antibodies NKI-beteb (Sanbio, Uden, The Netherlands) as a positive control. The MAbs against the investigated integrin subunits were chosen because these are reported to be expressed by melanocytes either *in vivo* or *in vitro* and the modulation of their expression pattern may alter melanocyte migration.[10,18,19]

Immunocytochemical detection of nitric oxide synthase

After acetone fixation, NHM monolayers on glass coverslips were pretreated with 3 per cent H_2O_2 in methanol (10 min, 4°C) and then incubated with mouse anti-brain NOS MAb (1:40) diluted in PBS, containing 10 per cent pooled normal human AB serum (CLB, Amsterdam, The Netherlands) or with the buffer alone for 1 h at room temperature. Specific binding of NOS was detected with biotinylated rabbit anti-mouse antiserum (DAKO; dilution 1:200) and StreptAB-Complex (DAKO, streptavidin plus biotinylated horseradish peroxidase). Peroxidase activity was visualized with amino-ethyl-carbazole (Sigma).

Melanin determination

Melanin content was mainly measured as described by Lee *et al.*[22] Melanin absorbance was measured at 400 nm and the melanin content was calculated by comparison with a standard curve plotted with synthetic melanin. Values are expressed in $\mu g/10^6$ cells.

Nitrite determination

Nitrite, a stable NO oxidation product, was determined spectrophotometrically using the Griess reaction by a microplate assay described by Ding *et al.*[23] The absorbance was measured at 540 nm with a reference wavelength of 690 nm. Nitrite concentrations were calculated using a $NaNO_2$ standard solution (0–100 μM).

Morphology

NHMs or MCs were seeded in 12-well plates (Falcon, Becton Dickinson, U.S.A.) with about 6×10^5 cells per well in the appropriate medium as described above. After 24 h, the medium was replaced with DMEM–BSA and then NO donors were added twice daily in the concentration range up to 10^{-2} M. DMEM–BSA and denitrosylated solutions of SIN-1 or GSNO were used as negative controls. The mitogen-deficient medium was changed every 24 h. Cell morphology was monitored twice daily by light microscopy for 3 days. The experiments were performed in triplicate.

Analysis of results

Concentration–response curves were calculated for the NO donor-induced inhibition of melanocyte adhesion to fibronectin. The curves were fitted to the experimental results using a computer program (Sigma plot 3.0, Jandel Co., San Rafael, CA, U.S.A.) and the following relationship: $E = E_{max}/[1 + (I/x)^n]$, where E is the effect of NO donors on the adhesion of melanocytes to fibronectin, expressed as inhibition of the adhesion in per cent of the control; E_{max} is the maximum effect; x is the concentration of NO donors; I is the concentration of NO donors giving 50 per cent inhibition of the adhesion (IC_{50}); and n is the Hill coefficient. Results are expressed as means ± SEM from the number of experiments indicated. Means were compared by using an unpaired Student's t-test. P values of less than 0·05 were considered as indicating significant differences.

RESULTS

Effects of NO-releasing compounds on the adhesion of melanocytes to fibronectin

The NO donor SIN-1 induced a concentration-dependent decrease in the adhesion of NHMs ($Mf9055P_{5–6}$, $Mf9415P_{4–5}$, $Mf9039P_{7–8}$) and of MCs (M14, Mel57) to fibronectin (Fig. 1 and Table I). The concentration–response curve of $Mf9415P_{4–5}$ (data not shown) did not differ significantly from that of $Mf9039P_{7–8}$. The adhesion of the pigmented M14 was more sensitive to the inhibitory effects of SIN-1 (IC_{50} at about 0·5 mM) than Mel57 and NHMs (IC_{50} between 1 and 2 mM SIN-1). $Mf9055P_{5–6}$ (IC_{50} at about 1 mM) with the highest melanin content, approximately 24 $\mu g/10^6$ cells (Table I), seemed to be more affected by SIN-1 than $Mf9039P_{7–8}$ and $Mf9415P_{4–5}$. The melanin content in $Mf9039P_{7–8}$ was at about 1 $\mu g/10^6$ cells. The other NO donor, the nitrosothiol GSNO, was less active than SIN-1 in the adhesion assay. GSNO at a high concentration of about 10 mM induced approximately 25 per cent inhibition of adhesion (Fig. 1). No inhibitory effects on adhesion were observed using either denitrosylated

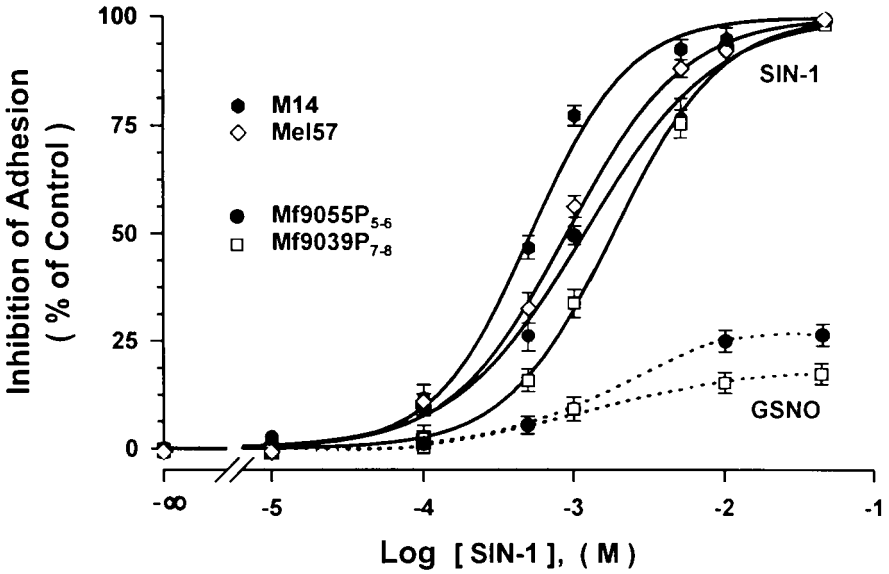

Fig. 1—Effects of NO-releasing compounds on the adhesion of melanocytes to fibronectin. The adhesion of MCs [■ M14; (◇) Mel57] and NHMs [(●) $Mf9055P_{5-6}$; (□) $Mf9039P_{7-8}$] (2×10^5 cells per well) to fibronectin was performed as described in the Materials and Methods section. Results are expressed as the percentage of adhesion in the absence of modulators. Values are means ± SEM from 4–6 different experiments in quadruplicate

solutions of SIN-1 or C87-3754, a weak NO donor,[13] as controls. There was also no change in the inhibition of adhesion in the presence of up to 2000 U/ml superoxide dismutase (SOD), an extracellular $O_2^{-\cdot}$ scavenger, and 500 U/ml catalase, indicating that $O_2^{-\cdot}$ generated from SIN-1 during decomposition[12] was not responsible for the effects observed (data not shown).

When the adhesion values of the investigated melanocytes to fibronectin (ranging from 40 to 70 per cent) were compared with their apparent IC_{50} of SIN-1, it appeared that adhesion in the absence of SIN-1 did not depend on the corresponding IC_{50} values. Control adhesion to plastic was less than 5 per cent.

Since integrins are the main determinants of melanocyte adhesion to fibronectin, we also measured the level of integrins before and after similar NO treatment of FACS analysis (data not shown). The results indicated that all five melanocytic cell lines expressed $\beta 1$ integrins, α_3, α_5, α_2, and α_6 subunits in variable degrees; however, the expression of the integrin subunits was not altered in the presence of 0·1 mM SIN-1 for 24 h (data not shown).

Cell proliferation and morphology

Cell viability was not affected during the investigation period or in the concentration range of the half-maximal effects. Proliferation was also measured as the percentage of BrdU-incorporated cells during an optimally evaluated time period ranging from 6 to 12 h (Table II). With 1 mM SIN-1, approximately 80 per cent inhibition of the proliferation of Mel57 cells was observed, whereas the proliferation rate of BrdU-incorporated M14 was not affected. All melanocytes proliferated without

Table I—Inhibitory potencies of SIN-1 on the adhesion of human melanocytic cells to fibronectin; on endogenous NO release during 24 h, determined as nitrite; and melanin content of the cells. The half-maximal inhibiting concentrations of SIN-1 (IC_{50}) were calculated as described in the Materials and Methods section. Values are means ± SEM from 3–6 different experiments in triplicate or quadruplicate

Melanocytes (cultured)	IC_{50} of SIN-1 (mM)	NO release (nmol/10^6 cells)		Melanin content (μg/10^6 cells)
		Basal	3 mM NMA	
NHMs				
$Mf9055P_{5-6}$	1·08 ± 0·11	16·5 ± 1·5	11·3 ± 1·1*	23·8 ± 1·7
$Mf9415P_{4-5}$	1·80 ± 0·17	7·3 ± 0·5	7·1 ± 0·4	3·2 ± 0·2
$Mf9039_{7-8}$	2·08 ± 0·07	8·0 ± 0·06	7·5 ± 0·4	1·2 ± 0·1
MCs				
M14	0·52 ± 0·03	1·4 ± 0·2	1·3 ± 0·1	1·8 ± 0·2
Mel57	0·89 ± 0·05	0·6 ± 0·1	0·7 ± 0·1	NP

*P<0·05 versus values in the absence of NMA.
NP = non-pigmented.

Table II—Effects of SIN-1 on the proliferation rates of Mel57 and M14 as determined by flow cytometric analysis of BrdU incorporation in the transformed melanocytes. Values are means ± SEM of three independent experiments

Cell culture	BrdU incorporation time (h)	% BrdU cells		
		Control	0·1 mM SIN-1	1·0 mM SIN-1
Mel57	6	21 ± 4·2	21 ± 3·6	4 ± 0·7* (80%)†
	12	51 ± 9·7	45 ± 8·1	8 ± 0·9* (80%)†
M14	6	50 ± 6·3	60 ± 9·9	40 ± 6·8
	12	58 ± 8·2	62 ± 8·7	55 ± 7·4

*$P<0·05$ versus values in the absence of SIN-1.
†Percentage of inhibition.

significant changes in their morphology during 8 h incubation with SIN-1 up to 3 mM. Concentrations above 5 mM induced morphological alterations in the NHMs, including loss of dendricity and rounder cell shapes in approximately 75 per cent of the cells (data not shown). GSNO had no toxic effects on the NHMs for any of the concentrations tested. The morphology of the tested MCs was not altered by SIN-1 or GSNO during the experiments. The denitrosylated form of SIN-1 showed no toxic effects on melanocytes.

Nitrite production

Endogenous NO release as determined by nitrite accumulation in the cell supernatant also supports the expression of cNOS in the investigated melanocytes (Table I). Nitrite accumulation was maximal 24 h after the change to mitogen-deficient conditions and was not altered in the presence of 500 U/ml INF-γ for 12, 24, or 48 h, respectively. The basal level of NO release in Mel57 was significantly lower than the NO levels in the normal melanocytes. This value for $Mf9055P_{5–6}$

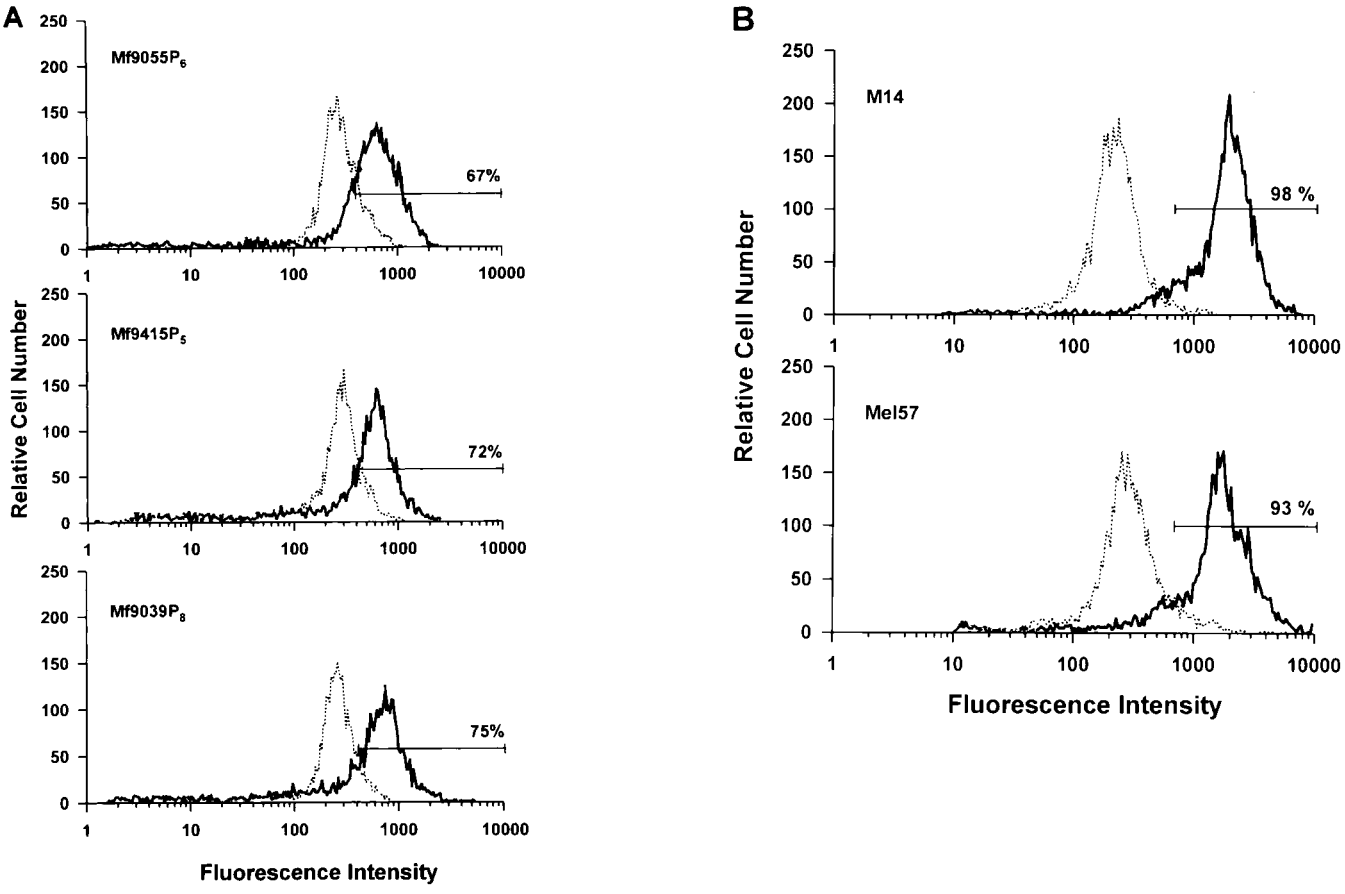

Fig. 2—Flow cytometric analysis of the expression of the neuronal isoforms of NOS in NHMs (A) and in MCs (B). Cell suspensions stained by anti-brain NOS MAb are shown as solid lines and those stained with the second antibody alone to determine background fluorescence as dotted lines. Data are from one representative experiment

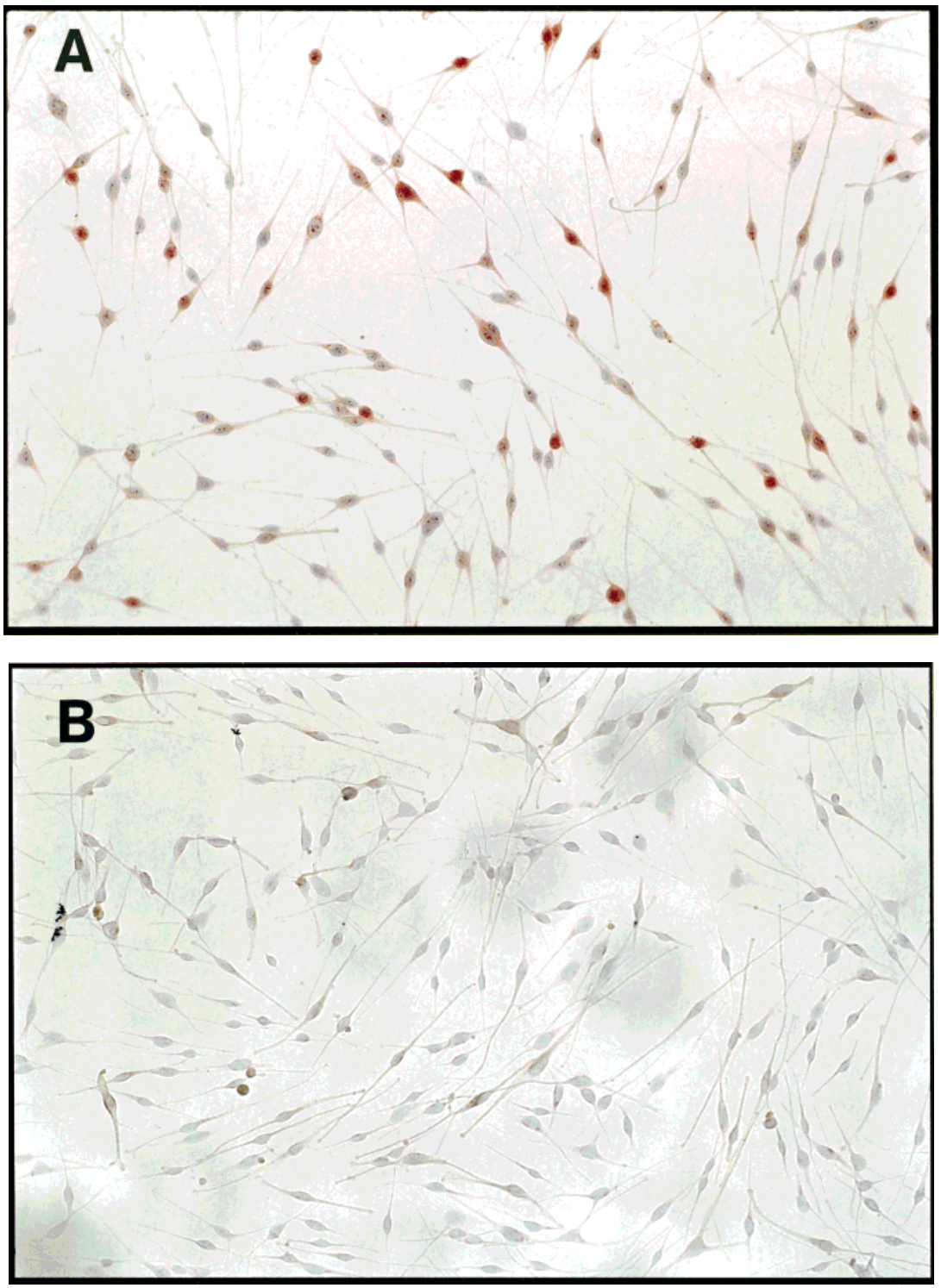

Fig. 3—Immunocytochemical analysis of the expression of the neuronal isoform of NOS in NHMs ($Mf9012P_{18}$) by indirect immunoperoxidase staining of adherent cells. Melanocytes stained with anti-brain NOS MAb (pink) are presented in A and cells with 10 per cent normal human serum as a control in B (× 50). Data are from one representative experiment

was approximately two-fold higher than those of $Mf9415P_{4–5}$ and $Mf9039P_{7–8}$. Interestingly, the IC_{50} for $Mf9055P_{5–6}$ was also approximately two-fold smaller than the IC_{50} for $Mf9039P_{7–8}$ and $Mf9415P_{4–5}$. In contrast, the basal level of NO release of M14 was approximately 12-fold lower than the NO level of $Mf9055P_{5–6}$, although M14 was about two-fold more sensitive to the inhibitory effects of SIN-1 than $Mf9055P_{5–6}$. The addition of 3 mM L-NMA, an inhibitor of NOS, significantly reduced NO release only in the highly pigmented $Mf9055P_{5–6}$, but not in the other melanocytes.

Expression of NOS in NHMs and MCs

Flow cytometric analysis for the expression of NOS in melanocytes showed that the unstimulated melanocytes were positive for endothelial as well as for neuronal NOS as a marker for cNOS. Positive staining was not observed using the anti-macrophage NOS MAb, even when preincubated melanocytes were used in the presence of 500 U/ml INF-γ for 24 h. Figures 2A and 2B show the expression of the neuronal NOS (cNOS) in NHMs and in MCs (M14, Mel57), respectively. All cell lines expressed cNOS in the range of 72–95 per cent. Expression of cNOS in NHMs was also confirmed by immunocytochemical methods as illustrated in Fig. 3.

DISCUSSION

These results demonstrate that NO-releasing compounds inhibit the *in vitro* adhesion of NHMs and MCs of different grades of pigmentation to the ECM component fibronectin. The expression of cNOS in all investigated melanocytes further supports the finding that NO may have a role in autocrine modulation of normal and transformed melanocytes, at least with regard to reattachment of released cells to the ECM.

The investigated sydnonimine SIN-1, a donor of NO redox species, induced a concentration-dependent reduction in the adhesion of melanocytes to fibronectin. This was an ideal ECM component for the present study, because most melanocytic cell lines adhere readily to fibronectin.[19,24] The apparent IC_{50} was in the range of 500 μM to 2 mM, and the IC_{25} in the range of 200–700 μM SIN-1. Although the IC_{50} values for the *in vitro* inhibitory effects of SIN-1 seem to be relatively high, the amount of $NO^{\cdot}$ produced by the iNOS of activated macrophages was found to be in a similar range.[25,26] SIN-1 is only a prodrug from which $NO^{\cdot}$ is released and therefore the available concentrations of $NO^{\cdot}$ will be much lower than those endogenously formed in macrophages, even if millimolar concentrations of SIN-1 are used.[13] Moreover, SIN-1 simultaneously forms $O_2^{-\cdot}$ and $NO^{\cdot}$ during decomposition;[12] these can react, with a diffusion-controlled formation of peroxynitrite.[27] Peroxynitrite by itself may modulate the signalling function of NO.[28] In addition, we observed no changes in the IC_{50} values in the presence of exogenous SOD and catalase, indicating that $O_2^{-\cdot}$ could not be directly responsible for the effects of SIN-1. Finally, considering GSNO as a potential NO^+-delivering compound and a much weaker inhibitor for melanocyte attachment to fibronectin, it seems that NO^+ and probably *S*-nitrosylation[14] are not involved in our system. It can therefore be assumed that the effects of SIN-1 observed in the present study reflect inhibition by $NO^{\cdot}$ of the interaction between melanocytes and ECM. Such effects might be dependent on the melanin content of the cells.

In the present study, it was demonstrated that the observed inhibition of melanocyte adhesion is a consequence neither of a decreased expression of fibronectin-binding integrins *per se*, nor of the downregulation of other adhesion molecules. Because integrins are the main determinants of melanocyte adhesion to fibronectin, and a very short time duration was used for the adhesion assay, the most reasonable explanation could be that integrin function is impaired by conformational changes induced by $NO^{\cdot}$. Nevertheless, the expression of cNOS in all tested melanocytes and the endogenous production of NO in cell supernatants support a function for NO in the pathobiology of the pigment cells. Interestingly, the levels of NO release in supernatants of MCs were about 12- to 25-fold lower than the NO levels of the most pigmented normal human melanocytes, despite the strong expression of cNOS in the MCs. The reasons for this discrepancy could be explained by differences in the activities of cNOS or by the endogenous presence of a NOS inhibitor in the MCs. Furthermore, the lack of inhibition on endogenous production of NO by L-NAMA in less pigmented cells might reflect the constitutive release of very low levels of NO in the cells and/or differences in the sensitivity of the respective NOS to L-NAMA.

In order to elucidate further the effect of NO, we also investigated the effect of SIN-1 on cell proliferation by measuring the changes of the percentage of BrdU-labelled cells in the growth phase. Such data could not be obtained with NHMs because of their very slow doubling times, since it is known that the demonstration of DNA synthesis by BrdU labelling of cells is dependent on the sampling time during the growth phase[29] of cell populations. This method was found to be applicable for evaluating the effects of SIN-1 only for MCs and not for NHMs. Consequently, we observed that proliferation of Mel57 alone was inhibited by 80 per cent, whereas MCs remained unaffected.

In conclusion, the results of the present investigation demonstrate that NO-releasing compounds such as SIN-1 can be used to study the pathophysiological effects of NO on pigment cells. In particular, the expression of cNOS in all investigated melanocytes supports the idea that NO can modulate adhesive processes in normal and transformed melanocytes.

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