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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 51CrO42−-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 redoxforms with distinct chemistries, including neutral nitric oxide (NO·), 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). 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 has been associated with inflammatory and immune responses. The presence of a NO receptor linked to a G protein has been demonstrated by several laboratories. 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. 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 cellular infiltrates in close proximity to normal human melanocytes (NHM s) has been attributed to the disappearance of these melanin-producing cells in vitiligo. Indeed, melanocyte stimulation and altered melanin production are generally accompanied by acute and chronic skin inflammation.
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· and S-nitroso-glutathione (GSNO)2,14 as a potential NO+ donor, on the adhesion of N H M s and M Cs 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 (D M E M ), and HEPES (N-2-hydroxyethyl-1-piperazine-N’-2-ethanesulphonic acid) were from I C N Biomedicals, Inc. (Costa M esa, C A., 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’-methyl-L-arginine (N M A); GSNO; and 5-bromo-2′-deoxyuridine (BrdU) were obtained from Sigma Chemical Co. (St. Louis, M O., U.S.A.); human interferon-γ (r–hINF-γ) was a gift from Dr P. van der Heide, T N O-Rijswijk, The Netherlands.

The N O donors (SIN-1 and C87-3754),13 kindly provided by Dr G rewé (H oechst A G., F rankfurt, G ermany), were freshly prepared before measurements.13 D enitrosylated solutions of SIN-1 as negative controls and GSNO were obtained by incubating 50 mM solutions in D M E M containing 0·25 per cent BSA for 24 h at 37°C. Synthesized standard melanin was a gift from A. Kammeyer (A cademic M edical Center, A msterdam University, T he N etherlands).

Culturing cell lines

N H M cultures, established from individual foreskins of different grades of pigmentation, were maintained essentially according to the method described previously by us.15 T he human M C lines were a kind gift from Professor Dr D. Ruiter (N ijmegen U niversity, T he N etherlands). The pigmented M 14 and the non-pigmented M el57 were cultured using D M E M 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 D M E M , supplemented with 0·25 per cent BSA, 10 mM H E P E S, pH 7·4 (D M E M – 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 N H M s and M Cs to poly-styrene 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 N H M s 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 Briefly, N a351CrO4− labelled N H M s and M Cs (2 × 105 cells per well) in D M E M – BSA were seeded immediately after addition of NO donors and were allowed to adhere for 75 min at 37°C in 5 per cent C O2 atmosphere. The non-adherent cells were washed off, the attached cells were lysed, and the radioactivity was measured. A s negative controls, D M E M – BSA without SIN-1 and also denitrosylated SIN-1 were added.

FACS analysis of nitric oxide synthase

For analysis of N O S expression, the melanocytes were grown as a monolayer in D M E M – BSA medium for 12 and 24 h in the absence (c N O S) and presence of 500 U/ml I N F-γ (I N O S). A fter washing with phosphate-buffered saline (PBS), harvested cells were fixed in acetone. Approximately 4 × 105 cells were preincubated for 10 min with PBS containing 0·4 per cent FCS and 0·01 per cent sodium azide (P F A), supplemented with 10 per cent pooled normal human A B serum (P F A N) (C L B, A msterdam, T he N etherlands) followed by incubation with mouse anti-brain (1:40), anti-macrophage (1:40), and anti-endothelial N O S (1:20) monoclonal antibodies (MAbs) (T ransduction Laboratories, L exington, K Y., U.S.A.) or preimmune IgG for 45 min at 4°C. A fter washing with P F A, cells were incubated with biotinylated F(ab)2 rabbit anti-mouse antisemir (D A K O a/s, G lostrup, D enmark; dilution 1:50) for 30 min at 4°C. Phyceroerythrin-labelled streptavidin (P E–S trept; D A K O; dilution 1:25) was added after a subsequent washing procedure and the cells were incubated for 30 min at 4°C. F luorescence of 5000 cells per sample of the stained melanocytes was then measured with a F A C Scan analyser (Becton Dickinson Immuno-cytometry Systems, U.S.A.). N egative 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 M el57 and M 14 but not with N H M s because the latter were found to have an extremely slow doubling time.20 T he M Cs were maintained for 12 h in D M E M – 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. Cells \((5 \times 10^5)\) were incubated with 10 \(\mu\)M 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 FACS.

**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 antisemum \((DAKO)\) and followed by PE-Strept as described above. All anti-integrin subunit MAsbs 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 MAsbs were used: 10G11 (anti-\(\alpha_2\), J143 (anti-\(\alpha_5\)), SA M1 (anti-\(\alpha_5\)), GoH3 (anti-\(\alpha_5\)), NK1-M7 (anti-\(\beta_3\)), 4B4 (anti-\(\beta_1\)), and C17 (anti-\(\beta_3\)). 3E1 (anti-\(\beta_3\)) was used as a negative control, and melanocyte-specific antibodies NK1- \(\beta_1\)-beteb (Sanbio, Uden, The Netherlands) as a positive control. The MAsbs 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.

**Immunocytochemical detection of nitric oxide synthase**

After acetone fixation, NHM monolayers on glass coverslips were pretreated with 3 per cent \(\text{H}_2\text{O}_2\) 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 \((DAKO;\text{dilution}1:200)\) and StreptAB-Complex \((DAKO,\text{streptavidin plus biotinylated horseradish peroxidase})\). Peroxidase activity was visualized with amino-ethyl-carbazole (Sigma).

**Melanin determination**

Melanin content was mainly measured as described by Lee et al. 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. The absorbance was measured at 540 nm with a reference wavelength of 690 nm. Nitrite concentrations were calculated using a \(\text{NaNO}_2\) standard solution (0–100 \(\mu\)M).

**M morphology**

NHMs or MCs were seeded in 12-well plates (Falcon, Becton Dickinson, U.S.A.) with about 6 \(\times\) 10\(^5\) cells per well in the appropriate medium as described above. After 24 h, the medium was replaced with D MEm - BSA and then NO donors were added twice daily in the concentration range up to 10\(^{-2}\) \(\mu\)M. D MEm - BSA and denitrosylated solutions of SIN-1 or \(\text{G} \text{S} \text{N} \text{O}\) 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: \(E = E_{\text{max}}/[1+(I/I_0)^n]\), 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; \(E_{\text{max}}\) is the maximum effect; \(I\) is the concentration of NO donors; \(I_0\) is the concentration of NO donors giving 50 per cent inhibition of the adhesion \((I_{50})\); and \(n\) is the Hill coefficient. Results are expressed as mean \(\pm\) 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 \((\text{M}9039\text{P}7-8, \text{M}9055\text{P}5-6, \text{M}9415\text{P}4-5, \text{M}9039\text{P}7-9, \text{M}14, \text{M}057)\) to fibronectin (Fig. 1 and Table I). The concentration-response curve of M 9415P4-5 (data not shown) did not differ significantly from that of M 9039P7-8. The adhesion of the pigmented M 14 was more sensitive to the inhibitory effects of SIN-1 \((I_{50}\text{at~about ~0.5~mM})\) than M 057 and NHMs \((I_{50}\text{between 1 and 2 mM SIN-1})\). M 9055P5-6 \((I_{50}\text{at~about ~1~mM})\) with the highest melanin content, approximately 24 \(\mu\)g/10\(^6\) cells (Table I), seemed to be more affected by SIN-1 than M 9039P7-8 and M 9415P4-5. The melanin content in M 9039P7-8 was at about 1 \(\mu\)g/10\(^6\) cells. The other NO donor, the nitrosothiol \(\text{G} \text{S} \text{N} \text{O}\), was less active than SIN-1 in the adhesion assay. \(\text{G} \text{S} \text{N} \text{O}\) 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...
solutions of SIN-1 or C87-3754, a weak NO donor, 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$_2^·$ scavenger, and 500 U/ml catalase, indicating that O$_2^·$ generated from SIN-1 during decomposition 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 $\alpha_1$ integrins, $\alpha_3$, $\alpha_5$, $\alpha_2$, and $\alpha_6$ subunits in variable degrees; however, the expression of the integrin subunits was not altered in the presence of 0·1mM SIN-1 for 24h (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 1mM SIN-1, approximately 80 per cent inhibition of the proliferation of M el57 cells was observed, whereas the proliferation rate of BrdU-incorporated M 14 was not affected. All melanocytes proliferated without modification.

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.

<table>
<thead>
<tr>
<th>Melanocytes (cultured)</th>
<th>IC$_{50}$ of SIN-1 (mM)</th>
<th>NO release (nmol/10^6 cells)</th>
<th>Melanin content (µg/10^6 cells)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>3mM NMA</td>
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<tr>
<td>N H M s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M f9055P$_{6-6}$</td>
<td>1·08 ± 0·11</td>
<td>16·5 ± 1·5</td>
<td>11·3 ± 1·1*</td>
</tr>
<tr>
<td>M f9415P$_{4-5}$</td>
<td>1·80 ± 0·17</td>
<td>7·3 ± 0·5</td>
<td>7·1 ± 0·4</td>
</tr>
<tr>
<td>M f9039P$_{7-8}$</td>
<td>2·08 ± 0·07</td>
<td>8·0 ± 0·06</td>
<td>7·5 ± 0·4</td>
</tr>
<tr>
<td>M Cs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M 14</td>
<td>0·52 ± 0·03</td>
<td>1·4 ± 0·2</td>
<td>1·3 ± 0·1</td>
</tr>
<tr>
<td>M el57</td>
<td>0·89 ± 0·05</td>
<td>0·6 ± 0·1</td>
<td>0·7 ± 0·1</td>
</tr>
</tbody>
</table>

*P <0·05 versus values in the absence of N M A.
N P = non-pigmented.
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 percent 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 Mf9055P5-6

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

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>BrdU incorporation time (h)</th>
<th>% BrdU cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0.1 mM SIN-1 1.0 mM SIN-1</td>
<td></td>
</tr>
<tr>
<td>Mel57</td>
<td>6 21 ± 4.2 21 ± 3.6 4 ± 0.7* (80%)†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 51 ± 9.7 45 ± 8.1 8 ± 0.9* (80%)†</td>
<td></td>
</tr>
<tr>
<td>M14</td>
<td>6 50 ± 6.3 60 ± 9.9 40 ± 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 58 ± 8.2 62 ± 8.7 55 ± 7.4</td>
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*P < 0.05 versus values in the absence of SIN-1.
†Percentage of inhibition.

NITRIC OXIDE AND MELANOCYTE ADHESION

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.

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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 M Ab, 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, M el57), 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. The apparent IC50 was in the range of 500 μM to 2 μM, and the IC25 in the range of 200–700 μM of SIN-1. Although the IC50 values for the in vitro inhibitory effects of SIN-1 seem to be relatively high, the amount of NO produced by the INOS of activated macrophages was found to be in a similar range.22,30 SIN-1 is only a produg from which NO is released and therefore the available concentrations of NO will be much lower than those endogenously formed in macrophages, even if millimolar concentrations of SIN-1 are used. Moreover, SIN-1 simultaneously forms ONOO− and NO 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 IC50 values in the presence of exogenous SOD and catalase, indicating that ONOO− 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-nitrosylation14 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+ 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. 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-NAM A 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-NAM A.

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 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 M el57 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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REFERENCES


15. Ivanova K, Schaefer M, Drummer C, Gerzer R.


17. Brüggen J, Sorg C, Macher E.

18. Danen EHJ, van den Wijngaard RMJGJ, Figdor CG.

19. Danen EHJ, van Muijen GN, van de Wiel-van Kemnade E, Jansen KFJ, Ruiter DJ, Figdor CG.

20. Le Poole IC, van den Berg FM, van den Wijngaard RMJGJ, et al.


22. Lee TH, Lee MS, Lu M-Y.

23. Ding AH, Nathan CF, Stuehr DJ.

24. McClenic BK, Mitra RS, Riser BL, NikoI I.

25. Padgett EL, Pruett SB.


27. Blough NV, Zafirou OC.

28. Tarpey MM, Beckman JS, Ishiropoulos H, Gore JZ, Brock TA.

29. Johansson MC, Baldetorp B, Bendahl PO, Faddei IA, Grodsson SM.

30. Morelli JG, Norris DA.

31. Morelli JG, John JJ, Zekman T, Norris DA.