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Nitric oxide differentially regulates pro- and anti-angiogenic markers in DLD-1 colon carcinoma cells

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Abstract Inducible nitric oxide (NO) synthase (iNOS) appears to be a marker of tumor progression in colon carcinogenesis. Here we investigated effects of NO on selected chemokines that differentially regulate angiogenesis, namely pro-angiogenic interleukin (IL)-8 as well as tumor-suppressive interferon-inducible protein-10 (IP-10) and monokine induced by interferon-γ (MIG). These chemokines are expressed by DLD-1 colon carcinoma cells after stimulation with IL-1β/interferon-γ. Expression of IL-8 was markedly upregulated by NO. Moreover, NO enhanced expression of vascular endothelial growth factor (VEGF). In contrast, expression of IP-10 and MIG was suppressed by NO. The present data are consistent with previous observations that link NO to enhanced tumor angiogenesis and imply that NO-mediated upregulation of IL-8 and VEGF as well as downregulation of IP-10 and MIG may contribute to this phenomenon.

Key words: Nitric oxide; Chemokine; Angiogenesis; Colon carcinoma cell

1. Introduction

Expression of inducible nitric oxide synthase (iNOS) has been recognized as a marker in human diseases associated with inflammation and immunoactivation [1]. iNOS expression has also been detected in a broad range of human malignancies including colorectal cancer [2]. In these tumors, iNOS not only is expressed in invading mononuclear cells, but also in the cancer cells [3]. Moreover, augmented levels of nitrotyrosine are detectable in the colon tumor microenvironment and imply upregulated NOS enzyme activity in vivo [4]. In vitro, expression of iNOS is induced in human epithelial-like DLD-1 colon carcinoma cells by incubation with the Th1 cytokine interferon-γ (IFNγ), particularly in combination with pro-inflammatory cytokines [5].

Although reports on the function of nitric oxide (NO) in tumor biology are not entirely uniform, several studies imply a role for NO in colon carcinogenesis. Notably, NO has been associated with augmented angiogenesis [2,6] and increased p53 mutation frequency in colorectal cancer [3]. To further examine the basis of NO-induced angiogenesis, we analyzed the influence of NO on expression of selected regulators of angiogenesis that are supposed to be expressed by colon carcinoma cells in an inflamed microenvironment. Specifically, effects of NO on expression of pro-angiogenic interleukin (IL)-8 [7] and vascular endothelial growth factor (VEGF) [8] as well as effects on anti-angiogenic interferon-inducible protein-10 (IP-10) and monokine induced by interferon-γ (MIG) [7] were investigated.

2. Materials and methods

2.1. Materials

Recombinant human IFNγ and IL-1β were from Pepro Tech Inc. (Frankfurt, Germany) and Cell Concepts (Umkirch, Germany), respectively. (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)-amino] diazen-1-ium-1,2-diolate (DETA-NO) was from Alexis Biochemicals (Grünberg, Germany).

2.2. Cultivation of DLD-1 cells

The human colon carcinoma cell line DLD-1 was from the Centre for Applied Microbiology and Research (Salisbury, UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco BRL, Eggenstein, Germany). Experiments were performed in the aforementioned culture medium in the absence of FCS. Unless otherwise stated, DLD-1 cells were preincubated with DETA-NO (1 mM) for 16 h. Thereafter, IL-1β/IFNγ was added. After another 8 h of incubation, cell-free supernatants were harvested and cells were lysed for RNA isolation. Under these conditions DETA-NO did not modulate viability of DLD-1 cells as detected by lactate-dehydrogenase assay (Boehringer Mannheim, Mannheim, Germany) (data not shown).

2.3. Determination of mRNA for IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Tri-reagent according to the manufacturer's instructions (Sigma). 1 ug of RNA were used for RT-PCR (GeneAmp RNA PCR kit using Amplitaq Gold, Perkin-Elmer Corp., Weiterstadt, Germany). The following sequences were performed for PCR: 94°C for 10 min (one cycle); 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min (with variable numbers of cycles); final extension at 72°C for 7 min. Sequences of the primers, numbers of cycles, and length of resulting amplicons: IL-8 (F): 5′-atg act tcc aag ctg gcc gtg gct-3′, IL-8 (R): 5′-ctc agc cct ctt caa aaa ctt ctc-3′, 28 cycles, 289 bp; GAPDH (F): 5′-acc aca gtc cat gcc atc ac-3′, GAPDH (R): 5′-tcc acc acc ctg ttg ctg ta-3′, 23 cycles, 452 bp. Duplicate PCRs without reverse transcription were performed and analyzed in parallel in order to control for possible contaminations of RNA isolates with genomic DNA. Identity of amplicons was confirmed by sequencing (310 Genetic Analyzer, Perkin-Elmer Corp.).

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon-γ; IL, interleukin; IP-10, interferon-inducible protein-10; MIG, monokine induced by interferon-γ; NO, nitric oxide; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor

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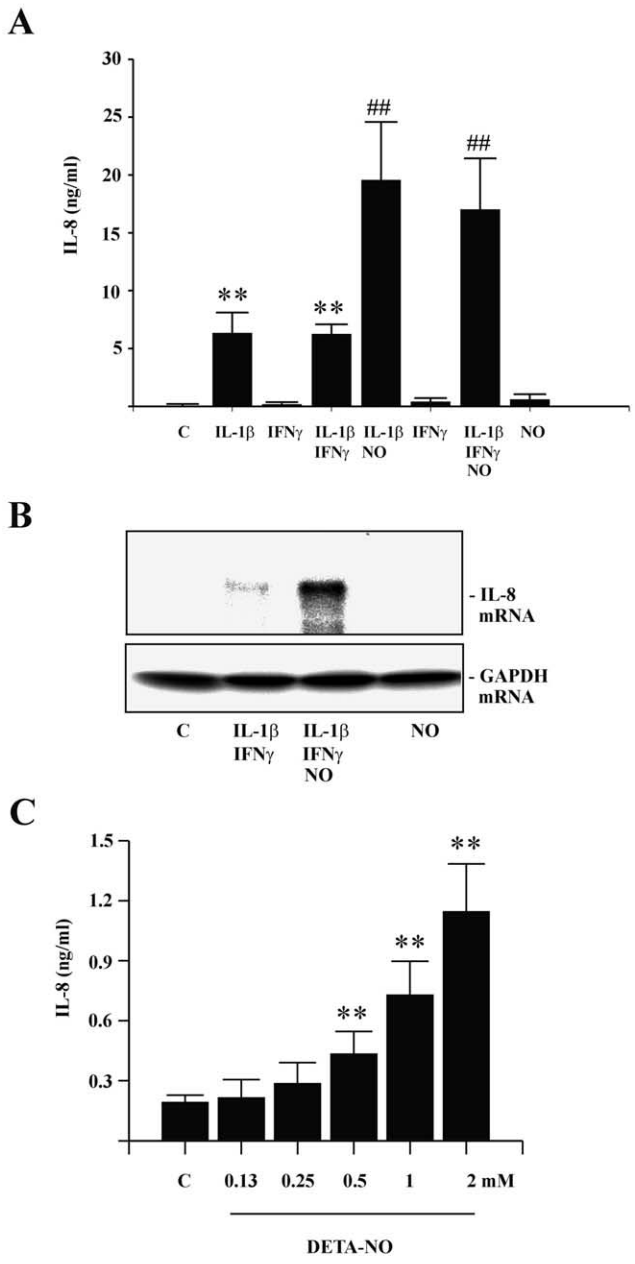

Fig. 1. NO enhances expression and release of IL-8 from DLD-1 colon carcinoma cells. A,B: DLD-1 cells were incubated as unstimulated control or with DETA-NO (1 mM). After 16 h, IL-1β (50 ng/ml), IFNγ (10 ng/ml) or IL-1β plus IFNγ were added as indicated. A: After 8 h, release of IL-8 was determined by ELISA. Mean IL-8 concentrations ± S.D. are shown ($n = 6$). $^{**}P < 0.01$ compared to unstimulated control; $^{\#\#}P < 0.01$ compared to stimulation with IL-1β/IFNγ alone. B: After an additional incubation period of 8 h, expression of IL-8 was determined by RNase protection assay. One representative of three independently performed experiments is shown. C: NO as a single stimulus mediates expression of IL-8. DLD-1 cells were stimulated with the indicated concentrations of DETA-NO. After 25 h, IL-8 was determined by ELISA (C). Data are expressed as mean IL-8 concentrations ± S.D. ($n = 6$). $^{**}P < 0.01$ compared to unstimulated control.

2.4. Determination of mRNA for VEGF and GAPDH by RNase protection assay

Assays were performed as previously described [9]. Human VEGF and GAPDH were cloned by PCR. The cDNA fragments correspond to nucleotides 347–633 for VEGF and 148–302 for GAPDH, respectively [10].

2.5. Detection of IP-10, MIG, and β-actin mRNA expression by Northern blot analysis

15 ug of RNA were separated on 1.4% agarose/formaldehyde gels and transferred to gene screen membranes (NEN Life Science Products, Boston, MA, USA). Filters were hybridized for 16 h at 42°C with ^{32}P-labelled cDNA inserts for detection of IP-10 or MIG. The following primers were used for isolation of human MIG and IP-10 cDNA using activated DLD-1 cells: 5′-gct ggt tct gat tgg agt gc-3′ (for) and 5′-tga cga gaa cgt tga gat ttt c-3′ (rev) for MIG; 5′-agt ggc att caa gga gta cc-3′ (for) and 5′-atc ctt gga agc act gca tc-3′ (rev) for IP-10. cDNA probes were labelled with [^{32}P]deoxycytidine triphosphate (dCTP) by random priming (Amersham Pharmacia Biotech, Freiburg, Germany). Stripped blots were rehybridized to the ^{32}P-labelled *Bam*HI/*Sal*I cDNA insert from clone pEX 6 coding for human β-actin [11].

2.6. Detection of cytokines by enzyme-linked immunosorbent assay (ELISA)

IP-10, IL-8 (Pharmingen, Hamburg, Germany), and VEGF (R&D Systems, Wiesbaden, Germany) in cell-free culture supernatants were quantified by ELISA according to the manufacturers' instructions.

2.7. Statistics

Data are shown as means ± S.D. and were analyzed by unpaired Student's *t*-test on raw data using Sigma Plot (Jandel Scientific).

3. Results

3.1. NO amplifies expression of pro-angiogenic IL-8 and VEGF in DLD-1 colon carcinoma cells

To further characterize consequences of enhanced NO production at the tumor microenvironment, expression of se-

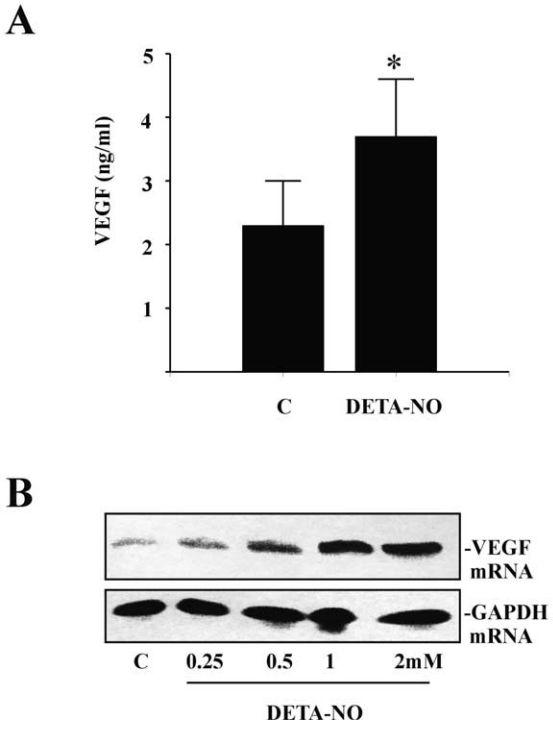

Fig. 2. NO mediates induction of VEGF in DLD-1 cells. DLD-1 cells were stimulated for 24 h with DETA-NO at 1 mM (A) or with DETA-NO at the indicated concentrations (B). Thereafter, release of VEGF was determined by ELISA (A) and mRNA expression was evaluated by RNase protection assay (B). Data are expressed as mean VEGF concentrations ± S.D. ($n = 6$). $^{*}P < 0.05$ compared to unstimulated control.

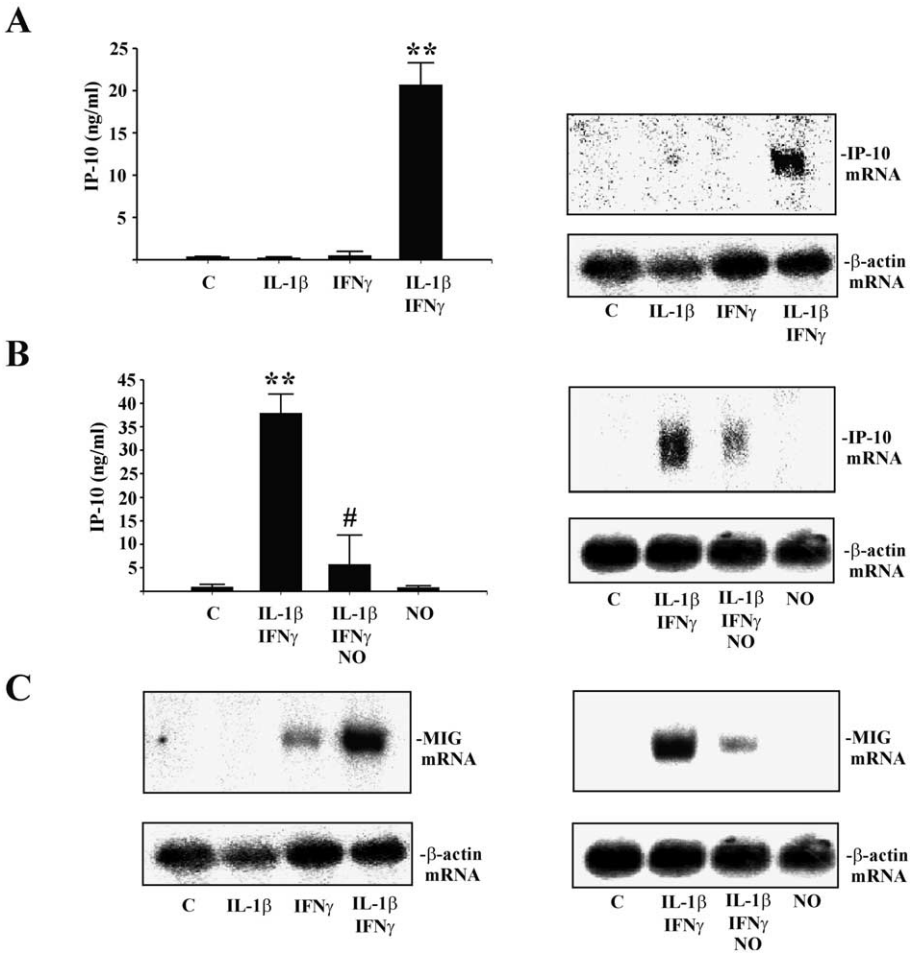

Fig. 3. NO suppresses IL-1β/IFNγ-induced IP-10 and MIG expression. A,C, left panels: DLD-1 cells were kept as unstimulated control or incubated with IL-1β (50 ng/ml), with IFNγ (10 ng/ml), and with IL-1β/IFNγ. After 8 h, release of IP-10 (A, left panel) and expression of IP-10 mRNA (A, right panel) and expression of MIG mRNA (C, left panel) were determined by ELISA and Northern blot analysis. Mean IP-10 concentrations ± S.D. ($n = 4$) are shown. $^{**}P < 0.01$ compared to unstimulated control. One representative of three independently performed Northern blot experiments is shown. B,C, right panels: DLD-1 cells were incubated as unstimulated control or with DETA-NO (1 mM). After 16 h, IL-1β (50 ng/ml)/IFNγ (10 ng/ml) was added as indicated. Following an additional 8 h incubation period, release of IP-10 (B, left panel) and expression of IP-10 mRNA (B, right panel) and MIG mRNA (C, right panel) were determined by ELISA and Northern blot analysis. Mean IP-10 concentrations ± S.D. ($n = 6$) are shown. $^{**}P < 0.01$ compared to unstimulated control; $^{\#}P < 0.05$ compared to IL-1β/IFNγ alone. One representative of three independently performed Northern blot experiments is shown.

lected regulators of angiogenesis were investigated. IL-8 has been recognized as important pro-angiogenic mediator [7]. Thus, effects of exogenous NO on the production of IL-8 by DLD-1 cells were investigated. As shown in Fig. 1A, IL-1β potently activated release of IL-8. Coincubation with IL-1β/IFNγ did not further upregulate IL-8 production. IL-8 was not inducible by IFNγ alone. Thus, IL-8 is primarily an IL-1β-dependent gene in DLD-1 cells. The role of NO in regulating gene expression in these cells was investigated by preincubation with the NO donor DETA-NO. We particularly selected DETA-NO since this donor spontaneously releases NO with a characteristically long half-life of 16.5–20 h [12,13]. This slow-releasing NO donor may thus resemble chronic iNOS activation. DETA-NO significantly augmented release of IL-8 by DLD-1. Augmented secretion of IL-8 was paralleled by upregulation of mRNA steady-state levels (Fig. 1B). NO significantly increased low level basal release of IL-8 (Fig. 1C) and also expression of mRNA as detected by RT-PCR analysis (data not shown). In accord with previous data we confirm that DLD-1 cells constitutively produce VEGF [14]. Basal expression and release of VEGF were upregulated by coincubation with NO (Fig. 2).

3.2. NO suppresses IL-1β/IFNγ-induced expression of the anti-angiogenic chemokines IP-10 and MIG

Among the chemokines expressed by IL-1β/IFNγ-activated DLD-1 colon carcinoma cells are the tumor-suppressive molecules IP-10 and MIG. In contrast to IL-8, expression of these two mediators required cellular activation by IFNγ (Fig. 3A,C). In these cells coincubation with IL-1β/IFNγ was necessary to detect a robust IP-10 production within a 8 h incubation period (Fig. 3A). Preincubation of DLD-1 cells with DETA-NO potently reduced expression of IP-10 (Fig. 3B) and MIG (Fig. 3C, right panel).

4. Discussion

Induction of iNOS protein and activity has been observed in colorectal cancer [2,3] and is supposed to be mediated by pro-inflammatory cytokines expressed at the tumor site [15–

17]. Upregulation of iNOS also has been observed in other human cancers, among them breast [18] and head and neck cancer [19]. Cell culture experiments suggest an anti-proliferative role of NO on colon carcinoma cells [20]. Furthermore, apoptosis of cancer cells driven by NO in vitro is a well-described phenomenon [21]. However, analysis of iNOS in human tumor tissues and murine tumor models may point to a different role of NO in tumor biology. Expression of iNOS in colon cancer is positively correlated with the frequency of mutations in the p53 tumor suppressor gene. This implies that NO provides a significant selection pressure for non-functional p53, a mechanism that may ultimately promote tumor growth [3]. It has also been reported that overexpression of iNOS in DLD-1 cells results in enhanced tumor growth after injection into nude mice. Tumors derived from these iNOS overexpressing carcinoma cells appear markedly more vascularized [6]. Notably, expression of iNOS and production of NO has been recognized as a marker of poor prognosis in colorectal cancer [22,23].

As angiogenesis is a chief parameter determining tumor growth, we investigated effects of NO on DLD-1 cells with respect to expression of four well-documented regulators of angiogenesis, namely angiogenic VEGF [24] and IL-8 as well as angiostatic IP-10 and MIG [7]. Due to divergent time kinetics (chemokine production: fast; iNOS expression and NO production much more delayed compared to chemokine production) it turned out to be impossible to investigate the function of endogenously produced NO in this experimental setting (data not shown). Therefore, in the present study the slow-releasing NO donor DETA-NO [25] was used in order to resemble iNOS-dependent high output production of NO in cancerous tissues. Here, we report that production of IL-8 in DLD-1 cells is augmented by coincubation with NO. Upregulation of IL-8 release by NO agrees with reports on human mesangial cells [26], endothelial cells [27], and monocytic cells [12]. It is interesting to note that IL-8 not only is as active as VEGF in mediating angiogenesis [28], but is also a growth factor for colon carcinoma cells [29]. In accord with previous data on glioblastoma and hepatocellular carcinoma cells [30], constitutive production of VEGF was enhanced by NO. Likewise, overexpression of iNOS can mediate amplification of VEGF [31] and reduced VEGF has been detected in tumors of iNOS-deficient mice [32]. In contrast, expression of the angiostatic chemokines IP-10 and MIG was suppressed by NO. A similar effect of NO has been observed in vascular endothelial cells [33]. These results suggest that NO may be able to specifically downregulate cellular responses to IFNγ in DLD-1 cells. This notion concurs with a report that demonstrates the capability of NO to interfere with activation of STAT-1 [34], a pivotal component of IFNγ signalling. By contrast, NO enhances a broad range of signaling pathways triggered by inflammatory cytokines like IL-1β and may thereby evoke amplification of IL-8 and VEGF expression [35].

Taken together, NO differentially regulated expression of modulators of angiogenesis in DLD-1 colon carcinoma cells. Production of tumor-promoting IL-8 and VEGF was amplified, whereas in the same cells expression of tumorstatic IP-10 and MIG was efficiently downregulated by NO. As IP-10 and MIG are supposed to be key mediators of IFNγ-mediated tumor regression [7], this regulatory function of NO may contribute to the phenomenon of tumor promotion by NO. The present data are in accord with reports on control of colon carcinogenesis in rodents by blockage of iNOS activity [36] and further support the concept of iNOS inhibition as a therapeutic strategy for the treatment of human cancer.

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