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Research Article

Nitric oxide modulates the expression of endothelial cell adhesion molecules involved in angiogenesis and leukocyte recruitment

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ABSTRACT

Tumor angiogenesis and immune response have in common to be cell recognition mechanisms, which are based on specific adhesion molecules and dependent on nitric oxide ($NO^{\bullet}$). The aim of the present study is to deepen the mechanisms of angiogenesis and inflammation regulation by $NO^{\bullet}$ to find out the molecular regulation processes that govern endothelial cell permeability and leukocyte transmigration.

Effects of $NO^{\bullet}$, either exogenous or produced in hypoxic conditions, were studied on microvascular endothelial cells from skin and lymph node because of their strong involvement in melanoma progression. We found that $NO^{\bullet}$ down-regulation of pseudo-vessel formation was linked to a decrease in endothelial cell ability to adhere to each other which can be explain, in part, by the inhibition of PECAM-1/CD31 expression. On the other hand, $NO^{\bullet}$ was shown to be able to decrease leukocyte adhesion on an endothelial monolayer, performed either in static or in rolling conditions, and to modulate differentially CD34, ICAM-1/CD54, ICAM-2/CD102 and VCAM-1/CD106 expression.

In conclusion, during angiogenesis and leukocyte recruitment, $NO^{\bullet}$ regulates cell interactions by controlling adhesion molecule expression and subsequently cell adhesion. Moreover, each endothelial cell type presents its own organospecific response to $NO^{\bullet}$, reflecting the functions of the tissue they originate from.

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Introduction

Among the numerous cellular (mutagenesis, angiogenesis, immune response, etc.) and molecular (HIF, VEGF, $NO^{\bullet}$, etc.) processes regulating the complex process of cancer, progression involves a stepwise series of genetic alterations to tumor cells. This results in tumor growth, invasion and ability to induce angiogenesis. Moreover, tumor gets around host immune system so that tumor cells migrate into the vasculature to other organs and form metastasis. Both mechanisms of angiogenesis and immune response are based

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Abbreviations: 1400 W, *N*-(3-(aminomethyl)benzyl)acetamide; DPTA-NONOate, dipropylenetriamine NONOate; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; cGMP, cyclic guanosine monophosphate; HIF, hypoxia inducible factor; HPLNEC.B3, human peripheral lymph node endothelial cell, B3 clone; HSkMEC, human skin microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; L-NAME, *N*(G)-nitro-L-arginine-methyl ester; L-NNA, *N*(G)-nitro-L-arginine; $NO^{\bullet}$, nitric oxide; PECAM-1, platelet endothelial cell adhesion molecule-1; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; VCAM-1, vascular cellular adhesion molecule-1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor

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on intercellular interactions: angiogenesis is mediated by endothelial cell (EC)–EC contacts underlying permeability; immune response implies recognition of ECs by leukocytes that migrate toward inflamed area. So, these phenomena are directly regulated by the EC adhesion molecules expression pattern. Furthermore, all of the above steps can be influenced by nitric oxide (NO$^•$) signaling.

In melanoma progression, tumor cell survival appears to be dependent on NO$^•$ [1]. Rather, there is some evidence that inducible NO synthase (iNOS), responsible for the production of high quantities of NO$^•$, displays a stronger expression and a higher activity level in malignant compared to benign tissue [2]. A high iNOS expression is also associated with a poor survival of melanoma patients [3]. Moreover, endothelial NOS (eNOS), producing a low and continuous flow of NO$^•$, is up-regulated during hypoxia. Indeed, Coulet *et al.* [4] identified a hypoxia-responsive element in the human eNOS gene promoter and Presley *et al.* [5] found an increased association between heat shock protein 90 and eNOS upon hypoxia. Recently, eNOS has been shown to be able, in anoxic atmosphere, to reduce nitrite anions to NO$^•$, independently of arginine [6,7]. Hypoxic production of NO$^•$ should also be catalyzed by xanthine oxidase, an enzyme structurally related to bacterial nitrate and nitrite reductases [8]. These new findings might explain the overproduction of NO$^•$ in low oxygen microenvironment created during carcinogenic process.

While the role of NO$^•$ in cancer biology has long been unclear because of conflicting data, today, some questions are beginning to be solved. In fact, the concentration of NO$^•$ seems to dictate the phenotypic response and the dichotomous nature of this radical [9]. For example, on breast cancer cells, low doses of NO$^•$ (1-300 nM) can mediate proliferative and protective effects, dependent on cyclic guanosine monophosphate (cGMP) signaling, Akt phosphorylation and HIF1α stabilization. But when NO$^•$ levels are sufficiently high to induce p53 phosphorylation (above 300 nM) or nitrosative stress (>500 nM), a cytostatic and even an apoptotic response can be induced [10]. During angiogenesis, NO$^•$ was shown to stimulate the proliferative and migratory functions of ECs in a cGMP-dependent manner [11], whereas high NO$^•$ concentrations inhibit angiogenic responses, through inhibition of PKC, ERK and c-Jun phosphorylation [12]. In contrast, the antitumor response of leukocytes is inhibited by NO$^•$, whatever the concentration, through inhibition of neutrophils [13] and T cells adhesion [14] on endothelium.

However, even if some molecular effects of NO$^•$ begin to be understood, the consequences of a NO$^•$ overproduction in cancer processes have been poorly studied at the cellular level for the modulation of cell recognition and adhesion molecule expression. Only few groups have shown that, in an inflammatory context, VCAM-1/CD106, ICAM-1/CD54 and E-selectin/CD62E seem to be down-regulated after NO$^•$ treatment [15,16]. The observed NO$^•$ effect depends on the activity and localization of NOS isoforms, on the concentration and duration of NO$^•$ exposure, and also on the cell sensitivity to NO$^•$.

The aim of the present study is to evaluate the effects of NO$^•$ on cell recognition mechanisms at the EC level. This process is involved in two steps of cancer progression: angiogenesis and leukocyte recruitment and we presented the consequences of NO$^•$ effect on both processes with the aim to elucidate the mechanisms of angiogenesis and inflammation regulation by NO$^•$. Its effects on cell interactions have been evaluated on two EC lines from different organs involved in melanoma: skin and peripheral lymph nodes. To disclose entirely the fundamentals of the processes, both cellular (cell adhesion either between ECs or between ECs and leukocytes) and molecular aspects (adhesion molecule expression) have been considered. The EC response to various concentrations of either exogenous (NO donors) or endogenous (produced in hypoxia) NO$^•$ suggests, first, an important role of NO$^•$ in both EC-EC interactions and leukocyte adhesion on EC, and second, points out an organospecific character of the EC response to NO$^•$.

Material and methods

Cell lines

The endothelial cell (EC) lines, human peripheral lymph node endothelial cells (HPLNEC.B3) and human skin microvascular endothelial cells (HSkMEC), were established in our laboratory according to the method previously described [17] and patented (C. Kieda, Centre National de la Recherche Scientifique patent 99-16169). As shown after immortalization by human telomerase reverse transcriptase [18], their phenotype was stable in terms of adhesion molecules and typical EC characteristics (e.g. the presence of angiotensin converting enzyme and von Willebrand factor) [17]. CEMT4 are human leukemic CD4+ T cells, purchased from the American Type Culture Collection (Rockville, MD).

Cell lines were cultured in OptiMEM with Glutamax-I (Invitrogen, Cergy Pontoise, France) supplemented with 2% Fetal Bovine Serum (BioWest, Nuaille, France), 40 μg/ml gentamycin (Invitrogen) and 0.05 μg/ml fungizone (Invitrogen). Cells were seeded at 2×10^4 cells/cm^2, 48 h before experiments and maintained at 37 °C in a 5% CO2/95% air atmosphere.

Cell treatments with NO donors or NOS inhibitors and hypoxia

NO donors (dipropylenetriamine NONOate or DPTA-NONOate, spermine-NONOate, from Cayman Chemical, Ann Arbor, Michigan) or NOS inhibitors (*N*(G)-nitro-L-arginine-methyl ester or L-NAME, *N*(G)-nitro-L-arginine or L-NNA, *N*-(3-(aminomethyl)benzyl)acetamide or 1400 W, from Cayman Chemical, Ann Arbor, Michigan) were added to cell cultures 48 h after seeding and incubation was allowed for 24 h or 48 h. Inactivated NO donors, used as negative controls, were obtained by dissolution of NO donors in phosphate-buffered saline (PBS) and incubation at 37 °C for at least 48 h before use on cells. In each experiment, decomposed NO donors were used in the same conditions than active NO donors and attest for the role of NO$^•$ in the studied mechanism. In fact, this excludes the involvement of nitrates, nitrites and residual backbones of the donor decomposition.

For hypoxia treatments, ECs were placed in a humidified atmosphere containing 1% of oxygen. This oxygen pressure was obtained by introducing 95% N_2/5% CO_2 gas mixture (Air Liquide, Paris, France) in an automated PROOX *in vitro* chamber (C-174; BioSpherix, Redfield, NY) under the control of a PROOX sensor-model 110 (BioSpherix).

In vitro angiogenesis assay

Before angiogenesis assay, cells were treated with either NO donors or NOS inhibitors for 6 h when incubated with Spermine-NONOate and for 24 h with other molecules. Angiogenesis was performed on 96-well plates coated with Matrigel™ (BD Biosciences, San José, CA). Cells were briefly treated with trypsin (Invitrogen), washed and were seeded at 3×10^4 cells/cm^2 in the presence of NO donors or NOS

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