Role of Iron in Tumor Cell Protection from the Pro-Apoptotic Effect of Nitric Oxide

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ABSTRACT

The host defense against tumor cells is in part based upon the production of nitric oxide (NO) by activated macrophages. However, carcinogenesis may involve mechanisms that protect tumor cells from NO-mediated apoptosis. In the present study, we have assessed the effects of exogenous NO on the proliferation and survival of human liver (AKN-1), lung (A549), skin (HaCat), and pancreatic (Capan-2) tumor cell lines, compared with normal skin-derived epithelial cell cultures. Except to the HaCat cell line, all of the other human epitheloid cells were sensitive to the antiproliferation effect of S-nitroso-N-acetyl-penicillamine or DETA NONOate, whereas tumor cells had low if any response to sodium nitroprusside. Growth inhibition with exogenous NO correlated with an increased apoptosis, but was not mediated by cyclic GMP, peroxynitrite generation, or poly(ADP-ribose) polymerase modulation, all of which involved in NO-mediated growth inhibition of normal skin-derived epithelial cell cultures. The simultaneous addition of iron-containing compounds protected tumor cells from NO-mediated growth inhibition and apoptosis. Intracellular iron quantification indicated that, as deferoxamine, exogenous NO significantly decreased intracellular ferric iron levels in tumor cells. Together, the current study reveals that intracellular iron elevation rescues tumor cells from NO-mediated iron depletion and subsequent growth inhibition and apoptosis.

INTRODUCTION

NO is a messenger molecule with complex biological activities including vasodilatation, neurotransmission, immunoregulation, and inflammation (1–4). NO is synthesized enzymatically from l-arginine by at least three different NOSs. The endothelial and the neuronal isoforms are constitutively expressed. The third isoform, NOS-II, has been to be induced with stimuli that include lipopolysaccharide and cytokines (2). Once expressed, NOS-II synthesizes large amounts of NO, which can lead to the inhibition of T-cell proliferation, has tumoricidal effect on the formation of hydroxyl radicals, suppression of the activity of host defense cells, and promotion of cancer cell multiplication (17, 18). Primary neoplasms develop at body sites of excessive iron deposits (18). The invaded host attempts to withdraw iron from the cancer cells via sequestration of the metal in newly formed ferritin (17). The host also endeavors to withdraw the metal from cancer cells via macrophage synthesis of NO (19, 20). The present study indicates, in various human tumor cell lines, that the pro-tumoral effect of iron may be in part related to its ability to rescue cells from NO-mediated growth inhibition and apoptosis.

MATERIALS AND METHODS

Chemicals. NO-releasing chemicals used in this study are SNAP, NOC18 (DETA NONOate; (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazin-1-ium-1,2-diolate; Alexis, Coger S. A., Paris, France), and SNP (Sigma-Aldrich, Saint Quentin Fallavier, France). NO donors were put in solutions and prepared immediately before use at 5–50 μM. DFX (2–100 μM; Novartis-Pharma, Rueil-Malmaison, France), 3AB (1 mM), bovine SOD (120 IU/ml), catalase (130 IU/ml), Fe(NO3)3, potassium ferrocyanide, Fe3+ Cl (10–200 μM), dibutyrylguanosine cyclic 3′-5′ monophosphate (400–800 μM), and camptothecin were purchased from Sigma-Aldrich. ODQ (Alexis) was used to inhibit the NO-sensitive guanylyl cyclase.

Cell Culture. The human pulmonary adenocarcinoma (A549), skin (HaCat), and pancreas carcinoma (Capan-2) cell lines were obtained from the American Type Culture Collection (Manassas, VA). The hepatic carcinoma cell line AKN-1 is described elsewhere (21). Tumor cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and glutamine (all of these from Life Technologies, Inc., Cergy-Pontoise, France). Normal skin-derived epithelial cell cultures (keratinoocytes) were obtained from skin biopsies as described elsewhere (22). For keratinocytes, we used a special serum-free culture medium supplemented with epidermal growth factor and bovine pituitary extracts (all of these from Life Technologies, Inc.). After expansion, the cells were transferred into plastic flasks and incubated with the serum-free culture medium. For various tests, cells were harvested after trypsin-EDTA treatment of culture surfaces, washed, counted, and suspended before cultures at 37°C in 5% CO2 atmosphere.

Cell Proliferation. Each cell line was seeded in 6-well culture plates at a density of 30,000 cells/ml/well for AKN-1, HaCat, Capan-2, and normal cells or 15,000 cells/ml/well for A549 cells, which had a rapid cell division rate compared with the other cells tested. After cell adhesion (4–24 h), the various reagents were added simultaneously as described under “Results.” After incubation, cells were harvested by trypsin treatment of plates, washed with culture media, and counted with trypan blue exclusion. The effect of various treatments on the cell nucleus was also assessed after cell centrifugation on slides, fixation with 2% paraformaldehyde, permeabilization with 0.1% Triton X-100, and PNA labeling. The DNA content of the isolated nuclei was determined by flow cytometry using the PI channel of the FACSCalibur (Becton Dickinson, Mountain View, CA). G1, S, G2+M, and death cell populations were obtained after CellQuest software (Becton Dickinson) analysis. The Cytometer data were analyzed using ModFit LT software (Verity Software, Topsham, ME).
Triton X-100, and coloration with DAPI (Vectorshied; Vector Laboratories, Burlingame, CA). Cells were analyzed using a fluorescent microscope. To assess cell proliferation, various cell lines were cultured on 96-well plates at 4000 cells/well/50 μl. After adherence (24 h), wells were supplemented with various chemicals in an additional 100 μl/well. [3H]thymidine was added 24 h after cultures, and radioactivity uptake was measured 24 h later. The levels of nitrates were measured in cell supernatants using the colorimetric Greiss method as described previously (22).

Cell Cycle and Apoptosis. To analyze the cell cycle, treated cells were washed in PBS containing 1% BSA. Pellets were then treated with 200 μl of lysis solution [calcium- and magnesium-free PBS, 0.5% NP40, 20 μg/ml propidium iodide, 0.2 mg/ml RNase A, and 0.5 mM EDTA (pH 7.2); all of these from Sigma-Aldrich] at room temperature in the dark for 20 min before measurement. DNA analysis was performed using the System II Coulter program (XL Coulter Instrument; Coultronics, Margency, France). To measure apoptosis, externalization of membrane phosphatidylserine was analyzed using annexin-V-FITC and propidium iodide kit (Immunotech, Marseille, France).

Intracellular Iron Quantification. Intracellular total and Fe2+ levels were measured using a standard analytic procedure as detailed elsewhere (23). Fe3+ annexin-V-FITC and propidium iodide kit (Immunotech, Marseille, France). apoptosis, externalization of membrane phosphatidylserine was analyzed using annexin-V-FITC and propidium iodide kit (Immunotech, Marseille, France).

RESULTS

Tumor Cells Differ in their Sensitivity to Exogenous NO. The effect of exogenous NO on continuous growth of tumor cell lines derived from various tissues was analyzed. SNAP was used as an exogenous NO source (see below). As shown in Fig. 1, proliferation of tumor cells was variably affected by NO addition. Although HaCat cells showed low or no sensitivity to NO, the proliferation of other tumor cells significantly decreased in the presence of exogenous NO (P < 0.001). SNAP breakdown and NO generation did not vary in all of the cell lines, as controlled by the quantification of the levels of nitrates in cell supernatants (data not shown). These experiments indicate that most tumor cells remain sensitive to the antiproliferation effect of NO.

Whether NO-sensitive cells respond to other formulations of exogenous NO was then investigated. In addition to SNAP, which is a rapid (<40-min half-life), relatively pure NO-releasing chemical, we added slower NO-releasing SNP and the very slow (>48-h half-life) NO-releasing donor, Deta NONOate (NOC18). The effect of these molecules on the growth of normal skin-derived epithelial cell (keratinocytes) cultures in response to epidermal growth factor was also quantified. As shown in Fig. 2, the addition of SNAP or NOC18 clearly inhibited the proliferation of epithelial cells, whereas SNP had a significantly low (P < 0.001), if any, effect on the growth of most tumor cell lines. Of interest, NO-releasing molecules differed in their effect on keratinocytes. Compared with tumor cell lines, keratinocytes presented significantly higher sensitivity to SNAP (P < 0.02) and less response to SNAP (P < 0.01), but had a similar response to NOC18. These data indicate that, by contrast to SNAP and NOC18, SNP had little or no effect on tumor cell growth. In addition, slow NO release by NOC18 seems to be very toxic for both tumor and normal skin-derived cell cultures and, therefore, was excluded for further analysis.

Effects of NO on Cell Cycle and Apoptosis in Tumor Cells. Because SNAP showed a higher effect on tumor cells, we have used this chemical to assay mechanisms of cell growth inhibition and antitumor effects of NO. After a 48-h cell incubation with SNAP (200 μM), a significant dose-dependent increase of apoptotic cells was observed in tumor cells (Fig. 3; P < 0.003), as quantified by analyzing...
phosphatidylserine externalization. This was further confirmed by the appearance of a sub-G₁ DNA peak without evidence of nuclear cell cycle modification (see below). Apoptotic cells also lost their adhesion capacity, and >95% of annexin-V⁺ cells were recovered in nonadherent fraction. This is further evidenced by DAPI staining, which showed increased chromatin condensation and nuclear fragmentation in nonadherent cell fraction but not in adherent tumor cells (Fig. 4) treated with SNAP. To mediate growth inhibition, NO was also shown to regulate various gene expression in target cells such as p53 and c-myc (24). Expression of p53 and c-myc was then quantified in mRNA or protein levels. We did not observe any constant variations in p53 or c-myc expression after incubation with SNAP (data not shown), which led us to conclude that modulation of p53 and c-myc expression may not account for the antitumor effect of NO.

Role of Intracellular Signals after NO Treatment of Tumor Cell Lines. NO derivatives inhibit cell growth through multiple intracellular targets (1–4). Most biological functions of NO are mediated through the induction of intracellular accumulation of cGMP after the activation of guanylyl cyclase (1). To assay the role of cGMP on tumor cells, we added ODQ (25), a specific inhibitor of NO-sensitive guanylyl cyclase, to NO-supplemented cultures. ODQ did not affect growth inhibition of tumor cells by SNAP, although it significantly inhibited the antiproliferative effects of SNAP in keratinocytes (Fig. 5). Accordingly and in contrast to keratinocytes, addition of the cGMP analogue was not toxic for tumor cells (Fig. 5). NO-mediated cytotoxicity also implies PARP activation and subsequent NAD⁺ depletion or the generation of the pro-apoptotic peroxynitrites (4). Inhibition of PARP activation through the use of 3AB had no effect on tumor cells but partially rescued keratinocytes from death (Fig. 6).
The addition of SOD and catalase, which decrease NO reaction with oxygen radicals, did not modify NO effects on tumor cells but increased the growth of keratinocytes (Fig. 6). Thus, in contrast to normal skin-derived cultures, NO-mediated inhibition of tumor cell growth involves neither the activation of guanylyl cyclase and subsequent generation of cGMP and PARP activation nor the formation of peroxynitrites.

Iron Supplementation Rescues Tumor Cells from Death in the Presence of Exogenous NO. The absence of growth inhibition after SNAP addition on tumor cells was intriguing and led us to clarify this response. In addition to NO, SNAP contains iron cyanide and sodium cyanide, making it different from other NO-releasing chemicals. We questioned the role of non-NO ferricyanide in this molecule and its effects on tumor cells. As SNAP, the addition of Fe$^{3+}$CN (200 μM) to tumor cells did not affect their growth (Fig. 7A). In contrast, the addition of ferricyanide to SNAP significantly reversed the antitumor effect of NO in all of the tumor cells tested (Fig. 7A). The addition of nonferric sodium cyanide (another SNP derivative) had no effect on SNAP-mediated tumor cell inhibition (50–200 μM). These data may explain the absence of antitumor activity of iron-containing NO donor, SNP. Interestingly, ferricyanide did not significantly reverse growth inhibition of NO-treated keratinocytes (Fig. 7). These data may explain the absence of antitumor activity of iron-containing NO donor, SNP. To further confirm the role of iron, we added Fe$^{3+}$Ci (200 μM) simultaneously with SNAP. Data in Fig. 7B clearly indicate that, similar to Fe$^{3+}$CN, Fe$^{3+}$Ci reversed the antiproliferation effect of SNAP. Then, we asked what was the effect of Fe$^{2+}$ on NO-mediated antitumor effect. As shown in Fig. 7B, Fe$^{2+}$ significantly reverses the effect of SNAP but remained less potent than the Fe$^{3+}$ compound in this respect. Surprisingly, dose-response analysis showed that only 2–10 μM Fe$^{3+}$ salt neutralized 200 μM SNAP (Fig. 7B), whereas ferrous salt required higher concentrations and remained less efficient in this respect. In addition to increased cell recovery, Fe$^{3+}$Ci also decreased the sub-G1 DNA peak (Fig. 8A) and significantly rescued tumor cells from NO-induced apoptosis (Fig. 8B; P < 0.012). Together, these data show the ability of iron, ferric compounds in particular, to protect tumor cells from the antiproliferation effect of NO.

Antitumoral Effect of Exogenous NO Correlated with Rapid Intracellular Iron Depletion. NO–iron interactions led us to investigate the effect of NO on intracellular iron concentrations. Fig. 9A indicates that the addition of SNAP resulted in a rapid decrease in intracellular iron levels, an effect comparable with that observed with the potent iron chelator, DFX. The rapid intracellular iron depletion in the presence of NO donor SNAP corroborates the short half-life of this compound. The addition of Fe$^{3+}$Ci did not allow iron depletion by NO, because it restored or increased intracellular iron levels (Fig. 9A). When compared with tumor cell growth, the decrease of intracellular total or ferric iron in the presence of SNAP or DFX always corroborated with lower cell recovery, whereas stabilization of Fe levels protect cells from iron depletion and the growth inhibitory effect of NO (Fig. 9B). Finally, NO-mediated iron depletion was not amplified by DFX, an observation that enforces the potent effect of NO in this respect.

**DISCUSSION**

NO, initially recognized as a member of the antitumor effector molecules of macrophages (5), was subsequently shown to be induced in various human or murine cells including tumor cells without any
significant antiproliferation activity (10, 11, 14). This implied that tumor cells acquire mechanism(s) that protect them from the pro-apoptotic effect of NO derivatives. The present work indicates that tumor cells from distinct tissues remained sensitive to the growth inhibitory effect of exogenous NO, dependent upon NO formulation. These data also showed, at least in the three cell lines tested, that cGMP generation after guanylyl cyclase activation by NO was not involved in the antitumor activity of NO. Activation of PARP and consequent NAD<sup>+</sup> depletion was implied in a variety of NO-mediated cell cytotoxicity (4, 26, 27). PARP inhibition through 3AB addition did not significantly alter tumor cell growth inhibition by NO. Finally, NO may react with oxygen radicals and generate pro-apoptotic radicals such as peroxynitrites. The addition of both catalase and SOD did not inhibit the antitumor effect of NO, although they partially rescued normal cell growth from NO effects. Together, these data revealed that, compared with normal skin-derived epithelial cell cultures, several NO-mediated pro-apoptotic pathways (1, 4) were no more functional in tumor cells.

The critical finding of this study is the ability of iron radicals to rescue tumor cells from the antitumor activity of NO. This was revealed by the failure of SNP to display a high or any growth inhibitory effect, together with the capacity of exogenous iron to protect tumor cells from the growth inhibitory effect of NO. In our hands, ferric compounds were more efficient than ferrous ones in inhibiting NO effects. This may be attributable to the higher affinity of ferric ions to NO-containing molecules. These findings may provide an explanation for the well-documented pro-cancer action of iron radicals (17), because authors (18, 28) have clearly shown that iron accumulation in many organs is correlated with the process of carcinogenesis. The iron effect is suggested to be attributable to in part interactions between iron and oxidative burst, including NO (28–31).

Iron may protect cells from oxidative radicals through the induction of antioxidants in these cells (30, 31), which then protect cells by neutralizing NO-mediated oxidation (32).

The present study in tumor cells corroborates recent conclusions drawn in hepatitis patients. In vivo, treatment of hepatitis patients with IFN-α correlated with an in vivo enhancement of NOS - II expression (33). Authors (34, 35) have subsequently observed that patients with high iron levels were less responsive to IFN-α treatment, compared to those with low in vivo iron concentrations. Accordingly, Kim et al. (36) have recently suggested that non-heme iron content determined whether cytotoxic levels of NO resulted in apoptosis versus necrosis in hepatic cells. Regarding the present study, iron availability in hepatic tissue may therefore rescue virus-infected cells from NO-mediated iron depletion and death. Recently, Amoroso et al. (32) have shown that SNP prevented cell death through the stimulation by iron ions of the activity of Na<sup>+</sup>-Ca<sup>2+</sup> exchanger in C6 glioma cells. NO affinity to iron may also lead to intracellular Fe loss, which is by itself cytotoxic for tumor cells. This is likely to be the principal mechanism of NO in the present study because, as observed with DFX (37), rapid decreases in intracellular iron and ferric levels induced apoptotic cell death after tumor cell incubation with NO. Accordingly, iron supplementation and increased intracellular iron levels may decrease in vivo tumor cell sensitivity to the antitumoral activity of NO. This corroborates multiple studies (17, 18) indicating that cell iron efflux could assist in prevention and management of cancer. Pharmaceutical methods for depriving tumor cells of iron are being developed in experimental and clinical protocols (38). Thus, the present study may help to define new therapeutic approaches combining NO and cytoreductive chemotherapy.

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REFERENCES

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