Induction of Iron-derived EPR Signals in Murine Cancers by Nitric Oxide

EVIDENCE FOR MULTIPLE INTRACELLULAR TARGETS*

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Neil R. Bastian§, Chang-Yeol Yim***, John B. Hibbs, Jr.,†, and Wolfram E. Samlowski††

From the Department of Internal Medicine, Infectious Diseases and Hematology/Oncology Divisions, and the University of Utah/Veterans Administration Medical Center Cancer Immunotherapy Program, University of Utah School of Medicine, Salt Lake City, Utah 84132, the Veterans Administration Medical Center, Salt Lake City, Utah 84148

The cell-mediated immune response to syngeneic tumors activates the cytokine-inducible nitric oxide synthase. We observed that syngeneic murine tumors exhibited EPR signals related to iron-nitrosyl complex formation. Three different EPR active iron-nitrosyl species were observed, an Fe(RS)₂(NO)₂ signal and two differentiable heme-nitrosyl complexes. Hemoglobin assays showed that the heme-nitrosyl signals were not derived from contaminating hemoglobin. Signal amplitudes were attenuated in mice treated with N°-monomethyl-L-arginine (MLA), an inhibitor of nitric oxide synthase. Tumors grown in vivo contained EPR signals while those grown in culture without continuing cytokine stimulation lost the signals after a few days. Cultured cells that were treated with cytokines, or that were cocultivated with cytokine-activated macrophages, regained EPR active complexes. These results show that the cell-mediated immune response to syngeneic tumors involves the induction of nitric oxide synthase. While nitric oxide synthesis is induced in both tumor infiltrating macrophages and in the tumor cells themselves, only tumor cells contributed to formation of heme-nitrosyl complexes. This result indicates the presence of a novel intracellular target for NO within tumor cells.

The discovery of the L-arginine:NO pathway provided a biochemical explanation for an unusual pattern of metabolic perturbation described previously in tumor cells cocultivated with cytotoxic activated macrophages (1, 2). Prominent metabolic changes induced in target cells by NO included inhibition of DNA replication (3, 4), protein synthesis (5, 6), and mitochondrial respiration (7). Subsequent studies suggested that the cytotoxic mechanism involved disturbance of intracellular iron homeostasis (8). This was based on observations that activated macrophages caused inhibition of complexes I and II of the mitochondrial electron transport system and the citric acid cycle enzyme, aconitase (8–11). These three enzymes are known to contain [4Fe-4S] prosthetic groups that are essential for their catalytic function. Further studies demonstrated that enzyme inhibition involved the [4Fe-4S] prosthetic group rather than the apoenzyme (10). It was also observed that tumor cells cocultivated with activated macrophages released a significant fraction of their intracellular iron in parallel with development of inhibition of DNA synthesis and mitochondrial respiration (5, 8, 10, 11).

NO biosynthesis is inhibited by several N-substituted L-arginine derivatives, including, N°-monomethyl-L-arginine (MLA) (1) but not by the respective D-arginine stereoisomers, thus providing a useful experimental tool for demonstrating which effector functions of the cell mediated immune response are mediated by the L-arginine: NO pathway (1, 2).

Commoner et al. (12) found that a g = 2.039 EPR signal appeared in the livers of rats which were fed various chemical carcinogens. This signal was due to formation of a paramagnetic Fe-NO-thiol complex in the tissues (13). Vanin et al. (14, 15) and Butler et al. (16) structurally characterized inorganic, EPR active Fe-RS-NO complexes and found them to have a general chemical formula of (Fe(RS)₂(NO))₂. The complexes were found to be unstable with either oxidation or polymerization leading to loss of the EPR signal. This iron-based EPR signal was recently demonstrated in activated macrophages and their target cells after the induction of the high output NO synthase (17–19). Identification of (Fe(RS)₂(NO))₂ complex formation in tumor cells links activated macrophage-induced inhibition of iron-containing enzymes with nitric oxide biosynthesis.

Heme-nitrosyl EPR signals have been described in tumors from various sources. Brennen et al. (20) described a three-line signal found in two murine tumors, a reticular cell sarcoma and a neuroblastoma. Subsequent papers by other groups (21) described a similar signal in several other tumors. Normal tissue was found to not contain the three-line signal, although, Maruyama et al. (22) described such a signal in normal tissues held at room temperature for 1–2 days. This three-line signal was subsequently identified as being caused by nitrosylated heme-containing proteins (23–27).

We describe here evidence that both the Fe(RS)₂(NO)₂ and the heme-NO EPR signals are products of the cell-mediated immune response to tumor cells in vitro. Furthermore, these signals can be induced in cultured tumor cells by cytokines or activated macrophages in vitro.

MATERIALS AND METHODS

Animals—Specific pathogen-free C3H/HeN (C3H), BALB/c, C57BL/6N (B6), and C57BL/10J (B10) mice (6–8 weeks of age) were

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** Visiting faculty member from the Department of Internal Medicine, Hematology/Oncology, of the Chonbuk National University Medical School, Chonbuk, Korea.

†† Visiting faculty member from the Department of Internal Medicine, Infectious Diseases and Hematology/Oncology Divisions, and the University of Utah/Veterans Administration Medical Center Cancer Immunotherapy Program, University of Utah School of Medicine, Salt Lake City, Utah 84132.

§ The abbreviations used are: MLA, N°-monomethyl-L-arginine; IFNγ, interferon γ; Fe(RS)₂(NO), iron-dithiol-dinitrosyl complex; IL-1, interleukin-1α; NO, nitric oxide; PBS, phosphate-buffered saline; TNF, tumor necrosis factor α.
obtained from Harlan-Sprague Dawley (C3H and BALB/c) (Indianapo-
olis, IN) and Frederick facility (B6 and B10) (Frederick, MD), and housed
in the Salt Lake City Veterans Affairs Medical Center (SLC-VAMC)
Animal Care Facility. Mice were maintained under guidelines estab-
lished by the SLC-VAMC Animal Care Facility Animal Care Committee,
which also adhered to all NIH and experimental protocols. Mice were age and sex
matched at the onset of each experiment. All experiments were performed
at least twice with highly concordant results.

Reagents—Murine interferon γ (IFNγ) and tumor necrosis factor α
(TNF) were obtained as a generous gift from Genentech (San Francisco,
CA). Interleukin-1α (IL-1) was a gift from Hoffman La Roche.

Tumor Cell Lines—A number of murine tumor cell lines were used in
these experiments, including RD-995, L5-10, B10.2, MetWA, MCA
106, S-180, and EMT-6. RD-995 and LR-351 were gifts from Dr. Robert
Kallman, Stanford University (Palo Alto, CA) and obtained from
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Iron-Nitrosyl EPR Signals Induced by NO

Tumors were grown subcutaneously and dissociated into single cell suspensions as described in the text. EPR spectra were taken on 140 μl of packed cells. Peak heights are reported as the average ± standard deviation of at least duplicate determinations for each tumor type. EPR parameters were as described in the text.

| Tumor type | Fe(RS)2(NO)2 | Heme-NO | No. Cells (x10^4) |
|------------|--------------|---------|------------------|
| RD-995     | 174 ± 8      | 58 ± 7  | 3.2              |
| LR 351     | 49 ± 12      | 74 ± 9  | 3.5              |
| MCA106     | 242 ± 7      | 30 ± 6  | 2.7              |
| S-180      | 19 ± 4       | 28 ± 13 | 3.2              |
| Meth/A     | ND*          | 30 ± 2  | 2.3              |
| B10.2      | ND*          | 8 ± 3   | 3.7              |

* ND, none detected.

**Comparison of EPR Signals Generated in Tumors in the Presence and Absence of MLA**—Since the cell-mediated immune response to tumors may activate the cytokine-inducible nitric oxide synthase, we investigated whether tumors growing in mice exhibited EPR signals related to Fe(RS)2(NO)2 complex formation. 3.2 x 10⁹ freshly dissociated RD-995 cells contained Fe(RS)2(NO)2 and Heme-NO EPR signals with relative peak heights of 174 and 58 units, respectively (Fig. 1, spectrum A). Freshly dissociated RD-995 cells from MLA-treated animals exhibited a 5.5-fold decrease in the amount of nitrate detected in the medium. Similarly, the Fe(RS)2(NO)2 and heme-nitrosyl EPR signals (Fig. 1, spectrum B) had peak heights of 45 and 31 units, respectively, in 3.2 x 10⁹ cells. These peak heights correspond to 3.9- and 1.9-fold decreases in the amount of Fe(RS)2(NO)2 and heme-nitrosyl complexes produced in the MLA-treated cells relative to untreated cells.

**Comparison of EPR Signals from Tumors Obtained in Vivo and in Vitro**—To determine whether the apparent in vivo nitrosylation was related to host cell infiltration of tumors, EPR signals generated from tumor cell lines cultured in vivo were compared with signals derived from the same line growing in vivo. While all of the tumors obtained in vivo contained one or more NO-derived EPR signals (Table 1), those grown in vitro without cytokine stimulation contained only the semiquinone radical signal as illustrated in Fig. 1, spectrum C. All six tumors grown in vitro contained heme-nitrosyl signals while only four of the six tumor types, MCA 106, RD-995, LR-351, and S-180, contained detectable Fe(RS)2(NO)2 signals.

**Induction of NO Synthesis and EPR Signal Formation by Cytokines**—To evaluate whether induction of endogenous nitric oxide synthesis in cultured tumor cells would generate EPR signals, cultured RD-995 tumor cells, which had lost their Fe-NO EPR signals were cultured in the presence of inductive cytokines for 48 h. Tumor cells were incubated with (a) 25 units/ml murine IFNγ plus 50 units/ml human TNFα; (b) 25 units/ml IFNγ plus 20 units/ml human IL-1; or (c) 25 units/ml IFNγ plus 50 units/ml TNFα plus 20 units/ml IL-1 (31). Tumor cells cultured in an equivalent volume of medium alone served as controls. After 48 hours of cytokine exposure, tumor cell supernatants were assayed for nitrite production and cells were assessed for development of EPR signals. Results, shown in Fig. 2, showed that nitrite production by the cells correlated well with Fe(RS)2(NO)2 EPR signal height, suggesting that the signals are related to induction of endogenous nitric oxide synthesis. Unlike macrophages, which do not respond to IL-1 (38, 39), induction of tumor cell NO synthesis was more potently induced by IL-1/IFNγ than by TNFα/IFNγ.

A heme-nitrosyl EPR signal also appeared in the IFNγ/TNFα-treated cells. The size of the heme-nitrosyl signal did not appear to change in cells treated either with IFNγ/IL-1 or with all three cytokines (Fig. 2), while the size of the Fe(RS)2(NO)2 signal increased substantially, especially in cells treated with all three cytokines (Fig. 2). This suggests that NO is bound to most of the accessible heme in the cells before the formation of Fe(RS)2(NO)2 complexes begins.

**Effect of Cytokine Levels on Iron-Nitrosyl Signals in Cultured EMT-6 Cells**—Murine EMT-6 adenocarcinoma cells grown in culture for multiple passages over a period of several years were exposed to cytokines in concentrations that varied over several orders of magnitude. Four of six control cultures exhibited a small heme-nitrosyl EPR signal and a very small Fe(RS)2(NO)2 signal (Fig. 3, spectra A and B). Control cells which contained EPR signals also contained low levels of nitrite and nitrate (Fig. 4) while medium from the two cultures not exhibiting an EPR signal did not contain detectable levels of either nitrate or nitrite. The Fe(RS)2(NO)2 EPR signal tripled in amplitude in EMT-6 cells treated with low levels of cytokines (0.02 units/ml IFNγ, 0.02 units/ml IL-1, 0.04 units/ml TNFα) while the heme-nitrosyl signal approximately doubled in size (Fig. 3, spectrum C). The nitrite and nitrate levels in the culture medium of these cells also increased slightly (Fig. 4). Nitrite and nitrate levels in the culture medium continued to increase as the cytokine levels were increased (Fig. 4). Fe(RS)2(NO)2 EPR signal heights also increased with increasing cytokine levels, reaching a maximum then dropping off slightly at higher cytokine levels (Fig. 4). The heme-nitrosyl signal on the other hand did not increase in size beyond that seen in samples containing the lowest levels of cytokines (Fig. 4). These results suggest that a heme compound(s) is the first target of cytokine induced NO with formation of Fe(RS)2(NO)2 complexes occurring at higher NO levels.

EMT-6 cells grown subcutaneously in BALB/c mice develop heme-nitrosyl EPR signals (data not shown). When tumors are removed from the animal and grown in culture, they continue
from erythrocytes within the dissociated tumor. Alternatively, the signal could arise from the reaction of nitric oxide with intracellular heme containing molecules in either host macrophages or tumor cells. The role of erythrocyte contamination in producing the heme-nitrosyl signal was excluded by subjecting freshly dissociated tumor to ammonium chloride erythrocyte lysis (32). This procedure did not diminish the heme-nitrosyl EPR signal which has a lower limit of detectability of approximately 2 µM. Direct measurements of hemoglobin in tumor samples using a colorimetric microtiter assay established that hemoglobin contamination of tumor cell suspensions was undetectable at the 1 µg hemoglobin/well (101 nm) sensitivity of the assay (data not shown). These studies showed that hemoglobin was not the source of the heme-nitrosyl EPR signal.

Comparison of EPR Signals from Tumors and from Tumor Infiltrating Macrophages—The next evaluations were designed to evaluate the relative contributions of host macrophages and tumor cells to the EPR signals. Macrophages grown in culture did not contain EPR active complexes in the absence of cytokines. Macrophage cultures which were activated by IFNγ and either lipopolysaccharide or TNFα were found to express only the Fe(RS)₂(NO)₂ signal as previously reported (17, 18). To determine whether either Fe(RS)₂(NO)₂ or heme-NO signals could be induced in tumor cells in vitro, RD-995 cells grown in vitro were cocultivated with activated macrophages. Activated macrophages were thoroughly washed to remove cytokines before addition to the RD-995 cultures. Cultures containing both RD-995 cells and activated macrophages developed heme-NO and Fe(RS)₂(NO)₂ EPR signals while those cultured alone or cocultivated with non-activated macrophages did not develop EPR signals. Activated macrophages cultivated alone developed an Fe(RS)₂(NO)₂ EPR signal but not a heme-nitrosyl signal. It was not possible to determine whether the Fe(RS)₂(NO)₂ signal seen in the cocultivated RD-995 and activated macrophage cells developed in the macrophages or in the tumor cells. The presence of MLA, a competitive inhibitor of nitric oxide synthase (1, 2), in the cocultivation medium, diminished formation of both EPR active complexes.

Discussion

Our current study demonstrates that the cell-mediated immune response is activated during progressive cancer growth in mice. We have also demonstrated that nitric oxide, one of the products of this response, interacts in a complex fashion with iron-containing molecules within the tumor cells. Our EPR

**FIG. 2.** Induction of NO synthesis by cytokines in cultured RD-995 tumor cells as shown by nitrite accumulation in the medium and EPR signals in the cells themselves. Cytokine concentrations were: 25 units/ml IFNγ, 20 units/ml IL-1, and 50 units/ml TNFα. Nitrite measurements are reported as mean ± S.D. on duplicate readings from three different batches of cultured cells for each treatment. EPR experiments were repeated twice and are reported as the mean ± standard deviation.

**FIG. 3.** EPR spectra of EMT-6 cells treated with varying levels of cytokines. A, control cells grown in culture for multiple passages in the absence of cytokines in which no NO₂ or NO₃ was present in the culture medium. B, control cells grown as in A but containing low levels of NO₂ and NO₃ in the culture medium, presumably due to endotoxin contamination in the culture medium. C, cells grown as in A but treated with 0.02 units/ml IFNγ, 0.02 units/ml IL-1, and 0.04 units/ml TNFα. D, cells grown as in A but with 20 units/ml IFNγ, 20 units/ml IL-1, and 40 units/ml TNFα. EPR parameters are as described under “Materials and Methods.” Instrument gains were 10 x 10⁶ for spectra A-C and 4.0 x 10⁶ for spectrum D.

**FIG. 4.** Nitrite and nitrite plus nitrate concentrations in culture medium of EMT-6 cells and Fe(RS)₂(NO)₂ and heme-NO EPR signal heights in EMT-6 cells treated with increasing levels of cytokines. IFNγ and IL-1 concentrations are given, and TNFα concentrations were twice those of the other two cytokines.
data indicates that nitrosylation of iron-dithiol groups to form Fe(RS)$_2$(NO)$_2$ complexes can take place in both tumor cells and macrophages. Two other EPR signals detected in tumor cells, related to the interaction of heme-bound iron with nitric oxide. These signals included a triplet signal centered at $g = 2.012$, with a hyperfine coupling constant of 1.7 millitesla which is typical of heme-nitrosyl complexes in which the iron atom is five coordinate (26, 27). A second heme-nitrosyl signal observed in some tumor samples, derived from six coordinate iron in which the hyperfine coupling between iron and NO is not seen (23). The type of EPR signal(s) formed in the tumors did not correlate with the progressor or regressor phenotype of the tumors.

A series of experiments were performed to establish that the heme signals were, in fact, derived from tumor cells. The possibility of hemoglobin contamination was excluded by erythrocyte lysis using Tris-buffered 0.15 M ammonium chloride, and by biochemical determinations of hemoglobin in tumor cell samples. The induction of heme-nitrosyl EPR signals in NO-treated tumor cells which had been maintained in culture for several years also rules out hemoglobin contamination as the source of this signal. The possibility that these signals were induced in host-derived macrophages was also evaluated. Heme-nitrosyl signals could not be detected in either unstimulated or cytokine-activated macrophages. Addition of cytokine-activated macrophages to cultured tumor cells induced formation of EPR signals in the tumor cells, demonstrating that nitric oxide diffused from the activated macrophages to tumor cell targets, resulting in nitrosylation of iron prosthetic groups. Addition of cytokines to cultured, EPR inactive, tumor cells caused induction of autolysis and heme-nitrosylation of iron-dithiol groups. Addition of cytokines to cultured tumor cells induced formation of EPR signals in the tumor cells.

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