Nitrosative Stress in Rotated Three-Dimensional Colorectal Carcinoma Cell Cultures Induces Microtubule Depolymerization and Apoptosis

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Abstract
Malignant cells undergo anoikis as they encounter fluid shear stress during transit to a metastatic site. We postulated that intracellular nitric oxide (NO) contributes to this cell death by comparing the growth of human colorectal carcinoma cells in low fluid shear stress rotated three-dimensional (Rotated 3-D) cultures with growth in static three-dimensional (Static 3-D) cultures on nonadherent surfaces and with two-dimensional monolayer (Monolayer 2-D) cultures. NO, loss of microtubules, and apoptosis increased significantly in Rotated 3-D cultures within 10 min and persisted at 24 h, whereas inhibition of NO synthase decreased apoptosis and intracellular NO and prevented tubulin degradation. Thus, fluid shear stress and three-dimensional growth increases NO synthase and NO to cause tubulin breakdown and induce anoikis. Intracellular NO in malignant cells entering the circulation may be a novel target for metastasis by colorectal carcinoma.

Introduction
Circulating tumor cells are a major problem in cancer (1, 2) because, even though only a small fraction survive, it is that small fraction that creates metastases to kill the host. Malignant cells in the circulation are not only exposed to potentially toxic host cells but must also survive in suspension in a fluid shear stress (3). Thus, metastatic potential may be proportional to the ability of a malignant cell to survive in an environment that has fluid shear stress without adhesion to a substrate. This condition is modeled in rotary suspension culture because cells are suspended within a low fluid shear stress flow field (4) and may be exposed to anoikis, the type of programmed cell death that occurs when adherent cells detach from extracellular matrix or adjacent cells and are suspended within an aqueous medium (5). The mechanisms involved in anoikis include loss of integrin-mediated survival signals (6) as well as increased oxidative stress (7). Thus, metastasis involves not only invasion, extravasation, and survival within the host parenchyma but also the ability to survive the effects of suspension within the circulation as well as the oxidative stress of implantation within the microcirculatory bed of an organ. We (8, 9) and others (10, 11) have shown that hepatic endothelium releases nitric oxide (NO) and reactive oxygen species (ROS) into the extracellular fluid that kill weakly but not highly metastatic cells as they arrest within an organ. Because oxidative stress is increased within cells undergoing anoikis, nitrosative stress secondary to increased levels of intracellular NO may also occur. We hypothesized that when metastatic precursor cells enter the circulatory flowstream, their intracellular NO and ROS increase, causing sufficient internal nitrosative and oxidative stress that most cells undergo apoptosis on implantation in a distant capillary bed. If this postulate is true, a future therapy to prevent metastasis may be to increase oxidative and nitrosative stress further within circulating cells. We tested this hypothesis in a bioreactor that suspends cells by rotation around the horizontal axis with a fluid shear stress that is as low as 0.2 dynes/cm² (4).

Materials and Methods
Cell Culture. The human colorectal carcinoma (CRC) cell lines are the weakly metastatic MIP-101 and Clone A and the highly metastatic CX-1 that have been described previously (12). The monolayer, the stationary three-dimensional (Static 3-D), and Rotated 3-D cultures were produced as described by Jessup et al. (13). Briefly, cells were cultured in complete medium of RPMI 1640 plus 8% heat-inactivated FCS plus antibiotics. Cells were free of Mycoplasma by monthly reverse transcription-PCR screening. Stationary three-dimensional cultures were established by incubating 2×10⁶ cells/ml in 10-cm Petri dishes coated with poly (2-hydroxyethyl methacrylate; poly-HHEMA) as described previously (13). The rotated three-dimensional (Rotated 3-D) cultures were produced with 2×10⁵ cells incubated in complete medium in 55-ml rotary cell culture system vessels (RCCS; Synthecon, Houston, TX) at 17–20 rpm as also described previously (13). In some experiments, antioxidants and NOS inhibitors were added. l-NAME (Sigma-Aldrich Corp., St. Louis, MO) was preincubated with MIP-101 cells at 10 mM for 16 h at 37°C and then were removed from the culture during the different culture conditions. The other inhibitors [l-NMMA (N⁵-monomethyl-l-arginine; 5 mM; Biomol, Plymouth Meeting, PA), 1400 W (100 nM; Biomol), and AG490 (7.5 μM; Calbiochem, San Diego, CA)] were added at the initiation of the different culture conditions at the concentrations specified and were present throughout the culture period.

Assessment of Apoptosis. Annexin V staining was used to detect cells that were committed to an apoptotic death (14). Cells (1×10⁶) were harvested, washed twice with 500 μl of PBS (pH 7.4) at 4°C, and resuspended in 100 μl of propidium iodide-Annexin V-FITC dual-staining solution as described previously (TACS Annexin V-FITC protocol; Trevigen, Gaithersburg, MD), and then were incubated in the dark for 15 min at room temperature. Four hundred μl of 1× binding buffer was added to the cell suspension, and the cells were analyzed by flow cytometry within 1 h. Caspase-3 activity was measured by labeling cells with cell permeant fluoroprobe PhiPhiLux (Ref. 15; G2D2; OncolImmunin, Gaithersburg, MD) according to the manufacturer’s instructions and then by performing static fluorometry as described immediately below.

Intracellular NO and ROS Static Fluorometry. Static fluorometry was used to assess the relative concentrations of NO and ROS. Cells were cultured under indicated conditions and 50 μg of either DAF-FM diacetate (4-aminomethyl-6-methoxy-2′,7′-difluorescein diacetate; to detect NO) or CM-H₂DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; to detect ROS; both dyes from Molecular Probes, Eugene OR) were dissolved in 50 μl of DMSO and were added to 10 ml of cells in complete culture medium. Because extracellular NO and ROS were minimal (see Fig. 2), cells were incubated for 30 min at 37°C and then were immediately visualized by epifluorescence on a Nikon Diaphot inverted microscope with excitation at 470–490 nm/emission at 520–560 nm for fluorescein. Images were captured on an integrating charge-coupled device (CCD; Optronics V) and were digitized in Photoshop (Adobe Systems Inc., Mountain View, CA). The fluorescence images were then registered with the phase contrast image so that individual cells within the field were identified in phase contrast and the...
corresponding region in the fluorescence image was measured for area and mean fluorescence intensity in ImageJ 1.30 (NIH, Bethesda, MD) on 100–300 individual cells/group. Total fluorescence was calculated as the product of area and mean fluorescence intensity, and the significance of differences in means of total fluorescence was determined by one-way ANOVA and the Fisher’s LSD with StatView v.5 (SAS Institute, Cary, NC).

**Western Blot Analysis.** Extracts were prepared from 50% to 70% confluent MIP 101 cell monolayers that were washed with PBS (pH 7.4) at 4°C and then were lysed at 4°C in a buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 40 mM β-glycerophosphate, 1 mM EGTA, 0.25% sodium deoxycholate, 1% NP-40, 50 mM sodium fluoride, 20 mM sodium PIP, 1 mM sodium orthovanadate, and protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μg/ml pefabloc). Cellular debris was removed by centrifugation at 14,000 rpm for 15 min. Supernatants were assayed for total protein content (Bio-Rad, Hercules CA), fractionated on 8% gradient polyacrylamide SDS–PAGE gels and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocking the membranes with 5% (w/v) nonfat dry milk or 4% BSA in Tris-buffered saline (pH 7.4), containing 0.5% Tween 20 (TBS-T) for 3 h, the membranes were incubated with the primary antibodies overnight at 4°C. The primary antibodies used were a rabbit polyclonal anti-β-tubulin (1:5000; Chemicon, Temecula, CA) and a mouse monoclonal anti-inducible NO synthase (anti-iNOS/NOS type II (BD Transduction Laboratories, San Diego, CA) that were visualized by enhanced chemiluminescence (Supersignal WestPico; Pierce, Rockford, IL) using horseradish peroxidase-linked donkey antirabbit or antimalleus IgG as the secondary antibodies, respectively (Amersham Pharmacia Biotech, Piscataway, NJ). The proteins were quantified by scanning the images into Photoshop with analysis carried out using Image J version 1.30.

**Confocal Microscopy.** MIP 101 cells were cultured on glass coverslips (7 × 10^3/18-mm coverslip) for monolayer cultures, as well as in Rotated 3-D and static three-dimensional (Static 3-D) cultures. Cells were fixed with 4% formalin for 20 min at room temperature in the dark and permeabilized with 0.2% Triton X-100 for 10 min. at room temperature in the dark. Colocalization was examined by immunostaining with anti-β-tubulin antibody (1:50; Chemicon, Temecula, CA), whereas the secondary antibody for β-tubulin was a FITC-conjugated anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Rabbit antihuman iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the secondary antibody was a donkey rhodamine-conjugated antirabbit antibody. Coverslips were mounted using the ProLong antifade kit (Molecular Probes). Confocal microscopy was performed on an Olympus Fluoview confocal microscope with a 60X/1.4 N.A. objective lens. Imaging was performed at the Microscopy and Imaging Shared Resources facility of the Lombardi Cancer Center, Georgetown University Medical Center (Washington, DC).

**Results and Discussion**

**Induction of Apoptosis in Rotated 3-D Cultures.** A rotational culture system designed by National Aeronautics and Space Administration (NASA), the Rotating Wall Vessel (RWV), was used to assess whether rotational suspension culture increases intracellular NO and/or ROS in metastatic precursor cells. The RWV is a fluid-shear stress of only 0.2 dynes/cm² (4). This fluid shear stress is considerably evident in Static 3-D cultures. Thus, 24-h Rotated 3-D cultures induce gross changes in microtubule structure that reflect depolymerization and gross rearrangement.

Recent data from Banan et al. (18) have shown that iNOS is up-regulated by oxidative stress and also is associated with depolymerization of microtubules. As a result, we postulated that intracellular NO and ROS increased in Rotated 3-D cultures and initiated microtubule depolymerization. MIP-101 cells were cultured for up to 24 h as monolayers, Static 3-D spheroids, and Rotated 3-D cultures, and were examined by static fluorometry with cell permeant fluorescent dyes for intracellular concentrations of NO and ROS. However, this was colocalized with β-tubulin in Rotated 3-D cultures than in either of the other two culture conditions (47% ± 5% of total iNOS in Rotated 3-D cultures associated with β-tubulin versus 37 ± 1% in Monolayer 2-D or 26 ± 7% in Static 3-D cultures; P = 0.036 by ANOVA). These results suggest that iNOS and β-tubulin interact as microtubules depolymerize in Rotated 3-D and lose their symmetrical distribution that is still evident in Static 3-D cultures. Thus, 24-h Rotated 3-D cultures induce gross changes in microtubule structure that reflect depolymerization and gross rearrangement.
Fig. 1. Rotated three-dimensional (Rotated 3-D) cultures display apoptosis and depolymerization of microtubules within 24 h. MIP-101 cells were cultured as monolayers [monolayer two-dimensional (Monolayer 2-D)], or as stationary, unrotated spheroids on a nonadherent surface [static three-dimensional (Static 3-D)], or as spheroids rotated in the rotating wall vessel (RWV) for 24 h (Rotated 3-D). Cells were harvested by trypsinization, were stained with Annexin V-FITC and propidium iodide (PI), and were analyzed by flow cytometry. The data indicate that apoptosis was low in nonrotated cultures (Monolayer 2-D in A, and Static 3-D in B) but that rotation markedly increased apoptosis and possibly necrosis (PI+/Annexin−; in C). Indirect immunofluorescence by confocal microscopy was performed in D–I for inducible nitric oxide synthase (iNOS) and β-tubulin. MIP-101 cells were cultured in Monolayer 2-D (D–F), in nonrotating Static 3-D (G–I), or in Rotated 3-D (J–L) cultures for 24 h. Cells were fixed, permeabilized, and stained for human tubulin with mouse anti-β-tubulin followed by FITC-conjugated goat antimouse antibody; and cells were fixed, permeabilized, and stained for human iNOS by rabbit polyclonal anti-human iNOS followed by rhodamine-conjugated donkey antirabbit antibody. Fluorescence was performed by laser scanning confocal microscopy with image processing by the MetaMorph software program. Monolayer 2-D cultures of MIP-101 cells demonstrate the filamentous microtubular network in cells attached to a substrate (F). Microtubules are retracted, short but evenly and symmetrically distributed just underneath the plasma membrane in the Static 3-D cultures (H). In contrast, the β-tubulin is clumped and asymmetric in the Rotated 3-D cultures (K). The iNOS follows the tubulin distribution in the Monolayer 2-D cultured cells (D) but appears to be distributed more evenly throughout the three-dimensional cultured cells (G and J). White bar in the combined images, 20 μm.

Both peroxides and superoxide anion; Fig. 2A). ROS is significantly increased in stationary three-dimensional cultures compared with Monolayer 2-D cultures but the concentration of ROS within Rotated 3-D cells is only slightly greater than the ROS in monolayer cultured cells (Fig. 2B). All of the cells contain ROS in the three culture conditions, whereas only 1% of monolayer cultured cells were positive for NO compared with 78% of Static 3-D cultures and 88% of Rotated 3-D cultures. Total fluorescence was then calculated as the product of the area of each cell times the mean fluorescence of that cell. When total fluorescence was compared among the three growth conditions, total intracellular NO concentration increased in stationary three-dimensional cultures compared with the Monolayer 2-D control cultures (Fig. 2B). Intracellular NO concentration increased further in Rotated 3-D cultures compared with the stationary three-dimensional cultures (Fig. 2B, Static 3-D). Because the primary source of NO is NOS, we then assessed whether inhibition of NOS decreased NO levels. An analysis of MIP-101 and other human CRC cell lines indicates that they contain inducible and endothelial NOS but negligible amounts of the brain-related NOS (data not shown). When MIP-101 cells were incubated with 1-NMMA, a general NOS inhibitor, intracellular NO levels in Rotated 3-D culture were eliminated (Fig. 2B). NO was also almost completely blocked by 1-NMMA treatment in stationary three-dimensional cultures (Fig. 2B, Static 3-D). Interestingly, intracellular ROS increased in Rotated 3-D cultures when NO was reduced by treatment with 1-NMMA (Fig. 2C). This increase may reflect the degree to which NO scavenges ROS within cells so that the concentration of ROS increases as the scavenger is removed (19). Thus, shape change that occurs with transition from Monolayer 2-D culture to three-dimensional growth either on a nonadherent surface without rotation or in a low shear stress rotational culture increases the concentration of ROS, which indicates that peroxides and/or superoxide anion were increased in three-dimensional growth compared with monolayer culture. These results confirm and extend the findings of Jacobson (7), who demonstrated that ROS was increased in cells as they detach from a substrate. To the best of our knowledge, this is the first report that intracellular NO is also increased in cells in three-dimensional growth with shear stress.

Inhibition of NOS but Not General Antioxidant Therapy Inhibits Apoptosis in Three-Dimensional Rotational Culture of MIP-101 Cells. Having demonstrated that rotational culture increased intracellular NO that could be inhibited by a NOS inhibitor, we tested the postulate that inhibition of NOS activity would increase
Fig. 2. Intracellular nitric oxide (NO) and reactive oxygen species (ROS) increased during stationary and rotational three-dimensional culture and are associated with protein nitration in MIP-101 cells. A, MIP-101 cells were cultured for up to 24 h at 37°C as monolayer two-dimensional (Monolayer 2-D), static three-dimensional (Static 3-D), or rotated three-dimensional (Rotated 3-D) cultures, and then were labeled with DAF-FM (4-amino-5-methylamino-2',7'-difluorescein diacetate) to identify intracellular NO or with CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'dichlorodihydrofluorescein diacetate) to detect intracellular ROS, and images were obtained with a ×40 objective on a Nikon Diaphot inverted microscope equipped for epifluorescence. Images are presented as phase contrast (P) with corresponding fluorescence (F). Static fluorometry measurements are presented for intracellular NO (B) and intracellular ROS (C) measured for at least 300 cells and are presented as mean ± SE in arbitrary units (A.U.) as described in “Materials and Methods.” The NO concentration within cells in Rotated 3-D cultures was twice the level in cells in Static 3-D cultures, and was more than 7-fold greater than within cells in monolayer culture [solid bars, Monolayer 2-D, P < 0.0001 by ANOVA versus either stationary culture (B)]. In contrast, the ROS within cells in Static 3-D cultures was 3-fold greater than in Monolayer 2-D and ~2-fold greater than ROS in the cells in the Rotated 3-D cultures [P < 0.001 for either three-dimensional culture versus Monolayer 2-D cultures by one-way ANOVA, solid bars (C)]. MIP-101 cells were incubated with 5 mM N⁴-monomethyl-L-arginine (L-NMMA) to inhibit NO synthase (NOS), and NO and ROS were determined. Inhibition of NOS decreased NO but increased ROS in Monolayer 2-D and Rotated 3-D cultures [open bars compared to untreated cells (solid bars)]. Thus, NO increases within MIP-101 cells that were exposed to three-dimensional culture are additionally increased by rotation, and the NO seems to be generated by NOS. Each experiment was repeated at least twice.

Fig. 3. Inhibition of nitric oxide synthase (NOS) improves the viability of MIP-101 cells in rotated three-dimensional (Rotated 3-D) cultures. MIP-101 cells were cultured for 24 h at 37°C as monolayers (M), or were cultured as static three-dimensional (Static 3-D) cultures (3-D S), or were cultured as rotating three-dimensional cultures (3-D R) in the Rotating Wall Vessel (RWV), in the presence (+NMMMA) or absence (−NMMMA) of N⁴-monomethyl-L-arginine (L-NMMA) as described in Fig. 2; the cells were extracted, and blots of the extracts were probed for inducible NOS (iNOS) and β-tubulin (A). β-Tubulin expression was decreased in Rotated 3-D cultures (3-D R) in the absence of NMMMA (+NMMMA), whereas β-tubulin expression was high in monolayer (2-D ML) and Static 3-D (3-D S) cultures. However, culture of cells with NMMMA decreased expression of iNOS in monolayer, in Static 3-D, and in Rotated 3-D cultures with a consequent increase in β-tubulin in the Rotated 3-D (3-D R) cultures. Expression was quantitated, and the relative expression of cultures was compared with the amount of either iNOS or β-tubulin present in 24-h cultures of monolayer cells. In B, MIP-101 cultures were treated with 10 μM L-N-acetylcysteine (NAC) or 5 mM NMMMA for 3 days. Cultures were analyzed by flow cytometry after propidium iodide (PI) and Annexin V staining as described in “Materials and Methods;” results were expressed as mean ± SE of the viable (PI-/Annexin V-) cell fraction in at least three independent experiments for each culture condition. Neither NAC nor NMMMA had a significant effect on the survival of MIP-101 in stationary two-dimensional (Monolayer 2-D) or three-dimensional (Static 3-D) cultures that already had high viability. However, NMMMA significantly increased viability in Rotated 3-D (Rotating 3-D) cultures, whereas NAC treatment did not. In C, MIP-101 cells were incubated in Rotated 3-D cultures and were treated with 100 nM 1400W, a specific iNOS inhibitor (Rotated 3-D + 1400W), or with 7.5 μM AG490, a JAK2 inhibitor that may protect cells from oxidative stress and was used as a control (Rotated 3-D + AG490). Treatment with 1400W confirmed that iNOS is the critical NOS, because the inhibition of iNOS also increased viability in the Rotated 3-D cultures in the RWV.

NITROSATIVE STRESS MEDIATES ANOIKIS THROUGH iNOS

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with the l-NMMA treatment described previously. Clearly, neither oxidant therapy had any effect on apoptosis in MIP-101 cells cultured under either two-dimensional or three-dimensional stationary cultures because viability (propidium iodide-Annexin-V unstained cells) was almost 90% in these cultures (Fig. 3B). However, Rotated 3-D culture increased Annexin V staining so that only ~20% of cells were viable. l-N-acetylcysteine treatment actually reduced viability slightly (Fig. 3B). In contrast, l-N-NMMA treatment decreased the Annexin V-staining of the Rotated 3-D culture so that 55% of the treated MIP-101 cells were viable (Fig. 3B). The iNOS-specific inhibitor 1400W achieved a similar increase in viability (Fig. 3C). These results indicate the essential role of iNOS in the apoptosis associated with Rotated 3-D culture in human CRC.

Short-Term Shape Change to Three-Dimensional Rotational Culture Induces Intracellular NO and ROS in CRC Lines. It is not clear how long malignant cells may persist in the circulation, but it is possible that they may circulate for longer than the time expected to transit from a primary to the metastatic site because malignant cells may need to traverse the first capillary bed that they encounter to implant in a more distant organ (21). A recent study in CRC patients (1) suggests that tumor cells may circulate in as many as 70% of stage II and III patients even though they appear to be clinically free of disease for 6 months or more. Although this type of clinical study does not really clarify how long tumor cells survive in the circulation, these results may be continuously shed from subclinical metastases rather than surviving for months in the circulation. The recent identification by Chang et al. (22) of vascular mosaicism that enables tumor cells to be part of the lining of tumor neovessels suggests that malignant cells may detach from the three-dimensional endothelial-tumor matrix and then persist in the sluggish circulation that comprises much of the vasculature of the primary tumor (23). These observations raise the possibility that circulating tumor cells may be exposed to low fluid shear stress environment in suspension without attachment to a matrix or other cells for hours and possibly days. However, it is not known how quickly intracellular NO and ROS are increased in malignant cells as they enter the circulation and whether this response occurs in other human CRC cell lines. Cells of two other human CRC cell lines [Clone A, a poorly differentiated weakly metastatic line, and CX-1, a moderately differentiated highly metastatic line (12)] along with MIP-101 cells, were grown as monolayers, were lightly trypsinized, and then were placed in either Rotated 3-D cultures or Monolayer 2-D cultures and collected for measurement of intracellular NO and ROS by static fluorometry at 10 min to 1.5 h. Each of the CRC lines increased intracellular NO and ROS within 10–45 min of Rotating 3-D culture and the NO or ROS of the Rotated 3-D culture is at least as much as, if not greater than, the corresponding value for Monolayer 2-D cultures. Interestingly, the total amount of reactive nitrogen and oxygen intermediates (the reactive species index, which is the product of NO and ROS normalized by the product of the initial intracellular concentrations of NO and ROS at time zero) is increased in the MIP-101 and Clone A cultures at 10 and 45 min, respectively, compared with the CX-1 cultures. The reactive species index in CX-1 does not become greater in rotational cultures than in monolayer cultures until the CX-1 cells have been exposed to flow shear stress for 1 h (Fig. 4). The difference between rotational and monolayer culture for the reactive species index in both of the weakly metastatic CRCs (MIP-101 and Clone A) is between 1.5- and 3.5-fold greater than in CX-1 cells (Fig. 4). Thus, NO and ROS are increased in rotational culture in the two weakly metastatic CRCs more quickly than either NO or ROS is increased in monolayer cultures or in the more highly metastatic CRC (CX-1; Fig. 4).

In summary, our data indicate that human CRCs increase their nitrosative and oxidative stress on undergoing shape change from a Monolayer 2-D shape to a spheroidal three-dimensional shape and on entering a low fluid shear stress environment. These stressors induce apoptosis that is associated with microtubule depolymerization and tubulin degradation. iNOS is a central participant in this type of anoikis. Although not tested in this work, we postulate that strategies that increase intracellular NO and ROS further may provide a novel approach to reduce metastasis by enhancing tumor cell apoptosis as they implant at sites of metastasis and are exposed to exogenous NO and ROS.
References


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