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 flowstream (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 nitroso-
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...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 PSDL 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% NP40, 50 mM sodium fluoride, 20 mM sodium PP, 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 acrylamide 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 in TBS-T containing 0.2% Triton X-100 for 10 min. at room temperature in the dark. Colocalization was examined by immunostaining with anti-β-tubulin antibody (1:5000; 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 antimouse 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 × 105/18-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 antitoxin 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-filled cylinder that rotates around the horizontal axis and suspends cells or particles up to 150 μm in diameter with a surface fluid shear stress of only 0.2 dynes/cm² (4). This fluid shear stress is considerably less than the fluid shear stress of a conventional stirred bioreactor or that of laminar blood flow over endothelium, which has a shear stress approaching 10 dynes/cm² (4). However, this shear stress is similar to that of cells traveling within the flow stream of the circulation. Our studies initially used the poorly differentiated human CRC cell line MIP-101, which is weakly metastatic in the intrasplenic injection experimental metastasis assay (12). MIP-101 cells were cultured in three-dimensions with rotation (Rotated 3-D) in the RWV, in three-dimensional spheroid cultures over a nonadherent surface (Static 3-D) or in standard monolayer culture on tissue culture plastic (Monolayer 2-D culture). Apoptosis was confirmed by flow cytometry with Annexin V-FITC staining (14) and static cell fluorometry for DEVD degradation by activated caspase-3 (15). MIP-101 cells cultured in the Rotating Wall Vessel contain 41.7% Annexin V + cells as early as 24 h after culture initiation (Fig. 1C), whereas the monolayer (Fig. 1A) and Static 3-D (Fig. 1B) cultures do not display significant levels of apoptosis (8.1 and 6.9%, respectively). Similarly, the Rotated 3-D cultures displayed significant caspase-3 activity (29 ± 8.2% of Rotated 3-D cells were caspase-3-active compared with only 1 ± 0.4% and 1.5 ± 1.2% for Monolayer 2-D and Static 3-D cultures (mean ± SE, with P < 0.004 for Rotated 3-D versus either of the other culture conditions). Interestingly, DNA damage was not detected after 24 h of culture because neither DNA ladders by DNA gel electrophoresis nor DNA strand breaks by single-cell COMET assay were detected in Rotated 3-D cultures at 24 h (data not shown). Thus, Rotated 3-D cultures of MIP-101 cells undergo apoptosis that involves activation of caspase-3 without significant initial DNA damage. This is consistent with protein alteration/degradation as the initiating cause of cell death.

**Three-Dimensional Rotational Culture Is Associated With Microtubule Depolymerization and Increased NO and ROS.** We had observed that MIP-101 cells in Rotated 3-D cultures arrested in G2-M, with alterations in the expression of G2-M cyclin and associated regulatory kinases that did not occur in Static 3-D or Monolayer 2-D cultures (16). Because the G2-M checkpoint kinases also regulate spindle formation and function, we hypothesized that alterations to microtubules may also occur in three-dimensional rotational cultures. Other investigators have observed altered microtubule structure in similar Rotated 3-D cultures (17). Examination of microtubule structure under confocal microscopy after 24 h of culture revealed that the microtubules of MIP-101 cells in Rotated 3-D cultures were clumped and located just under the plasma membrane in an asymmetric fashion (Fig. 1D), whereas the microtubules of Monolayer 2-D cultures contained intact fibrils (Fig. 1D) distributed throughout the cells and Static 3-D cultures contained short microtubules distributed symmetrically around the cells under the plasma membrane (Fig. 1D). In contrast, iNOS was associated with microtubules in monolayer culture but evenly distributed throughout the cytoplasm of three-dimensional cultures, either rotating or static (Fig. 1D). When the images were analyzed in Imagej for the distribution of β-tubulin and iNOS, iNOS displayed two distributions: one compartment was distributed with β-tubulin and the other was distributed throughout the rest of the cell (Fig. 1, D–L). The β-tubulin had a fixed amount of iNOS associated with it because 81–84% of β-tubulin was associated with iNOS under the three different culture conditions (Fig. 1, D–L, data not shown). However, in the three conditions, more of the iNOS 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.

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. Monolayer cultures of MIP-101 cells did not contain detectable levels of intracellular NO but low concentrations of ROS (which is composed of...
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 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 brain-related NOS (data not shown). When MIP-101 cells were incubated with L-NMMA, a general NOS inhibitor, intracellular NO levels in Rotated 3-D culture were eliminated (Fig. 2B). NO was also almost completely blocked by L-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 L-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...
tubulin expression and decrease apoptosis in the Rotated 3-D cultures. NO and superoxide anion (O$_2^-$) spontaneously form the relatively long-lived reactive species peroxynitrite (20), which inhibits cell functions through protein nitration. We first assessed whether inhibition of NO synthase would affect the amount of tubulin that was present in the cell. When untreated MIP-101 cells were analyzed for iNOS and β-tubulin content on Western blots, tubulin levels were markedly decreased in Rotated 3-D cultures without a significant change in the iNOS amount (Fig. 3A). In contrast, when MIP-101 cells were treated with L-NMMA, iNOS was decreased in monolayer and stationary three-dimensional (Static 3-D) cultures and was essentially absent in Rotated 3-D cultures (Fig. 3A). Interestingly, the MIP-101 cells treated with L-NMMA in Rotated 3-D cultures had as much β-tubulin as did the cells growing under the other two conditions (Fig. 3A). Thus, the inhibition of iNOS was associated with the maintenance of the amount of β-tubulin within the cell.

We then assessed whether NOS inhibitors affected apoptosis. As a control for L-NMMA, MIP-101 cells were pretreated with the general antioxidant N-acetylcysteine for 16 h at 37°C to increase intracellular glutathione as a general antioxidant defense. This was compared with untreated cells, 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 the absence of L-NMMA (A), β-tubulin expression was high in monolayer (2-D ML) and Static 3-D (3-D S) cultures. However, culture of cells with NMAA 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 cells were treated with 10 μM N-acetylcysteine (NAC) or 5 mM NMMA 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 NMMA 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, NMMA 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.
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-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 because the cells 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 a 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 Rotated 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 spherical 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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