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Walker Carcinosarcoma Cells Damage Endothelial Cells by the Generation of Reactive Oxygen Species

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The passage of circulating tumor cells across vessel walls is an important step in cancer metastasis and is promoted by endothelial injury. Because Walker carcinosarcoma 256 (W256) cells generate oxygen-derived free radicals after cellular activation, the authors tested the hypothesis that these cancer cells can damage endothelial monolayers by producing such reactive oxygen species. To confirm that oxygen-derived radicals can damage endothelial cells, ^{3}H-2-deoxyglucose-labeled human endothelial cell monolayers were exposed to xanthine oxidase in the presence of 0.2 mmol/l xanthine. ^{3}H-2-deoxyglucose release was observed after the addition of xanthine oxidase in concentrations ranging from 6.5×10^{-3} to 52×10^{-3} units/ml. The extent of damage correlated with xanthine oxidase-dependent chemiluminescence ($r = 0.91$). Chemiluminescence assays in the presence of 5×10^{-5} M luminol confirmed activation of the W256 cells by 1×10^{-6} M chemotactic peptide fMLP. When fMLP-activated W256 cells were incubated with endothelial monolayers, concentrations of 2×10^{6} to 6×10^{6} W256 cells/ml were found to cause a 27% increase in the specific release of 2-deoxyglucose after a 90-minute incubation. A small but significant increase in ^{3}H-2-deoxyglucose release also was observed in the absence of fMLP. Detection of ^{3}H-2-deoxyglucose release in the presence of activated or unactivated tumor cells was dependent on preincubating the endothelial cell monolayer with 1 mM buthionine sulfoximine, an inhibitor of glutathione synthesis. Under these conditions, the specific release of ^{3}H-2-deoxyglucose was increased from nondetectable levels to 21%, in the presence of 6.5×10^{-3} units of the oxidase. Cultured W256 cells promoted isotope release from endothelial cell monolayers when activated with phorbol myristate acetate. Catalase (1000 units/ml) inhibited the tumor cell-induced release of ^{3}H-2-deoxyglucose by 84% whereas superoxide dismutase, even at concentrations of 1 mg/ml, had no effect. A requirement for cell contact was shown because addition of cell-free supernatants from fMLP activated tumor cells did not cause ^{3}H-2-deoxyglucose release and because pretreatment of W256 cells with 1 μM cytochalasin B inhibited their ability to promote isotope release even while increasing tumor cell-generated chemiluminescence threefold. Electron microscopy revealed that fewer cytochalasin B-treated W256 cells were attached to the endothelial cell monolayer than in untreated controls. It is concluded that the W256 tumor cells can damage endothelial cells directly via a mechanism involving production of reactive oxygen species. (Am J Pathol 1989, 134:787–796)

Cancer cells frequently disseminate from primary tumors via the bloodstream. The entrapment of intravascular tumor cells in microvascular networks is probably a prerequisite for their growth into metastatic lesions.[1,2] Factors involved in cancer cell arrest include the physical constraints placed on circulating tumor cells as they enter vessels of small diameter and the expression of adhesion molecules on the surface of endothelial cells.[3–5] Since the successful establishment of secondary tumors seems to be associated with prior translocation of cancer cells into extravascular tissue, however, tumor cell arrest may not be sufficient for the establishment of secondary tumors. Most cancer cells do not survive long periods within the blood stream.[1]

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Microvascular walls, consisting of endothelium and basement membrane, act as a barrier to tumor cell extravasation. Although cell migration and the secretion of degradative enzymes have been implicated in cancer cell damage to basement membrane,[6,7] the mechanisms involved in tumor cell passage through the endothelium have been less well documented. Observations from studies *in vitro* and *in vivo* suggest that this might involve active retraction of endothelial cells after cancer cell attachment[8] and/or mechanical damage due to cell proliferation.[2,9]

Several *in vivo* models of metastasis implicate reactive oxygen species in endothelial cell damage and the subsequent promotion of metastasis. These include the activation and sequestation of neutrophils in pulmonary capillaries,[10] and exposure of animals to agents such as bleomycin,[11] high concentrations of oxygen,[12] or X-ray.[13,14] These data and our previous observation that activated Walker 256 carcinosarcoma cells produce reactive oxygen species[15] suggested that free radicals released from cancer cells might induce endothelial damage. Here we report that cells from the W256 carcinosarcoma cell line can cause endothelial cell damage *in vitro* by the generation of reactive oxygen metabolites.

Materials and Methods

Materials

The following materials were purchased from the Sigma Chemical Company, St. Louis MO: bovine serum albumin; catalase, from bovine liver; cytochalasin B, from *Helminthosporium dematioideum;* N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP); luminol (5-amino-2,3-dihydro-1,4-phthalazinedione); phorbol 12-myristate, 13-acetate; and superoxide dismutase (SOD) from bovine liver. 2-deoxy-D-[1-^{3}H]glucose was obtained from Amersham Corporation, Oakville, Ontario at a concentration of 1.0 mCi/ml, (specific activity of 17.0 Ci/mmole). All media were obtained from GIBCO, Burlington, Ontario. The chemotactic peptide fMLP was dissolved in absolute ethanol to appropriate stock concentrations. Luminol was similarly prepared using dimethyl sulphoxide as the solvent. All other agents were dissolved in appropriate aqueous buffers.

Cell Lines

Pathogen-free, adult (200 g) male Long-Evans rats were maintained according to principles set out by McMaster University for the care and use of laboratory animals. The Walker carcinosarcoma 256 cell line described previously[15] was maintained *in vivo* as an ascites tumor. For experiments, tumor ascites fluids were harvested in heparinized Hanks' balanced salts solution (5 IU Heparin/ml) and erythrocytes removed by 2 to 3 cycles of osmotic lysis. Tumor cells were then washed in RPMI containing 0.5 mg/ml bovine serum albumin (assay medium) and were resuspended in this medium at the desired concentration. Cell suspensions prepared by this method contained 98 ± 1% tumor cells identifiable on Wright-stained cytospin preparations with a mean tumor cell viability of 95% after 6 hours as estimated by trypan blue exclusion and intact cell morphology.

Endothelial Cell Culture and Assays of Endothelial Damage

Human umbilical cord endothelial cells were obtained by collagenase treatment and maintained in culture by a method modified from Jaffe et al.[16] Briefly, the cells were harvested by collagenase treatment and maintained in medium 199 plus 20% human heat-inactivated serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 100 μg/ml pituitary-derived endothelial growth factor. The cells were either passaged once into T25 flasks and then seeded onto fibronectin coated plastic discs, contained in 24-well tissue culture dishes, or alternatively seeded directly into these multiwell dishes after collagenase treatment. Cells were identified as being endothelial in origin by their cobblestone morphology as well as their expression of Factor VIII antigen. All cells used in experiments were either at passage one or two.

The endothelial cells were assayed for damage as follows. Confluent monolayers were labeled overnight by the addition of 1 μCi/well 2-deoxy-[1-^{3}H]-glucose-6-phosphate.[17] Eighteen to twenty-four hours later the media were removed and the endothelial cell monolayers were washed four times with 1 ml of RPMI plus 0.5 mg/ml BSA. In some cultures 1 mM buthionine sulfoximine was added to the cells the night before the assay and then included at this same concentration in all washes as well as the assay medium itself. The assay for endothelial cell damage was initiated by adding various concentrations of xanthine oxidase in 0.5 ml RPMI with 0.5 mg/ml BSA and 0.2 mmol/l xanthine. Alternatively, varying numbers of W256 cells were added in a total volume of 0.5 ml RPMI plus 0.5 mg/ml BSA. In some experiments the tumor cells were then activated by adding 1×10^{-6} M fMLP. After a 90-minute incubation at 37 C in a humidified environment containing 5% CO_2, the medium was removed from each well. The endothelial cell monolayer was washed once with 0.5 ml RPMI plus 0.5 mg/ml BSA and this was added

to the medium. Any cells present in these supernatant fluids were removed by centrifugation for 3 minutes at 200*g*. The discs on which the endothelial monolayers had been grown were then removed from the culture dishes, and placed into 0.2 ml of Beckman tissue solubilizer. The radioactivity retained in the monolayer was then determined along with that in the medium using a Beckman LS 1801 scintillation counter. The release of the ^{3}H-2-deoxyglucose into the medium was calculated as a percentage of the total radioactivity in each well. In controls, considerable variation was found in the release of ^{3}H-2-deoxyglucose between different endothelial cell preparations. Percent specific release was therefore determined by the formula: $A - B/B \times 100$, where A equals the percent release found under experimental conditions and B equals the percent release found in controls, ie, medium without xanthine oxidase or tumor cells. Percent release was calculated as a mean of four to six replicates. Percent specific release is given as a mean plus or minus standard error.

Chemiluminescence Apparatus and Assay

Details concerning the construction of the chemiluminescence apparatus have been published previously.[15] Briefly, it consists of an EMI 9588A red-sensitive photomultiplier tube that is mounted vertically and monitored by a model C10 photon counter (Thorn EMI-Gencom instruments). Above the photomultiplier tube there is a light-tight aluminum chamber into which a 6-well culture plate (Linbro plastics #FB6) can be placed. The lids of the culture dishes are replaced with a specially designed cover that includes six miniature stirring motors driving Teflon paddles at 17 rpm to ensure agitation and holes through which air or reagents can be added. During experiments the temperature in this chamber was maintained at 37 C.

Before each experiment, all materials were kept in the dark for a period of 1 hour. Two milliliters of tumor cell suspension (5×10^6 cells/ml) were added to each culture well in the photometer and allowed to equilibrate for 4 minutes. Luminol was then added to obtain a final concentration of 5×10^{-5} M luminol. This was incubated for another 3 minutes before adding fMLP. Light emission was recorded for a period of 8 to 10 minutes. Tracings were evaluated by measuring the peak chemiluminescence (counts per minute) or by calculating the area under the chemiluminescence light curve over 8 minutes continuous recording (integral chemiluminescence). For this purpose we used a Microplan II Image Analysis System (Laboratory Computer Systems Inc., Cambridge, MA).

Preparation of Tissue Cultures For Scanning Electron Microscopy

Cultures were rinsed in serum free medium and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.5, at 37 C. Primary fixation was continued for 30 minutes at 22 C. Cultures were postfixed in 1% OsO_4 in 0.1 M sodium cacodylate at 4 C for 30 minutes, rinsed in water, and dehydrated through graded ethanol (50%, 70%, 90%, 95%). They were then critical-point dried from absolute ethanol in a critical point dryer (Ladd Research Industries) using CO_2 as the transition fluid. The dried preparations were coated with 20 nm of gold in a sputter coater, and viewed on a Philips 501B scanning electron microscope at 15 or 30 kV.

Data Analysis

All experiments were performed at least twice. Statistical differences between the experimental and control groups in the ^{3}H-2-deoxyglucose release assay were determined by the Student's *t*-test for unpaired samples.

Results

Effect of Xanthine Oxidase and Walker Carcinosarcoma 256 Cells on Chemiluminescence and 2-deoxyglucose Release

Figure 1 illustrates typical chemiluminescence traces, obtained when 5×10^6 W256 cells were incubated with 10^{-6} M fMLP or when 6.5×10^{-3} units of xanthine oxidase were incubated with 0.2 mmole xanthine. A detailed characterization of the W256 tumor cell response has been published previously.[15] Background production of low level chemiluminescence was observed in studies of unactivated tumor cells whereas no chemiluminescence was observed in the presence of xanthine oxidase alone. In contrast to xanthine oxidase chemiluminescence, W256 cell chemiluminescence was sustained for greater than 20 minutes. Xanthine oxidase chemiluminescence declined to one third its initial peak value by 10 minutes (Figure 1).

In the presence of 0.2 mmole xanthine, chemiluminescence was dependent on the presence of added xanthine oxidase in concentrations ranging from 6.5×10^{-3} units to 52×10^{-3} units/ml (Figure 2). Increasing xanthine oxidase concentrations to 104×10^{-3} units had no further effect on chemiluminescence. In the presence of fMLP 5×10^6 tumor cells/ml produced a level of chemilumines-

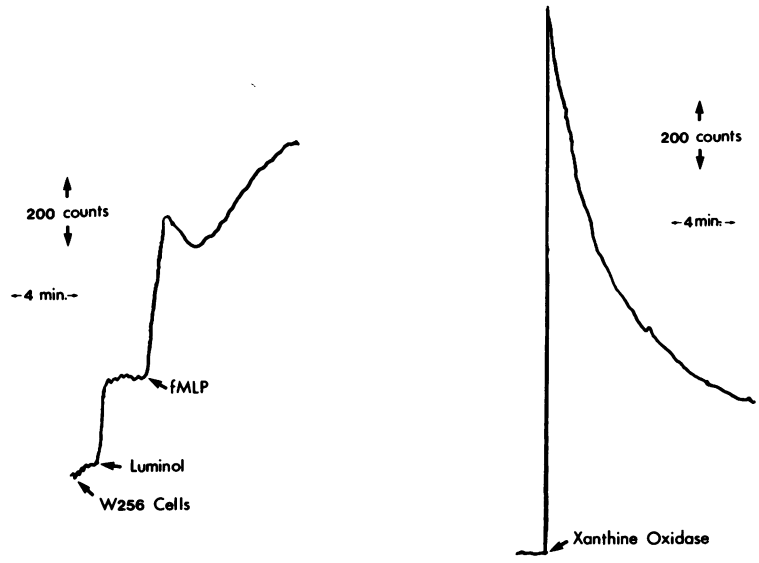

Figure 1. *Representative chemiluminescence produced by W256 cells or xanthine oxidase.* ***Left:*** *5 × 10^6 W256 cells were stimulated with 1 × 10^{-6} M fMLP.* ***Right:*** *0.2 mmol/l xanthine was incubated with 6.5 × 10^{-3} units/ml xanthine oxidase. Five hundred millimoles luminol was present in both experiments.*

cence approximately equivalent to that produced by 6.5 × 10^{-3} units/ml xanthine oxidase. Xanthine oxidase concentrations ranging from 6.5 × 10^{-3} to 104 × 10^{-3} units were therefore choosen to assess the release of ^{3}H-2-deoxyglucose from human umbilical cord endothelial cells. The release of ^{3}H-2-deoxyglucose from human umbilical cord endothelial cells incubated with xanthine oxidase also was observed at concentrations of xanthine oxidase between 6.5 × 10^{-3} and 52 × 10^{-3} units (Figure 2). Increasing the xanthine oxidase concentration to 104 × 10^{-3} units had no further effect on ^{3}H-2-deoxyglucose release. Specific ^{3}H-2-deoxyglucose release reached levels of approximately 50% at these concentrations of xanthine oxidase. ^{3}H-2-deoxyglucose release correlated directly with xanthine oxidase generated chemiluminescence (r = 0.91). Linearity of ^{3}H-2-deoxyglucose release with increasing xanthine oxidase concentration was dependent on the presence of 5 mg/ml soybean trypsin inhibitor in the media during the course of the assay.[17] Commercially prepared xanthine oxidase typically contains trypsin as a contaminant.[18] In the absence of soybean trypsin inhibitor approximately 50% of the endothelial cells detached from the surface of the culture dish, even at xanthine oxidase concentrations of 13 × 10^{-3} units/ml.

The increase in tumor cell-dependent ^{3}H-2-deoxyglucose release from human umbilical cord endothelial cells was observed only when the assays were conducted in the presence of 1 mM buthionine sulfoximine (BSO) on cells that had been precultured in 1 mM BSO. Both overnight pretreatment of the endothelial cell monolayer and the continued presence of BSO in the reaction media appeared nessesary for maximum responses. Under these conditions the specific release of ^{3}H-2-deoxyglucose found in the presence of 6.5 × 10^{-3} units xanthine oxi-

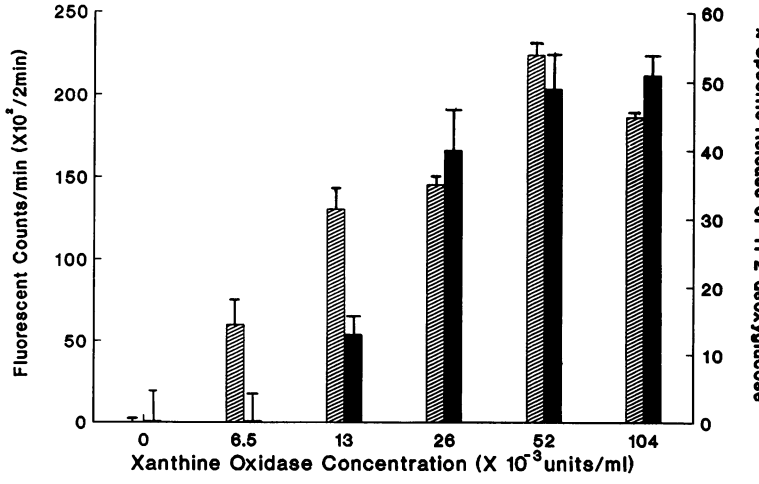

Figure 2. *The effect of xanthine oxidase concentration on chemiluminescence and on the release of ^{3}H-2-deoxyglucose from human endothelial cells is shown. All measurements were made in the presence of 0.2 mmole/l xanthine in RPMI 1640 medium with bovine serum albumin (0.5 mg/ml). Assays for ^{3}H-2-deoxyglucose release were conducted in the presence of 5 mg/ml soybean trypsin inhibitor. Assays of chemiluminescence were performed in the presence of 500 mM luminol. Isotope release in control cultures was 41 ± 1%.*

Table 1. *The Effect of Buthionine Sulfoximine on Xanthine Oxidase-Promoted ^{3}H-2-deoxyglucose Release*

Buthionine sulfoximine concentration	Percent specific ^{3}H-2-deoxyglucose release
None	ND
10 μM	ND
100 μM	12 ± 6
1.0 mM	21 ± 3*

The endothelial cell monolayer was pretreated overnight with varying concentrations of buthionine sulfoximine. The assay medium contained xanthine (0.2 mmol/l), xanthine oxidase (6.5×10^{-3} units/ml), soybean trypsin inhibitor (5 mg/ml), buthionine sulfoximine at the given concentrations in RPMI 1640, and bovine serum albumin (0.5 mg/ml). Isotope release in control cultures was 35 ± 2%. ND: not detected.

* $P < 0.05$ when compared with release of isotope from endothelium alone.

dase was increased from nondetectable levels to 21%. This increase was dependent on the concentration of BSO (Table 1).

Figure 3 illustrates the effect of cancer cell concentration and cell activation state on the specific release of ^{3}H-2-deoxyglucose from human umbilical cord endothelial cells. Maximum values for ^{3}H-2-deoxyglucose release were observed when fMLP-activated W256 cells were incubated with the monolayers. ^{3}H-2-deoxyglucose specific release increased from undetectable levels at a cell concentration of 1.0×10^6 W256 cells/ml to a maximum of 30% at a cell concentration of 6.0×10^6 cells/ml (Figure 3). In the absence of fMLP, there was a small but significant increase in ^{3}H-2-deoxyglucose release at cell concentrations greater than or equal to 4.0×10^6 cells/ml. The incubation of fMLP with endothelial cells in the absence of tumor cells failed to cause an increase in the specific release of ^{3}H-2-deoxyglucose. At concentrations greater than 2.0×10^6 tumor cells per ml, the endothelial cell monolayer appeared completely covered by tumor

Table 2. *The Effect of Culture Time on Tumor Cell-Promoted ^{3}H-2-deoxyglucose Release*

Length of assay (minutes)	Percent specific ^{3}H-2-deoxyglucose release: Unactivated W256 cells	Activated W256 cells
0	-	-
45	9.1 ± 9.9	13.9 ± 1.8*
90	3.2 ± 3.2	22.4 ± 3.2*
180	6.2 ± 0.8*	6.2 ± 1.9*

Isotope release in control cultures was 21 ± 1%, 31 ± 3%, and 47 ± 2% for 45, 90, and 180 minutes, respectively.

* $P < 0.05$ when compared with release of isotope from endothelium alone.

cells as viewed by light microscopy (data not shown). Maximum levels of ^{3}H-2-deoxyglucose release were thus obtained with a W256 to endothelial cell ratio between 5 to 7:1.

In the above experiments, ^{3}H-2-deoxyglucose release was measured after a 90-minute incubation of W256 cells with endothelial cell monolayers. After 90 minutes of incubation the specific release of ^{3}H-2-deoxyglucose, promoted by activated W256 cells, was maximum (Table 2). In the absence of tumor cells, background release of ^{3}H-2-deoxyglucose increased linearly with time. At 180 minutes, approximately 50% of the retained ^{3}H-2-deoxyglucose had been released into the surrounding media. This resulted in a significant decrease in the observed ^{3}H-2-deoxyglucose release, which was specific for the presence of tumor cells (Table 2).

Because in most experiments, W256 tumor cells were obtained in the form of an ascites, we assessed the contribution of host leukocytes to ^{3}H-2-deoxyglucose release. Cytospins were prepared and differential cell counts performed for each experiment. No significant correlation was found between the extent of ^{3}H-2-deoxyglucose re-

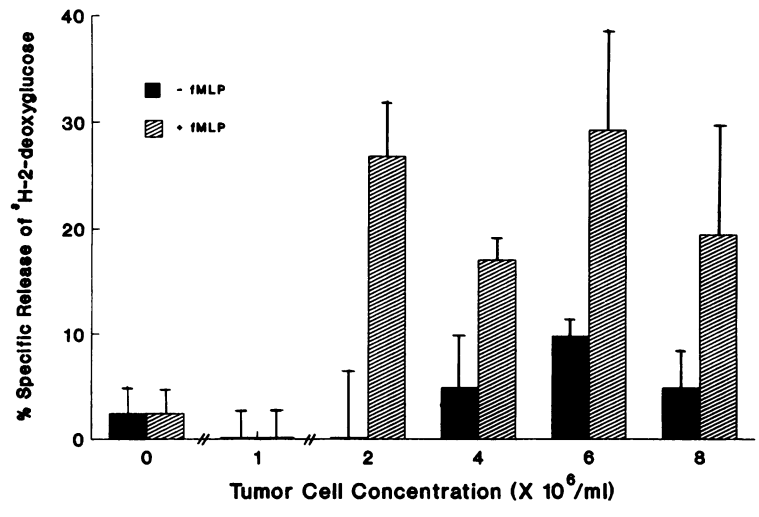

Figure 3. *The effect of W256 cell concentration on the release of ^{3}H-2-deoxyglucose from umbilical cord endothelial cells. Endothelial cells were cultured overnight with 1 mM buthionine sulfoximine and 1 μCi/ml ^{3}H-2-deoxyglucose. W256 cells were cocultured with the endothelium for 90 minutes in the presence or absence of 1 × 10^6 M fMLP in RPMI 1640 plus bovine serum albumin (0.5 mg/ml). Isotope release in control cultures was 42 ± 1%.*

Table 3. *The Effect of Cultured Tumor Cells on Tumor Cell-Promoted ^{3}H-2-deoxyglucose Release*

Endothelial treatment	Exp. 1	Exp. 2
Phorbol-12-myristate, 13-acetate	-	1.0 ± 4.3
Unactivated W256 cells	6.3 ± 4.3	7.8 ± 1.2
Activated W256 cells	19.1 ± 5.2*	28.8 ± 3.6*

W256 cells obtained from an ascites tumor were cultured in BGJ medium with 10% fetal calf serum and 1% antibiotics for 3 days before the experiment. The culture flask was changed after the first and secnd day to remove adherent leukocytes. W256 cells were then activated with 1 × 10^{-6} M phorbol myristate acetate since fMLP fails to activate cultured W256 cells.[15] Isotope release in control cultures was 34 ± 2% and 39 ± 1% for experiments 1 and 2, respectively.

* $P < 0.05$ when compared with release of isotope from endothelium alone.

lease and the number of contaminating neutrophils found in a given preparation (r = 0.154). Further, cultured W256 cells stimulated with 10^{-6} M phorbol myristate acetate (PMA), although devoid of contaminating neutrophils, still induced ^{3}H-2-deoxyglucose release from endothelial cells to the same extent as did W256 cells isolated as an ascites. Cultured W256 cells still promoted low levels of ^{3}H-2-deoxyglucose release from endothelial cell monolayers in the absence of PMA (Table 3).

The Effect of Catalase and Superoxide Dismutase

The release of ^{3}H-2-deoxyglucose associated with the addition of 2.0 × 10^6 activated tumor cells per ml to labeled endothelial monolayers was unaffected by the simultaneous addition of superoxide dismutase at concentrations of 0.1 or 1.0 mg/ml (Table 4). In contrast, the addition of 1000 units/ml of catalase resulted in the nearly complete inhibition of this response. At catalase concentrations of 100 units/ml there was no observable effect on tumor cell-promoted ^{3}H-2-deoxyglucose release (Table 4). The complete inhibition of tumor cell-promoted ^{3}H-2-deoxyglucose release observed in the presence of 1000 units/ml of catalase was reduced to approximately 40% inhibition when tumor cell concentrations were increased from 2.0 × 10^6 tumor cells to 6.0 × 10^6 tumor cells per ml (Figure 4).

The Requirement for Cell Contact

Table 5 compares the effect of adding intact tumor cells to endothelial monolayers with the effect of adding the supernatant of these cells. W256 cells were incubated in medium for 30, 60, or 90 minutes in the presence or absence of 10^{-6} M fMLP. The cells were then pelleted and the supernatant assayed for its ability to increase ^{3}H-2-deoxyglucose release by incubation with endothelial monolayers for 90 minutes. In control cultures, tumor cells from the same population were incubated directly with the endothelium as described above. In the absence of tumor cells the addition of supernatant to endothelial cells failed to increase ^{3}H-2-deoxyglucose release.

To more fully explore this effect, tumor cells were preincubated with 1 μM cytochalasin B before being assayed for their ability to promote ^{3}H-2-deoxyglucose release. Table 6 illustrates the percent inhibition of ^{3}H-2-deoxyglucose release from endothelial monolayers exposed to cytochalasin B-treated W256 cells. The results from these two experiments clearly indicate that ^{3}H-2-deoxyglucose release is reduced substantially when the tumor cells are pretreated with cytochalasin B. This is in direct contrast to the observed chemiluminescence response of cytochalasin B treated W256 cells; the pretreatment of W256 cells with 1 μM cytochalasin B typically increases the level of chemiluminescence threefold.[15] Scanning electron micrographs of tumor cells adherent to washed endothelial cell monolayers demonstrated 60 to 80% fewer adherent W256 cells when the tumor cells were pretreated with cytochalasin B. Those tumor cells that were attached in this case had a distorted morphology with a reduction in the number of cytoplasmic processes (Figure 5).

Discussion

Previous studies indicated that fMLP-activated W256 cells generate reactive oxygen species.[15] We have tested the possibility that oxygen radical production by these cells could damage endothelial cell monolayers because vessel wall damage can be caused by the free radicals released from activated leukocytes.[10,19] By using ^{3}H-2-de-

Table 4. *The Effect of Superoxide Dismutase and Catalase on Tumor Cell-Promoted ^{3}H-2-deoxyglucose Release*

		Superoxide dismutase		Catalase	
Cells	Control	0.1 mg/ml	1 mg/ml	100 U/ml	1000 U/ml
Unstimulated W256	0.8 ± 3.9	-	-	-	-
Stimulated W256	19.2 ± 4.2*	17.2 ± 5.6*	18.4 ± 2.3*	25.1 ± 9.5*	3.3 ± 4.0

Superoxide dismutase and catalase were added to the endothelial cultures before adding W256 cells. Isotope release in control cultures was 24 ± 1%.
* $P < 0.05$ when compared with release of isotope from endothelium alone.

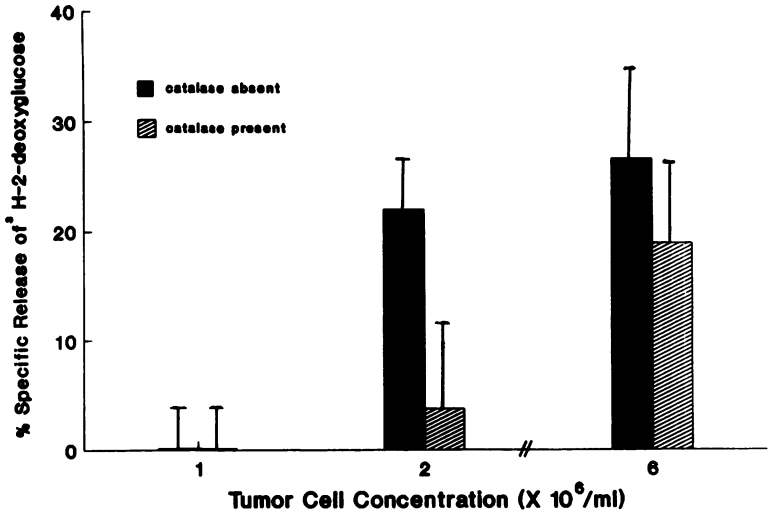

Figure 4. *The effect of tumor cell concentration on catalase-mediated inhibition of ^{3}H-2-deoxyglucose release. Activated W256 cells were added to endothelial cultures in the presence or absence of 1000 units/ml catalase. Isotope release in control cultures was 33 ± 1%.*

oxyglucose release from prelabeled endothelial cells as an index of cell damage,[17,20] we found that fMLP-activated W256 cells are capable of directly damaging endothelial cells *in vitro*. The mechanism appeared to involve the generation of reactive oxygen species since: 1) pretreatment of endothelium with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase synthesis, was required to detect apprecible ^{3}H-2-deoxyglucose release; 2) catalase inhibited release of the isotope; 3) detectable isotope release required the presence of tumor cells (supernatants were ineffective); and 4) damage was maximal when the tumor cells had been activated with fMLP and correlated with tumor cell-generated chemilumenescence.

Studies of W256 cell-generated chemiluminescence suggest that hydrogen peroxide, superoxide anion, and hydroxyl radical are all generated after W256 cell activation.[15] Polymorphonuclear leukocytes also produce these oxygen metabolites when activated.[21,22] Some studies with PMNs have indicated that hydrogen peroxide is the reactive oxygen metabolite most likely to be responsible for endothelial cell damage.[20,23] Other studies have indicated that superoxide anion and hydroxyl radical are also cytotoxic.[24] Two observations support the conclusion that hydrogen peroxide is predominately responsible for endothelial cell damage by the W256 cell line. First, in the presence of glutathione peroxidase, hydrogen peroxide is directly reduced by glutathionine and is effectively scavenged.[25] Therefore, because ^{3}H-2-deoxyglucose release was observed only when the endothelial cell monolayer had been pretreated with buthionine sulfoximine, which depletes the cells of endogenous glutathione, cells would be more susceptible to hydrogen peroxide-mediated damage.[25] Second, inhibition of tumor cell-dependent ^{3}H-2-deoxyglucose release by the addition of catalase and not superoxide dismutase to the assay suggests that hy-

Table 5. *Inability of Cell-Free Supernatants to Induce Release of ^{3}H-2-deoxyglucose from Labeled Endothelial Cells*

Conditions	t = 30	t = 60	t = 90
Intact unactivated W256 cells	-	-	6.0 ± 2.0
Intact activated W256 cells	-	-	16.0 ± 1.0*
Supernatant of unactivated W256 cells	0.0 ± 4.6	2.3 ± 2.9	3.2 ± 2.0
Supernatant of activated W256 cells	3.2 ± 3.7	3.5 ± 3.9	0.0 ± 3.5

W256 carcinosarcoma cells in suspension culture at a concentration of 5 × 10^6 cells/ml were activated with 1 × 10^{-6} M fMLP for 30, 60, or 90 minutes. The cells were then pelleted and the cell-free supernatants added to the labeled endothelial cell monolayers to assess ^{3}H-2-deoxyglucose release. In control conditions intact cells from the same original population were added to endothelial cultures. Isotope release in control cultures was 36 ± 2%. t = time in minutes.

* $P < 0.05$ when compared with release of isotope from endothelium alone.

Table 6. *The Effect of Cytochalasin B Treatment of W256 Cells on Release of ^{3}H-2-deoxyglucose from Endothelial Cell Monolayers*

	Inhibition of ^{3}H-2-deoxyglucose release after cytochalasin B treatment	
W256 population	(Exp. 1)	(Exp. 2)
Unactivated W256 cells	56%*	23%
Activated W256 cells	55%*	13%*

W256 cells were treated with 1 μM cytochalasin B for 10 minutes, washed, and tested for their ability to promote ^{3}H-2-deoxyglucose release, as in Figure 3. Inhibition of release was calculated with reference to release caused by W256 cells that had not been treated with cytochalasin B.

* $P < 0.1$ when compared with isotope release promoted by untreated W256 cells.

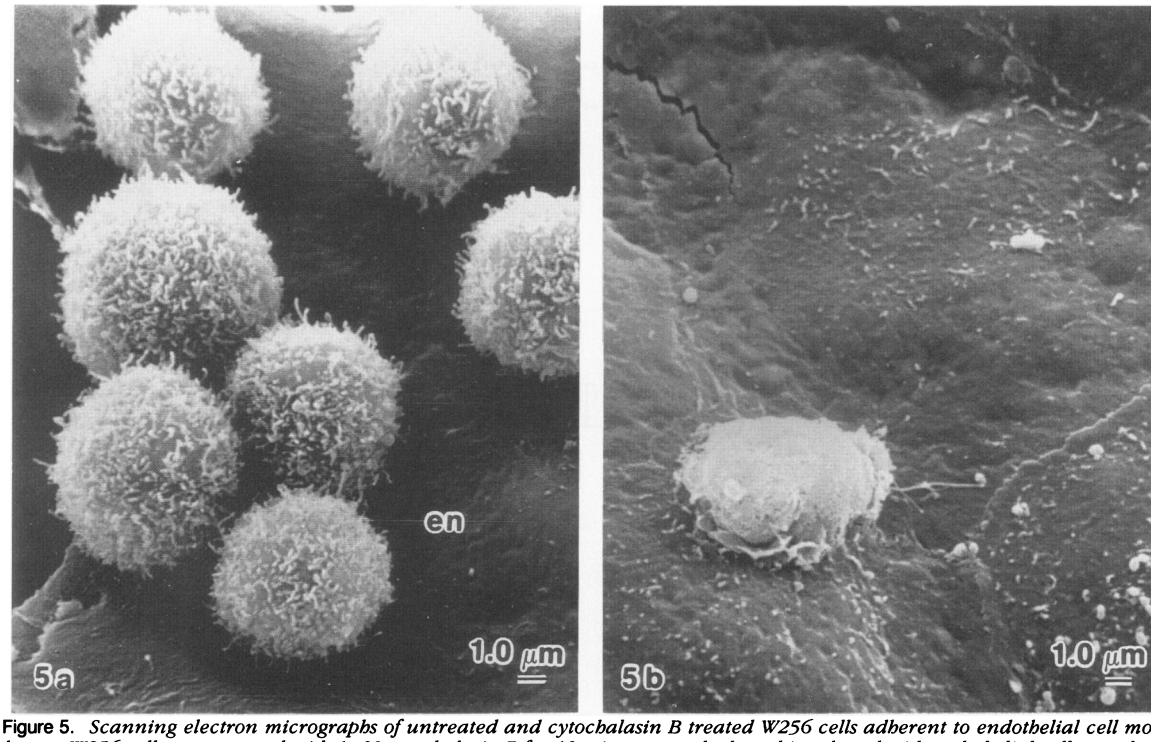

Figure 5. *Scanning electron micrographs of untreated and cytochalasin B treated W256 cells adherent to endothelial cell monolayers. W256 cells were treated with 1 μM cytochalasin B for 10 minutes, washed, and incubated with endothelial cell monolayers for 90 minutes. The discs on which the endothelial monolayers were grown were then removed, washed in RPMI medium to remove nonadherent W256 cells, and fixed in 2% glutaraldehyde and 0.1M cacodylate buffer in preparation for electron microscopy.* **A:** *Untreated W256 cells attached to the endothelial cell monolayer.* **B:** *A cytochalasin B-treated W256 cell still attached to the endothelial cell monolayer.*

drogen peroxide and not superoxide anion is responsible for endothelial cell damage.

The treatment of neutrophils with cytochalasin B has been shown to increase proteolytic enzyme release,[26,27] as well as generation of reactive oxygen metabolites.[21,22,28] Because W256 cell chemiluminescence is increased threefold by cytochalasin B treatment,[15] we expected that endothelial damage would also be greater. However, pretreatment of tumor cells with cytochalasin B greatly reduced their ability to cause ^{3}H-2-deoxyglucose release. Sacks et al reported a similar effect after cytochalasin B pretreatment of neutrophils that was suggested to be the result of decreased neutrophil adhesion to the endothelial cell monolayer.[29] Similarily, we found a reduction in the number of activated tumor cells adherent to the endothelial cell monolayers after cytochalasin B pretreatment. A reduction in the adherence of activated W256 cells to plastic dishes after cytochalasin B treatment also has been reported.[30] This experiment and the observation that supernatants from activated W256 cells do not cause endothelial damage suggest that direct contact between the W256 cell and endothelium may be required for maximum endothelial damage. These data are consistant with the proposal of Sacks et al that the damaging agents are short lived reactive oxygen species. Hydrogen peroxide stability is greatly diminished in the presence of even trace amounts of metals such as iron. Under these circumstances hydrogen peroxide undergoes a Fenton type reaction, resulting in the production of hydroxy radicals.[31]

A similar mechanism of endothelial cell damage has been reported when human neutrophils are activated by phorbol esters or C5a.[17,20,23,25,29] Because the W256 ascites typically contains a 1 to 2% contaminating neutrophil population, we were concerned that this might be responsible for the cell damage observed in our assays, but there is substantial evidence to the contrary. First, there was no correlation between the magnitude of the chemiluminescence response, the degree of endothelial damage, and the number of neutrophils found in a given W256 cell preparation. Second, cultured W256 cells, free of neutrophil contamination, still demonstrated chemiluminescence[15] and were able to promote the release of ^{3}H-2-deoxyglucose from endothelial cells after PMA activation. Third, fMLP activation of 2×10^{6} rat neutrophils (greater than 100 times the maximal number of such cells present in the ascites fluid) produced less than 2 nmoles superoxide in 1 hour as assayed by ferricytochrome c reduction.[27] The activation of 5×10^{6} W256 cells produces approxi-

mately 2 nmoles superoxide anion in 5 minutes when assessed by the same method.[15] Endothelial cell damage as assayed by ^{3}H-2-deoxyglucose release was already maximum when 2×10^6 W256 cells were added to the assay. Because rat neutrophils make up only 1 to 2% of the W256 cell preparation, it would seem that the observed damage in our experiments is unlikely to be due to the activation of contaminating leukocytes.

In these experiments, W256-mediated damage was dependent on the pretreatment of the endothelial cell monolayers with the glutathione redox cycle inhibitor, buthionine sulfoximine. Harlan et al also have reported a similiar requirement to detect neutrophil-mediated oxidative damage of endothelial cells.[32,33] It should be recognized however, that a requirement for BSO pretreatment *in vitro* does not nessarily infer a requirement for BSO *in vivo. In vitro,* endothelial cell damage by W256 tumor cells may be limited by a number of factors inherent to the assay itself, including a relatively short duration of tumor cell–endothelial interaction due to the background efflux of ^{3}H-2-deoxyglucose, as well the limited availability of oxygen due to a high number of respiring cells/well. Further, because these experiments were conducted with human umbilical cord endothelial cells, it is unknown to us whether damage to microvascular endothelial cells would require BSO pretreatment.

In summary, we have performed *in vitro* experiments that show that activated W256 cells cause endothelial cell damage, detected by the release of ^{3}H-2-deoxyglucose. This injury was dependent on direct contact between the effector and target cells and could be related to oxygen radical generation by the cancer cells. *In vivo,* drug, neutrophil, or x-ray-induced endothelial injury are known to promote metastasis of circulating cancer cells.[10,11,14] Free radical mechanisms are probably involved in the genesis of these injuries. Therefore, we suggest that cancer cells also could facilitate their own passage through vessel walls by generating reactive oxygen species *in vivo.* This possibility is currently being studied.

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