Trocar Site Recurrence is Unlikely to Result from Aerosolization of Tumor Cells

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PURPOSE: This study was undertaken to investigate the ability of a high-pressure CO_2 environment to aerosolize tumor cells in both *in vitro* and *in vivo* models. (An aerosol is defined as a stable gaseous suspension of insoluble particles.) Also, this study was designed to determine if rapid desufflation is capable of transporting fluid laden with tumor cells. METHODS: The four *in vitro* aerosol experiments were performed in an 18.9-l plastic vessel fitted with two 7-mm ports and a compliant latex balloon affixed to the top. After CO_2 insufflation, the vessel was desufflated through a sterile soluset containing 25 ml of culture media that was subsequently emptied into a culture dish, incubated for two weeks, and periodically assessed for growth. At the bottom of the vessel, one of the following was placed: Study 1 and 2, a suspension of B16 melanoma or colon 26 tumor cells in liquid culture media; Study 3, colon 26 cells in saline solution; Study 4, several pieces of solid colon 26 tumor. In Studies 1 to 3, cell preparations were subjected to the following high-pressure CO_2 conditions (pneumo): 1) static pneumo of 15 and 30 mmHg (10 minute dwell); 2) a continuous flow (CF) of CO_2 (1O l) while maintaining a pressure of 15 or 30 mmHg in the vessel. In Study 4, only the 30 mmHg static and CF conditions were tested. Between 6 and 12 determinations were performed for each condition and cell preparation. *In vivo* aerosol experiments consisted of Spraque Dawley rats that received intraperitoneal injections of 10–5 B16 cells in 0.1 ml of liquid media. Two laparoscopic ports were placed in the abdomen, one each for insufflation and desufflation. Study groups were: 1, static CO_2 pneumo of 15 mmHg; 2 and 3, continuous CO_2 flow (10 l) at a stable pneumo pressure of 5 and 10 mmHg. Desufflation was performed *via* the same collecting device and handled in an identical manner to the *in vitro* experiments described above. The *in vitro* balloon experiment was designed to investigate the ability of desufflation to transport fluid-containing tumor cells; latex balloon model was used. To prevent complete loss of volume on desufflation, a wire coil was placed inside the balloon. Twenty ml of media containing 20×10^{-6} B16 cells was placed in the bottom of the balloon. The balloon was insufflated with 1 to 2 l of gas. There were three study groups that differed in the degree to which the cell suspension was agitated before desufflation. Study conditions were as follows: 1) no agitation; 2) moderate agitation to coat the lower walls and coil; 3) maximum agitation to coat the entire balloon. To verify the viability of tumor cells, at the end of each *in vitro* and *in vivo* study, a sample of tumor cells or peritoneal washing was incubated in sterile media. These samples served as positive controls. RESULTS: *In vitro* aerosol studies consisted of the following. At the end of two weeks of incubation, no tumor growth was noted in any of the 124 test dishes. The 14 control samples all demonstrated tumor growth. *In vivo* aerosol studies consisted of the following. Zero of 18 experimental dishes grew tumor. All three peritoneal washing samples demonstrated growth. *In vitro* balloon studies consisted of the following. Zero of 12 test dishes in Groups 1 and 2 demonstrated growth, whereas five of six dishes did so in Group 3 (maximally agitated before desufflation). Again, positive controls all grew tumor cells. SUMMARY: We were unable to demonstrate aerosol formation in any of the *in vitro* and *in vivo* studies performed. In the balloon experiment, desufflation-related transport of tumor cells was demonstrated but only when the entire balloon surface was coated with the tumor cell suspension before desufflation. CONCLUSION: Aerosols of tumor cells are not likely to form. Free intraperitoneal tumor cells are most likely found in liquid suspension. Desufflation is a potential means of transport of cell-laden fluid. [Key words: Laparoscopic-assisted colectomy; Port site tumors; Aerosolization; Laparoscopy]

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At least 33 port site tumor recurrences after laparoscopic-assisted colon resection (LACR) have been reported in the literature.[1-3] This issue of port site tumors is at the center of controversy regarding appropriateness of laparoscopic methods for curative colorectal cancer resection. The true incidence of port site tumors is unclear at this time; estimates based on relatively small series range from 0 to 21 percent.[1, 4] Several randomized prospective trials are presently underway that will, hopefully, answer this question.

The etiology of port site tumors is unknown. Direct spread of tumor cells to the port site is the most likely mechanism because, for anatomic reasons, the lymphatic and hematogenous routes of spread are not likely. Direct spread of tumor cells to the wound

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might occur 1) at time of specimen removal, 2) *via* removal of contaminated instruments, and 3) *via* seeding of port wounds by free intraperitoneal tumor cells. Jones *et al.*,[5] in an animal study that used tumor cell suspensions, implicated the pneumoperitoneum in the etiology of these lesions. They demonstrated a threefold increase in port site tumors in a pneumoperitoneum group compared with a group undergoing laparotomy alone. There are several theoretical ways in which a pneumoperitoneum *via* desufflation might facilitate direct spread of tumor cells. An aerosol of free tumor cells might form in the high-pressure CO_2 environment and be carried to the port wounds during desufflation. Alternatively, liberated tumor cells in liquid suspension may be transported to the port during desufflation.

In a previous preliminary study, our laboratory investigated the ability of a high-pressure pneumoperitoneum to aerosolize B16 melanoma cells from a liquid suspension in an *in vitro* model.[5] Under the conditions of that study with the B16 tumor cell line, aerosolization of cells did not occur. The purpose of this current study is twofold: 1) to further investigate the potential mechanism of aerosolization *via in vitro* and *in vivo* models, and 2) to assess the ability of desufflation to transport tumor cells *via* a liquid suspension.

MATERIALS AND METHODS

In Vitro Aerosol Experiments

The purpose of this set of experiments was to determine if aerosols of tumor cells (stable gaseous suspension) form in a high-pressure $C0_2$ environment and are transported *via* desufflation.

Experimental Apparatus. These studies were performed in an 18.9-liter plastic cylindrical vessel with a 22.5-cm diameter base and a 40-mm round aperture at the top to which a compliant latex pouch was attached with an airtight seal (Fig. 1). Two 7-mm ports were placed 25 cm above the base of the vessel. One of the ports was attached to an automatic insufflator (Meditron Devices Inc, Hackensak, NJ) that was used to generate the high-pressure CO_2 environment. The second port was used for desufflation and was connected *via* a 5-cm long tube (internal diameter, 0.5 cm) to a collection device that consisted of a soluset containing 25 ml of culture media. At the completion of each determination, each soluset was drained back through the desufflation tubing into a sterile culture dish that was placed in an incubator and evaluated for tumor growth periodically for two weeks.

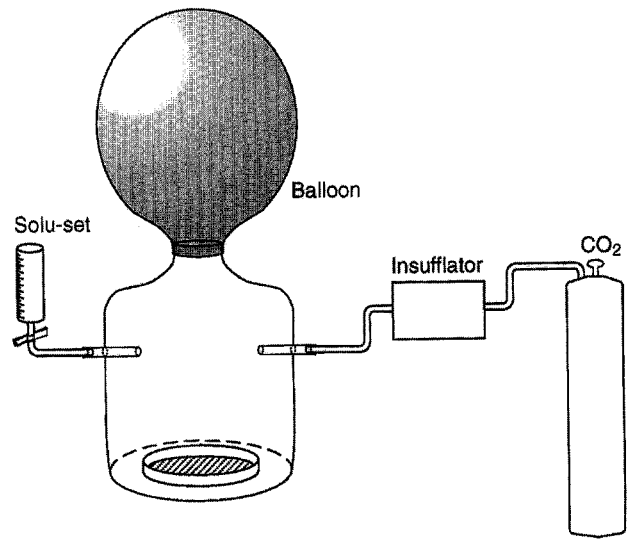

Figure 1. Experimental apparatus for Studies 1 and 2.

Tumor Cell Lines. Both B16 melanoma and colon 26 tumor lines were tested. The following four different cell preparations were tested: Group 1, B16 melanoma cells suspended in liquid culture media; Group 2, colon 26 cells suspended in liquid culture media; Group 3, colon 26 cells in a saline suspension; and Group 4, solid pieces of colon 26 tumor harvested from a mouse. All liquid tumor cell suspensions contained 20 million cells in a 20-ml volume (10^{-6} cells/ml). Tumor cell preparations were placed in a culture dish at the base of the experimental vessel at the start of each study.

Liquid culture media consisted of Dulbecco's modified Eagle's medium supplemented with 10 percent fetal bovine serum and 5 percent penicillin and streptomycin (Gibco Labs., Grand Island, NY). All culture dishes were incubated at 37°C and 5 percent CO_2 concentration.

For B16 melanoma, tumor cells were harvested from a stock culture with a trypsin solution, centrifuged (10 minutes at 2,000 rpm), and resuspended in culture medium. The concentration of cells was calculated with a hemacytometer (Fischer Scientific, Pittsburg, PA) and then appropriately diluted with addition of media to achieve a final concentration of 1×10^{-6} cells/ml.

The colon 26 tumor cell line is maintained in our laboratory *via* serial subcutaneous tumor transplants in BALB/c syngeneic mice. Tumor cells for Groups 2 and 3 were obtained by reflecting the skin over the tumor implant and excising the lesion. After mechan-

ical dissociation, the tumor was then chemically dissociated with a mixture consisting of collagenase (1.5 percent), DNAase (1 percent), and trypsin (2.5 percent) for one hour. The resulting suspension was filtered through a wire mesh and then centrifuged for 10 minutes (2,000 rpm). The resulting pellet is washed twice with sterile culture media and then resuspended in culture media or phosphate-buffered saline. Concentration of cells was calculated with a hemacytometer (Fischer Scientific) and then appropriately diluted with addition of media to achieve a final concentration of 1×10^{-6} cells/ml.

The solid colon 26 tumor specimens (150 mg each) used for Group 4 were obtained from the BALB/c mice as described previously and cut into three to four pieces before being placed in the experimental vessel. Cell viability of each tumor cell preparation was determined to be greater than 85 percent by trypan blue exclusion. Sensitivity studies were conducted to determine minimum cell concentration that would result in detectable growth. Small samples of serial dilutions of B16 melanoma cells were placed into dishes containing culture media and incubated (10^{-6} cells to 10^{-0} cells/ml). After two weeks, dishes containing as few as ten cells demonstrated tumor growth (Fig. 2).

Study Conditions. Cell preparations in Groups 1 to 3 were each subjected to four different test conditions, which were 1) static pneumo of 15 mmHg with a 10-minute dwell, 2) static pneumo of 30 mmHg (10 minutes), 3) a continuous flow of CO_2 (10 liters) while maintaining a pressure of 15 mmHg inside the vessel, and 4) a continuous flow pneumo of 30 mmHg (10 liters). A static pneumoperitoneum was created by occluding the outflow port during insufflation. A continuous flow of CO_2 was achieved by leaving the outflow port open during insufflation. Intraperitoneal pressure was maintained at the desired level (either 15 or 30 mmHg) *via* constant insufflation during continuous flow studies. Group 4 was subjected to a 30 mmHg static pneumo (10 minutes) and 30 mmHg continuous flow pneumo (10 liters) conditions only. Between 6 and 12 determinations were performed for each condition and cell preparation. A cell viability control culture was obtained at the end of each set of determinations by culturing a sample of cell preparation from base of the vessel.

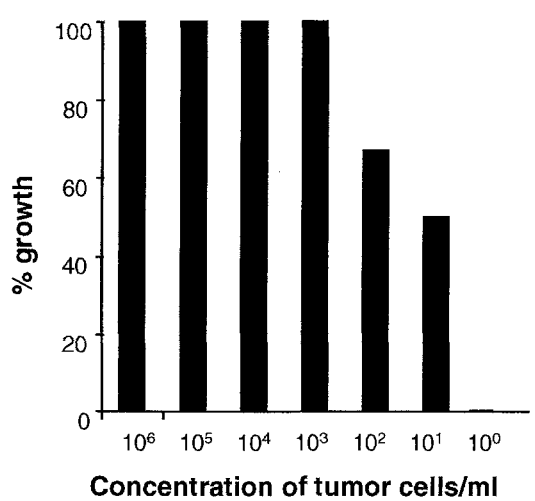

Figure 2. Percent of culture dishes with tumor growth after transfer of suspension fluid to sterile dishes on trocar tips, by concentration of suspension fluid.

In Vitro Nonviable Cell Aerosol Experiments

To determine if nonviable tumor cells were aerosolized, the following two studies were performed using the same experimental vessel described above. In the first study, B16 cells (20×10^{-6} cells) were placed in the bottom and a static 30 mmHg CO_2 pneumo established. After a brief dwell period, the vessel was desufflated directly onto a glass slide *via* the desufflation port. The slide was fixed, Giemsa-stained, and then examined by a pathologist for presence of cells. This was repeated five times. In the second study, which used the same test conditions, gas was desufflated through normal saline in the previously described collection device. Saline was subsequently centrifuged and the precipitate resuspended and then placed on a glass slide. Slide was fixed and Giemsa-stained. A total of five determinations were performed.

In Vivo Experiments

The purpose of these three studies was to determine whether desufflation is capable of transporting free viable intraperitoneal tumor cells through a port in an *in vivo* animal model. Male Spraque-Dawley rats (350–400 gm) were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (1.6 mg/kg). Abdomens were shaved and prepared with betadine. Animals then received a left lower quadrant intraperitoneal injection of 0.1 ml of a suspension of B16 melanoma cells (10^{-6}/ml). Two 1-inch long 14-gauge angiocatheters were placed in the abdomen and served as port sites. One port was used for insufflation, whereas the second was used for desufflation, which was performed through the same collection

device as described above (Fig. 3). Three study conditions were tested. In Study 1, a static CO_2 pneumoperitoneum of 15 mmHg was established, and then desufflation was performed through the collection device. This insufflation/desufflation cycle was repeated five times for each collection device. Culture media in the collection device was then emptied into a sterile culture dish and incubated. This process was repeated six times using a new collection device for each trial. In Study 2, a continuous flow pneumoperitoneum (total flow volume, 10 liters) with a stable intraperitoneal pressure of 5 mmHg was tested. In Study 3, test conditions were a continuous flow pneumoperitoneum (10 liters) at a pressure of 10 mmHg. A total of six determinations were performed for both Studies 2 and 3. At the end of each study, a peritoneal washing sample was obtained and cultured as a cell viability control.

In Vitro Balloon Experiments

The purpose of these studies was to investigate the ability of rapid desufflation to transport fluid containing tumor cells. A highly compliant balloon containing a 6-cm wire coil was used for these experiments. The coil was used to prevent complete collapse of the balloon during desufflation. With the coil in place, the residual volume of the system was 70 ml. The balloon opening had a 1.7-cm diameter. After disinfection, 20 ml of a B16 melanoma cell suspension (total of 20×10^{-6} cells) was introduced into the balloon. The balloon was insufflated with air to a volume of 1 to 2 liters. The three study groups differed in degree to which cell suspension was agitated before desufflation. In Group 1, suspension was not agitated and was left at bottom of the balloon. In the second group, moderate agitation was performed so the bottom half of the inflated balloon was coated with the suspension. In the third group, the entire inner surface of the balloon was coated with tumor cells by maximally swirling and agitating the balloon. The balloon was positioned so the opening of the balloon was upright and opposite the pooled culture media in the bottom. A sterile culture dish was positioned upside down 3 cm from the mouth of the balloon, and the balloon was desufflated onto the exposed dish. The dish was then filled with 25 ml of sterile culture media and incubated for two weeks. Each plate was inspected daily for presence of tumor cells. A total of six determinations were performed for each study group. At the end of each study, a sample of cell suspension at bottom of the balloon was obtained and cultured. This served as a cell viability control.

RESULTS

In Vitro Aerosol Experiments

As a positive control, an aerosol of methylene blue was created in the experimental apparatus under conditions of a 30 mmHg continuous flow CO_2 "pneumoperitoneum." Shortly after creating the aerosol, fluid in the collecting device turned blue thus demonstrating that the aerosol was transported *via* desufflation through the short tubing to the collection device.

Results for each of the four cell preparation groups after being subjected to a static and continuous pneu-

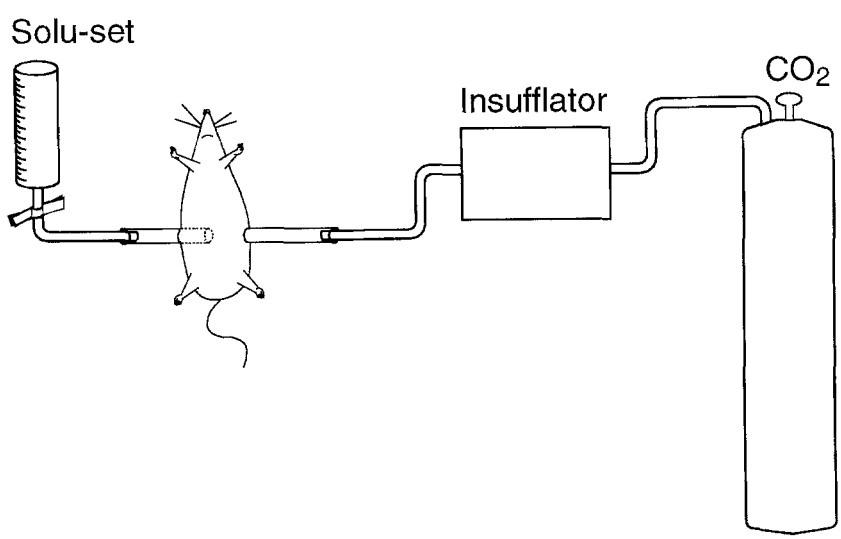

Figure 3. Experimental setup for Study 3.

moperitoneum of 15 and/or 30 mmHg are presented in Tables 1 and 2.[5] At the end of two weeks of incubation, no tumor growth was noted in any of the 124 test dishes. In contrast, all 14 cell viability control samples demonstrated tumor growth.

In Vitro Nonviable Cell Aerosol Experiment

None of the ten slide preparations after Giemsa staining demonstrated cells of any type.

In Vivo Experiments

Results for the three rat insufflation model studies are shown in Table 3. After two weeks of incubation, 0 of 18 test dishes demonstrated tumor growth, whereas all three peritoneal washing samples demonstrated growth.

In Vitro Balloon Experiments

Results are shown in Table 4. No tumor growth was observed in the 12 culture dishes from the first or second study groups (no agitation and moderate agitation). However, in the third group in which the entire inner surface of the balloon was coated with the cell suspension, tumor growth was documented in five of six test dishes. The three cell viability control samples all demonstrated tumor growth.

DISCUSSION

To date, there have been reports of at least 33 port site tumor recurrences following LACR for malignant disease.[1-3] It is the impression of most surgeons that incisional tumor metastases following open surgery are exceedingly rare. Hughes *et al.*[6] in a retrospective review of open colectomy patients reported a 0.6 percent incidence of incisional tumors following open colectomy. True incidence of port site implantation following LACR is unknown. In published LACR series that for the most part are relatively small, incidence ranges from 0 to 21 percent.[1, 4] Several randomized and prospective trials are underway that will, hopefully, answer this question.

Etiology of port site tumors is unknown. On anatomic grounds, it is highly unlikely that lymphatic and hematogenous routes of spread could account for these abdominal wall tumors. Direct spread is the most likely route of port site seeding. The most obvious means of direct spread is *via* specimen removal through an unprotected wound. Removal of instruments contaminated with tumor cells is another mechanism. Jones *et al.*[5] implicated the pneumoperitoneum in the etiology of port tumors. They demonstrated a threefold increase in port site tumors in a tumor cell suspension hamster model when pneumoperitoneum was performed and compared with a laparotomy-alone group. It is not yet clear how the pneumoperitoneum facilitates port site tumors. One possible pneumoperitoneum-related mechanism is desufflation.

Table 1.
Results of Static Large Bottle Experiments

Cell Type	No. of Trials	Pressure	Tumor Growth
B16 media	12	15	0/12
	12	30	0/12
C26 media	8	15	0/8
	8	30	0/8
C26 saline	8	15	0/8
	8	30	0/8
C26 solid	6	30	0/6
Controls	7		7/7

Table 2.
Results of Continuous Flow Large Bottle Experiments

Cell Type	No. of Trials	Pressure	Tumor Growth
B16 media	12	15	0/12
	12	30	0/12
C26 media	8	15	0/8
	8	30	0/8
C26 saline	8	15	0/8
	8	30	0/8
C26 solid	6	30	0/6
Controls	7		7/7

Table 3.
Aerosolization: *In Vivo* Results

Conditions	No. of Trials	mmHg	Tumor Growth
Static pneumo	6	15	0/6
Continuous flow	6	5	0/6
Continuous flow	6	10	0/6
Controls	3		3/3

Table 4.
Balloon Experiment Results

Study Group	Tumor Growth
No agitation	0/6
Lower wall coated	0/6
Diffuse coating	5/6

Desufflation may carry free intra-abdominal tumor cells to the unprotected port wound at time of port removal at the end of a case or *via* inadvertent port dislodgement during a case. Slow leakage of gas around a port can occur, especially in a long case and might also provide a means of transport for tumor cells. Likewise, small leaks occur at time of instrument exchange. Where are liberated intra-abdominal tumor cells found? There are three possibilities. An aerosol of tumor cells may form in a high-pressure CO_2 environment. Alternatively, cells may be found in liquid suspension or simply sitting on the surface of the bowel or peritoneal surface. This study attempted to answer two questions. Do aerosols of tumor cells form? Second, can desufflation transport tumor cell-laden fluid?

In the series of *in vitro* aerosol experiments described above, we were not able to demonstrate that aerosols of viable cells form when either solid tumors or liquid suspensions of cells are subjected to high-pressure CO_2 environments. No tumor growth was observed in any of the 124 test culture dishes. This was true for both a static and continuous flow "pneumoperitoneum." Because it was possible that cells had become aerosolized but were not viable, we evaluated the desufflated gas for cells. None were found. The *in vivo* rat model studies demonstrated that when liquid suspensions were subjected to pneumoperitoneum, viable cells were not transported out of the abdomen *via* an aerosol or fluid suspension.

The *in vitro* balloon experiment demonstrated that desufflation was capable of transporting tumor cell-laden fluid but only when the entire balloon surface was coated with the suspension. Results in this *in vitro* model support the hypothesis that in the human setting desufflation may transport cells in a liquid suspension to the port sites. *In vivo*, the abdomen is a humid and moist environment that is filled with bowel. Much of the intestine may be sitting in intraperitoneal fluid. Unlike our balloon models, in humans desufflation is much more likely to result in turbulent gas flow during rapid desufflation. It is logical to assume that such turbulent flow increases the chances that fluid coating the viscera or lying free may be transported to the port.

If, in fact, desufflation is one mechanism for port site seeding, then surgical technique may have an impact on the incidence of recurrences. Rapid desufflation *via* inadvertent port dislodgement can be avoided by securing the ports to the abdominal wall. Chronic leaks around ports can be limited by minimizing the number of instrument transfers. When it is necessary to desufflate the abdomen, it should be done *via* a suction instrument positioned so that its tip is well away from the abdominal wall.

If intraperitoneal fluid may contain liberated tumor cells, then careful suctioning should remove cells and may serve to limit the incidence of port site seeding. At the end of the case, irrigation of the abdomen and ports followed by meticulous suctioning should help remove free cells that may be sitting on peritoneal surfaces or the ports themselves.

This group of studies has not addressed the question of how tumor cells become liberated in the abdomen. It has been shown during the course of bowel resections that tumor cells may be found in the blood suctioned from the abdomen.[7] It is logical to assume that the surgical technique used during a resection will influence the number of cells liberated. Poor technique such as grasping the bowel near the tumor or excessive manipulation of the tumor may serve to spill tumor cells. Macro or micro perforations of the bowel from retraction would also provide opportunity for cell spillage. Improving the surgical technique and carefully avoiding excessive tumor manipulation should lower the chances of liberating tumor cells and subsequent seeding of the port.

CONCLUSIONS

Aerosols of tumor cells are not likely to form in a high-pressure CO_2 environment. Therefore, desufflation-related seeding of port wounds *via* a stable suspension of tumor cells in CO_2 gas is not a likely cause of port tumors. Under certain conditions, in an *in vitro* model, rapid desufflation can transport tumor cells in liquid suspension. This may be one mechanism for formation of port site tumors.

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