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INTRODUCTION

BRCA1 is a multifunctional tumor suppressive protein. Knockout of WT BRCA1 in breast cancer cells resulted in an increase in cell proliferation, anchorage-independent growth, cell migration, invasion and a loss of p21/Waf1 and P27Kip1 expression. In BRCA1 knocked-down cells, the expression of survivin was significantly up regulated with a decrease in cellular sensitivity to paclitaxel. Cells that harbor endogenous mutant or defective BRCA1 (such as MDA-MB-436 and HCC1937) were highly proliferative and expressed a relatively low levels of p21/Waf1 and p27Kip1 and high level of survivin and were resistant to paclitaxel. Thus, mutated BRCA1 or loss of WT BRCA1 upregulates the malignant cell behavior. However, it is still not clear how tumor cells expressing mutant BRCA1 have enhanced tumorigenicity *in vivo*.

Hypothesis: We hypothesize that:1) adhesion of BRCA1mutated cells to endothelial cells activates several distinct signaling pathways to induce MMP gene expression and increased ROS levels. We hypothesize that oxidative stress induces by adhesion of cells with mutated BRCA1 to HBMEC results in alterations in the integrity of the BBB and changes of the Tight-junctions, leading to transmigration of tumor cells across the BBB and colonization of these cells in the brain forming breast cancer metastasis in the brain.

Specific Aims: 1) Elucidate the molecular mechanisms and signaling pathways by which adhesion of breast cancer cells expressing mutated BRCA1, as compared to breast cancer cells expressing WT-BRCA1, induces ROS production in human brain microvascular endothelial cells; 2) Examine the effects of oxidative stress on tight junction expression (ZO-1, ZO-2, occludin and claudin-5), permeability and integrity of the brain endothelium using in vitro and in vivo models; and 3) Determine the protective effects of PARP inhibitors and/or selenium in preventing BBB-induced damage by oxidative stress, and in inhibiting breast cancer metastasis to the brain. Further, since selenium has anti-cancer properties that are linked with protection against oxidative stress and Poly (ADP-ribose) polymerase (PARP) inhibitors have shown activity against BRCA1 and BRCA2 deficient cancers, we will therefore analyze their therapeutic potential to inhibit damage to the BBB and transmigration of tumor cells across the BBB.

BODY

RESULTS—Year 1

Cocultures of HBMEC and human astrocytes as an in-vitro human blood-brain barrier model: The astrocyte-endothelial cocultures were established as a BBB model system using the transwell coculture system (Corning Costar, Cambridge, MA). Briefly, HBMEC were grown to confluence in 12-well plastic tissue culture plates, after which the culture medium was changed to media containing 10% FCS. Concurrently, astrocytes were grown to confluence on semiporous 0.45 nm transwell inserts. Cocultures were initiated by transferring inserts containing astrocytes directly into wells containing confluent HBMEC. After 48 h incubation, endothelial cells were collected for further analysis. These cocultures were also tested for the maintenance of a tight endothelial permeability barrier by determination of the paracellular permeability of the BBB monolayers for ³H-inulin and ¹⁴C-sucrose.

Effects of ROS on permeability changes in HBMEC: To examine if oxidative stress induced by adhesion of HCC1937 cells to HBMEC could be the cause of HBMEC impairment, we examined the effects of ROS on HBMEC in cocultures of breast cancer cells with HBMEC using the DCF-DA fluorescence assay. H₂O₂ (100μM), a ROS-donating agent was used as positive control. Adhesion of HCC1937 to HBMEC showed increase in ROS levels as compared to control, and this increased in ROS formation was abrogated by the antioxidant uric acid, UA (Table 1).

Table 1: Adhesion of HCC1937 cells induced oxidative stress on brain endothelial cells	
Adhesion of cells	Fluorescence Intensity (x10 ³)/mg Protein in HBMEC

	No Treatment	UA
HCC1937	23 ± 4	17 ± 4
HCC1937/WT BRCA1	15 ± 3	14 ± 2
MCF-7	14 ± 3	12 ± 3
MCF-7/mutated BRCA1	22 ± 2	18 ± 4
Control PBS	6 ± 1	5 ± 3
H(2)O(2) /HBMEC	27 ± 4	13 ± 2

Table 1: Cultures of breast cancer cells with either untreated or pretreated with the antioxidant UA (50μM). As positive control, HBMEC were treated with ROS donors for 2 hours H(2)O(2). The breast cancer cells were then added to HBMEC monolayers for adhesion. After 6 hours, the tumor cells were removed, and HBMEC monolayers were washed and ROS production in HBMEC was examined using the kit for detection of ROS based on DCF-Dais converted to highly fluorescent DCF by ROS. The fluorescence was detected at excitation 488 nm and at 525nm emission spectra using a florescence plate reader. Results were expressed as specific mean fluorescence intensity (MPI) per mg protein. Based on these results, the adhesion of HCC1937 with HBMEC induced ROS generation in HBMEC.

Next, we examined the effects of ROS on permeability changes of HBMEC. HBMEC integrity was analyzed by TEER and pattern of tumor cell migration across HBMEC. As shown in Table 2, significant increase in HBMEC permeability was observed by ROS and these changes were inhibited in the presence of UA antioxidant, uric acid, indicating the involvement of ROS in loss of the HBMEC integrity. The functional changes paralleled enhanced tumor cell migration across HBMEC (Table 2). Thus, we suggest that ROS induced adhesion of BRCA1 carrier cells to HBMEC resulted in modification of cytoskeleton organization and loss of HBMEC integrity and increased tumor migration across the HBMEC.

Table 2: Effects of ROS on TEER and cell migration across HBMEC

Cells:	Treatment	TEER of HBMEC (% of Control)	% Tumor cell migration across HBMEC
<u>HCC1937:</u>	No Treatment	82 ± 3*	22 ± 3*
	UA	96 ± 2	7 ± 2
<u>HCC1937/WT BRCA1:</u>	No Treatment	91 ± 4	13 ± 3*
	UA	94 ± 5	5 ± 1
<u>HBMEC:</u>	H(2)O(2)	82 ± 4*	18 ± 3*
	UA+ H(2)O(2)	96 ± 2	7 ± 4
	PBS	100%	0%

Table 2: TEER measurement across the HBMEC monolayer assessed the BBB tightness. TEER values recorded in ohms. Results are from two experiments in triplicate. Results were expressed as mean percent of controls. *p<0.05 as compared to HBMEC control. The transmigration of cells was assessed after 6 hours. The % of cell transmigrates out of the total input. *p<0.05 as compared to control.

Effects of ROS and PARP inhibitors on adhesion, transmigration and permeability of tumor cells across HBMEC

Adhesion: HCC1937 cells expressing mutant BRCA1 were found to adhere strongly to HBMEC (Fig. 1A). This adhesion was inhibited by COX-2, ROS and PARP inhibitors, although the inhibition by ROS inhibition was significant.

Interestingly, HCC1937 cells expressing WT BRCA1 were found to adhere much less to HBMEC, as compared to HCC1937 cells expressing mutant BRCA1 (Fig. 1B and Fig. 3). This adhesion was increased in the presence of ROS inhibitor and PARP inhibitor, indicating that HBMEC integrity is not impaired and these tumor cells are responsive to both inhibitors. However, in tumor cells expressing MT BRCA1, high levels of ROS is secreted from these cells, which in turn causes impairment of HBMEC.

Transmigration: Next, we examined the effects of ROS and PARP inhibitors on migration of HCC1937 cells expression either WT or mutant BRCA1. As shown in Fig. 3, there was a significant difference in the morphology of transmigrating HCC1937/WT cells, as compared to HCC1937/MT cells. While the HCC1937/WT BRCA1 cells showed morphology of epithelial breast cells, the mutant cells showed much smaller and rounded shaped cells. The transmigration of HCC1937/MT BRCA1 was modestly inhibited in the presence of ROS and PARP inhibitors (Fig. 3), while the transmigration of WT/BRCA1 was significantly lower, as compared to HCC1937/MT BRCA1 (Fig. 3A+3B). Further, this transmigration of HCC1937/WT BRCA1 was reduced by ROS inhibitor. Fig. 3B showed the quantitative analyses of the transmigration data.

Permeability: Next, we analyzed the effects of these inhibitors on permeability of HBMECs by TEER assay. As shown in Table 3, the permeability of HBMEC was increased in the presence of HCC1937/MT cells, as compared to WT cells. Changes in permeability as analyzed by TEER were reduced in the presence of either COX-2, PARP or ROS inhibitors.

Table 3: Effects of ROS and PARP inhibitors on TEER

Cells	Treatment	TEER of HBMEC (% of Control)
HCC1937/MT BRCA1	-	83±3**
	ROS inhibitor	93±4
	PARP inhibitor	94±3
Cells	Treatment	TEER of HBMEC (% of Control)
HCC1937/WTBRCA1	-	90±3*
	ROS inhibitor	94±5
	PARP inhibitor	95±2
HBMECs	-	100

Table 3: TEER measurement across the HBMEC monolayer assessed the BBB tightness. TEER values recorded in ohms. Results are from three experiments in triplicate. Results were expressed as mean percent of controls. *p<0.05 as compared to HBMEC control; **p<0.005 as compared to HBMEC control.

Effects of mutant BRCA1 on tight junctions expression:

To assess changes in tight junction expression (ZO-1 and claudin-5) on HBMEC following coculture with HCC1937 cells expressing WT or mutant BRCA1, immunostaining of these TJs was performed and quantitative analysis of TJ expression in BMECs was determined using the Volocity software. As shown in Fig. 4A, significant reduction in ZO-1 expression was observed in the presence of HCC1937/MT BRCA1 and HCC WT/ BRCA1. ROS inhibitor blocked the decrease in ZO-1 expression in the presence of HCC1937/MT BRCA1 but not with HCC1937/WT BRCA1. COX-2 inhibitor had no effects on ZO-1. Further expression of claudin-5 in HBMECs was decreased in the presence of HCC1937/WT BRCA1, and this decrease was modestly inhibited in the presence of COX-2 and ROS inhibitors (Fig. 4B). HCC1937/MT-BRCA1 also caused decrease in claudin-5 expression (Fig. 4C).

Taken together, these studies show increased aggressiveness in vitro of HCC1937/MT-BRCA1 cells that are associated with oxidative stress, which may result in increased metastasis of these tumors in brain.

Key research accomplishments:

- 1) Adhesion to HBMECs by tumor cells expressing mutated BRCA1, as compared to WT-BRCA1, is significantly increased and is mediated by ROS production by tumor cells, resulting in alteration of HBMEC permeability.
- 2) Transmigration of HCC1937 expressing mutant BRCA1 is enhanced, as compared to tumor cells expressing WT BRCA1.
- 3) Permeability changes in HBMECs are more pronounced in the presence of MT-BRCA1, as compared to WT-BRCA1 and these changes can be inhibited by ROS and PARP inhibitors.

Reportable outcomes: None.

Conclusion: Antioxidant therapy and PARP inhibitors may be viable therapeutic approaches for inhibiting metastasis to brain in BRCA mutated breast cancers, based on the *in vitro* results.

References: None

Appendices: None