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TITLE: Oxidative Stress Increases the Blood Brain Barrier Permeability Resulting in Increased Incidence of Brain Metastasis in BRCA Mutation Carriers

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Oxidative Stress Increases the Blood Brain Barrier Permeability Resulting in Increased Incidence of Brain Metastasis in BRCA Mutation Carriers

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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/Waf and p27Kipl expression. Further, 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/Waf and p27Kipl and high level of survivin and were resistant to paclitaxel. Thus, mutated BRCA1 or loss of wt BRCA1 up-regulates the malignant cell behavior. However, it is still not clear how tumor cells expressing mutant BRCA1 have enhance tumorigenicity in vivo.

15. SUBJECT TERMS- Breast Cancer
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I. INTRODUCTION

BRCA1 is a multifunctional tumor suppressive protein. Knockout of wt BRCAI 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. Further, in BRCAI 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 BRCAI (such as MDA-MB-436 and HCC1937) were highly proliferative and expressed a relatively low levels of p21/Waf1 and p27Kip1 and high levels of survivin and were resistant to paclitaxel. Thus, mutated BRCAI or loss of wt BRCA1 up-regulates the malignant cell behavior. However, it is still not clear how tumor cells expressing mutant BRCAI have enhance tumorigenicity in vivo.

II. HYPOTHESIS

We hypothesize that adhesion of BRCAI mutated cells to endothelial cells activates several distinct signaling pathways to induce MMP gene expression and increased ROS levels. We hypothesize that oxidative stress induced by adhesion of cells with mutated BRCA1 to HBMEC results in alterations in the integrity of the blood-brain barrier (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 reactive oxygen species (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.

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.

III. SUMMARY OF RESULTS

Year 1 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 reactive oxygen species (ROS) production in human brain microvascular endothelial cells.

Results

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 $^3$H-inulin and $^{14}$C-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. H(2)O(2) (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>
<thead>
<tr>
<th>Adhesion of cells</th>
<th>Fluorescence Intensity (x10$^3$)/mg Protein in HBMEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC1937</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>HCC1937/WT BRCA1</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>MCF-7</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>MCF-7/mutated BRCA1</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Control PBS</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>H(2)O(2)/HBMEC</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>

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.
Effects of ROS and PARP inhibitors on permeability of tumor cells across HBMEC

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. 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.
Year 2
Aims

2) Examine the effects of oxidative stress on HBMEC-tight junction expression (ZO-1 and claudin-5) *in vitro* and *in vivo*.

3) Determine the protective effects of PARP and ROS inhibitors, as well as COX-2 inhibitors in preventing BBB induced change and breast tumor colonization in brain.

Results

Since the BBB function is maintained by the presence of a continuous tight junction (TJ) complex, we investigated the effects of HCC1937 cells expressing mutant BRCA1, as compared to HCC1937/WT-BRCA1 expressing WT BRCA1 in HBMECs 3D cocultures of human astrocytes together with HBMEC system, on HBMEC-TJ integrity. While HBMECs expressed a linear and continuous distribution at cell-cell boundaries with normal ZO-1 and claudin-5 distribution (Fig. 1A), the cocultures of HCC1937 expressing mutant BRCA1 resulted in significantly damaged and less continuous, non-linear appearance in HBMECs or completely missing at several cell-cell boundaries (Fig. 1C). Interestingly, HCC1937/BRCA1 was capable of maintaining some TJ structures in HBMECs (Fig. 1B), and the damage of BMEC-TJs was less severe as compared to that of HCC1937.

The addition of ROS inhibitor or COX-2 inhibitor directly to HBMEC cultures was toxic and caused apoptosis of HBMEC at 50nM (Fig. 2A) or at 25nM (data not shown). In cocultures of HBMECs and HCC1937 (Fig. 3B) and HCC1937/WT-BRCA1 (Fig. 3A), ROS inhibitor by itself was unable to prevent damage of TJ structures in HBMECs (Fig. 3). However, ROS inhibitor showed some protective effects on HBMEC-TJs ZO-1 and claudin-5, as compared to cocultures with HCC1937 cells. Further, PARP inhibitor at 40µM was also toxic to HBMEC cocultures (Fig. 2B).

Since we observed some protective effect with HCC1937/WT-BRCA1, we examined the expression of TJ structures (ZO-1 and claudin-5) in the presence of HCC1937/WT-BRCA1 in more details. The presence of TJs ZO-1 and claudin-5 on HBMECs was less damaged (Fig. 4B) as compared to control HBMECs (Fig. 4A). Interestingly, these changes in TJs were minimized in the presence of COX-2 inhibitor (Fig. 4C). These data strongly suggest that mutated BRCA1 expressing tumor cells are more aggressive in causing damage to BMECs and the BBB. Interestingly, we observed that HCC1937 cells express high level of COX-2 by RT-PCR (Fig. 5A) and western blotting (Fig. 5B), while HCC1937/WT-BRCA1 had no expression of COX-2 (Fig. 5B). Thus, WT-BRCA1 inhibits COX-2 expression in tumor cells.

Next, we performed in vivo studies on the effects of MT-BRCA1 expressing tumor cells on the BBB integrity, TJ complexes in BMECs, and mammary tumor colonization in brain. Mice were injected with GFP-HCC-1937 or with GFP-HCC-1937/WT BRCA1 to the carotid artery (10^6 cells). After 7 weeks, the mice were euthanized and the BBB permeability in the brain of these mice was analyzed. As shown in Table 4, there were significant changes in the BBB in both groups. Neither inhibitors for ROS or PARP were able to prevent changes in the BBB integrity with both HCC1937 and HCC1937/WT although the changes were more dramatic with HCC1937 cells. Since COX-2 was more effective in vitro in preventing BBB permeability and TJ changes (Fig. 4C), we examined the potential of COX-2 inhibitor to inhibit BBB permeability.
changes and tumor colonization in brain. We employed 4T1-BrM5 mammary epithelial cells that express MT-BRCA1 and injected them into the mammary fat pads of BALB/c mice. Within 2 weeks, tumor growth in the mammary fat pads was observed as shown in Table 5A. COX-2 inhibitor inhibited tumor growth in vivo in the mammary fat pads (note different scale between panels A and B in Table 5). Further, COX-2 inhibitor decreased BBB permeability (10 mice per group per treatment), as analyzed by Evans blue test (data not shown). Further, in vivo imaging of the brain samples from these mice showed reduced colonization of 4T1-BrM5 tumor cells in brain by COX-2 inhibitor (1 out of 10 mice had tumor colonization in brain, Fig. 6B) as compared to brain tumor colonization without treatment (10 out of 10 mice had tumor colonization in brain, Fig. 6A). Further, while tumor colonization of 4T1BrM5 was observed on day 10 (Fig. 6A) in Balb/c brain, we were able to observe tumor colonization in one mouse treated with COX-2 inhibitor only after 5 weeks (Fig. 6B).

Figure and Table Legends

Figure 1: BRCA1 mutant expressing cells induced changes in the distribution of ZO-1 and claudin-5 TJ proteins in HBMECs
3D cocultures of HBMECs with human astrocytes were either cocultured in the presence of HCC 1937 expressing MT BRCA1 (HCC MT) or wild type BRCA1 (HCC WT) for 6 hours and fixed immediately with 2% paraformaldehyde. Cells were then probed for ZO-1 and claudin-5 expression and visualized by indirect immunofluorescence using Alex Fluor 568 (red) conjugated secondary antibody (Molecular Probes). Nuclei were counterstained with DAPI (blue). Arrowheads indicated intact claudin-5 and ZO-1 TJs as indicated. Images shown are from a representative axial plane and are representative of over 30 fields examined in at least three independent experiments. Scale bar = 20 µm.

Figure 2: Effects of COX-2 inhibitor and ROS inhibitor of BMEC-TJs
HBMEC cocultures were preincubated with PARP inhibitor (30mM) or with ROS inhibitor (10mM selenium) for 6 hours. Immunostaining of TJs ZO-1 and claudin-5 was performed as described in Fig. 1.

Figure 3: Effects of ROS inhibitor on BMEC-TJs in the presence of HCC 1937 cells expressing mutant BRCA1 or WT BRCA1
HBMEC cocultures were pretreated with ROS inhibitor (50mM) for 1 hour. HCC-MT/BRCA1 or HCC-WT/BRCA1 cells were then added to HBMEC cocultures for 6 hours. The immunostaining of ZO-1 and claudin-5 TJs on BMECs were performed as described in Fig. 1.

Figure 4: Effects of HCC-BRCA1/WT and COX-2 inhibitor in vitro on BMEC-TJs
3D cocultures of HBMECs with human astrocytes were established and cocultured with HCC 1937 cells expressing WT BRCA1 in the absence of presence of COX-2 inhibitor (30 mM COX-2 inhibitor celecoxib). TJs immunostaining of ZO-1 and claudin-5 were performed as detailed in Fig. 1.

Figure 5: Expression of COX-2 in HCC1937 and HCC1937/WT-BRCA1 by RT-PCR (Panel A) and Western blotting (Panel B)
A. For RT-PCR, we obtained mRNA from both cell lines. RNA concentration was 0.211 µg/µl for HCC1973 and 0.264 µg/µl for MCF-7. RT-PCR analysis was run with 158 bp primer (Zattelli et al.) at annealing temperature of 52 °C using Qiagen One step RT-PCR kit was used for
the analysis.
B. Western blot analysis using COX-2 antibody from Santa Cruz (cat#sc-19999, lot F0510 product) with 72 kd.

**Figure 6: Internal images of murine mammary epithelial tumor (4T1BrM5-GFP) metastasis in brain**
Murine breast metastasis in the mouse brain was imaged by GFP expression. Images show metastatic lesions in the brain.
A. Series of internal fluorescence images of metastatic lesions in the brain in mice administered with 4T1BrM5-GFP mammary tumor cells into mammary fat pads at day 10 or 17 as compared to WT control brain (C1, C2)
B. Series of internal fluorescence images of metastatic lesions in the brain in mice administered with 4T1BrM5-GFP cells and COX-2 inhibitor taken at week 5 following treatment. The GFP positive signal in the mammary tumor is shown as a positive control.

**Table 4: Quantitative analysis of TJs in vivo in brain:** The changes in BBB TJs were analyzed 7 weeks following administration of HCC 1937/MT (HCC/MT) - BRCA1 or HCC 1937/WT(HCC/WT)-BRCA1 cells in the absence or presence of ROS inhibitor selenium (5mg/kg) or PARP inhibitor AZ0228 (10mg/kg) i.v. one hour before tumor cell injection. Quantification of CD31 positive BMEC expression and TJ proteins claudin-5 and ZO-1 (n=12, random sections, 10 mice per group) was performed. The TJs of the samples were analyzed, quantitated and compared to control brain. The TJs in control brain were defined as 100%. Percent changes in treated samples, as compared with control brain were calculated. Data are presented as mean +/- 1:SD *p < 0.05, **p < 0.005.

**Table 5:** BALB/c mice were administered either vehicle control (Panel A) or COX-2 inhibitor celecoxib at 10 mg/kg (Panel B), one hour before injection of 4T1-BrM5 tumor cells (100,000 cells) into the mammary fat pads (10 mice per group per treatment). Live mice were then sacrificed at day 21 and tumor volume in the mammary fat pads was measured. Some of the mice died during the experiment. Therefore, we have no data for those mice, as indicated.
<table>
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<th>Treatment</th>
<th>Quantification of TJ change (%)</th>
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<tbody>
<tr>
<td></td>
<td>ZO-1</td>
<td>Claudin-5</td>
</tr>
<tr>
<td>WT-control brain</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>HCC/MT BRCA1 + vehicle control</td>
<td>$42 \pm 8^{**}$</td>
<td>$22 \pm 7^{*}$</td>
</tr>
<tr>
<td>HCC/MT BRCA1 + ROS inhibitor</td>
<td>$37 \pm 11^{**}$</td>
<td>$17 \pm 6^{*}$</td>
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<tr>
<td>HCC/MT BRCA1 + PARP inhibitor</td>
<td>$41 \pm 14^{**}$</td>
<td>$26 \pm 7^{*}$</td>
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<tr>
<td>HCC/WT BRCA1 + vehicle control</td>
<td>$28 \pm 9^{**}$</td>
<td>$21 \pm 11^{*}$</td>
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<tr>
<td>HCC/WT BRCA1 + ROS inhibitor</td>
<td>$30 \pm 8^{**}$</td>
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<tr>
<td>HCC/WT BRCA1 + PARP inhibitor</td>
<td>$27 \pm 8^{**}$</td>
<td>$19 \pm 8^{*}$</td>
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**TABLE 4**
Figure 1
Figure 2
Figure 3
Figure 4

4A: HBMECs

4B: HBMEC / HCC-BRCA1 WT

4C: HBMEC / HCC-BRCA1 WT / COX-2 inhibitor
Figure 5

5A. COX-2 RT-PCR

1 µl  2 µl
HCC1937
MCF-7

5B. Western blot analysis of COX-2 expression in HCC1937/MT-BRCA1 and HCC1937/WT-BRCA1
Figure 6

A. WT-Control Brain

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<th>C2</th>
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<tr>
<td>WT</td>
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<td>mouse2</td>
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<tr>
<td>10-OFP-4T1 BrM5</td>
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<td>mouse2</td>
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B. COX-2 inhibitor - 5 weeks in vivo imaging

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Mammary tumors (positive control)</th>
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<tr>
<td>Top view</td>
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<td>Bottom view</td>
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Year 3 (No Cost Extension)
No data generated

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 HCC-1937 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 BRCA, as compared to WT BRCA and these changes can be inhibited by ROS and PARP inhibitors.
4) *In vivo* results showed that COX-2 inhibitors may be viable therapeutic approaches for inhibiting metastasis to the brain in BRCA1 mutated breast cancers.

Reportable outcomes
A manuscript is in preparation.

Conclusion
COX-2 inhibitors may be viable therapeutic approaches in addition to chemotherapy. In addition, they may minimize the risk for breast cancer metastasis to the brain in patients with mutated BRCA1.