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The Role of Breast Cancer Derived Prostaglandin E2 in the Elaboration of a Therapeutic Immune Response

Stephen L. Eck, Ph.D.

University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

We postulated that the production of prostanoids, principally prostaglandin E2 (PGE2), by human breast cancers contributes to the lack of a significant immune response to breast cancer cells. We have conducted a series of experiments to show that human breast cancer cells commonly secrete soluble agents that directly inhibit T lymphocytes. Following up on our preliminary data, we demonstrated that one of the major inhibitory factors made by breast cancer cells is PGE2. Specifically, tumor conditioned media containing PGE2 is able to inhibit T cell proliferation. When PGE2 is depleted from the media or when PGE2 synthesis is inhibited, tumor conditioned media no longer inhibits T cell proliferation. We also demonstrate that over expression of cyclooxygenase alone is sufficient to induce PGE2 synthesis and T cell inhibition. Finally, we have found that membrane proteins in breast cancer cells also contribute to the lack of the immune response to breast tumor derived antigens.

Breast Cancer immunotherapy, B7-1, immunosuppressive factors, PGE2

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Date: July 14, 1999

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Human breast cancer derived PGE₂ inhibits B7-1 induced T cell proliferation

Key words: breast cancer, immunotherapy, B7-1, immunosuppressive factors, PGE₉

Introduction

The principal goal of this study is to understand why breast cancer cells are able to evade the host immune system despite the presence of tumor antigens and tumor antigen-specific T lymphocytes. We postulated that the production of prostanoids, principally prostaglandin E₂ (PGE₂), by the tumor directly contributes to the lack of a significant immune response to breast cancer cells. We have conducted a series of experiments to show that human breast cancer cells commonly secrete soluble agents that directly inhibit T lymphocytes. Following up on our preliminary data, we demonstrated that one of the major inhibitory factors made by breast cancer cells is PGE₂. While there have been isolated reports of prostaglandin synthesis by human breast cancers, those studies used only a few cell lines. Therefore, it was important to establish that this is a general phenomenon of human breast cancer cells. Previous investigators have postulated a variety of effects that are mediated by breast cancer produced PGE₂. Most of the prior work has focused on the direct effects of PGE₂ on the growth of human breast cancer. We believe that an equally important function of PGE₂ is to directly alter or suppress the immune response to breast cancer cells. We have shown that the expression of cyclooxygenase (COX) and the resultant production of PGE₂ are sufficient to abrogate the T cell response to tumors cells in a vaccination model. Furthermore, this can be block by inhibiting the COX mediated production of PGE₂.

The initially proposed first year Aims and related Tasks are shown and indicated in the relevant portions of the results sections below.

Aim #1 We will determine the frequency of PGE₂ production in human breast cancers and determine whether inhibition of PGE₂ synthesis eliminates the T cell inhibitory factor in breast cancer media.

Task 1. Analyze tumor conditioned media from human breast and determine the frequency of T cell inhibitory factors. (Months 1-6)

Task 2. Prepare lipid extracts of the tumor condition media and establish that this fraction contains all or part of the inhibitory factors. (Months 1-12)

Task 3. Quantify the PGE₂ levels in the breast cancer conditioned media and correlate their levels with the inhibitory effects produced the conditioned media and authentic PGE₂. (Months 1-12)

Task 4. Demonstrate that addition of cyclooxygenase inhibitors blocks PGE₂ synthesis with concomitant decline inhibitory activity. (Months 7-12)

Task 5. For those specimens producing PGE₂ and TGFβ we will determine whether blocking both agents has an additive effect. (Months 10-14)

Task 6. We will characterize three murine mammary cancer cell lines (SCK cells, T2994 and MT901 cells) for PGE₂ and TGFβ production and T cell inhibition. (Months 9-12)
Body of the Report

Breast carcinoma cells produce high levels of agents known to alter T cell responses including transforming growth factor β (TGFβ, 1), interleukin 10 (2), and PGE₂ (3). PGE₂ exerts heterogeneous effects on diverse lymphocyte subpopulations. Whereas it increases the expression of IL-12 receptors on lymphocytes (4), promotes immunoglobulin synthesis (5), it also inhibits IL-17 production (6), and reduces cytotoxic activity (7) and lymphocyte proliferation (8-10). In the mouse thymus PGE₂ production plays a critical role in T cell maturation (11,12). Furthermore, PGE₂ has been shown to inhibit differentiation of lymphokine activated killer cells (LAK), suppress of natural killer cell (NK) activity (13-15), and down-regulate humoral immune responses (16). The inhibition of T cell proliferation in lymphocyte cultures by PGE₂ is mediated in part by the down-regulation of MHC class II expression on antigen presenting cells (LAK), suppression of cytokine production (20-21). PGE₂ is produced by cyclooxygenase (COX) mediated oxidation of arachidonic acid and has been found in some human breast cancer cell lines (22). There are two isoforms of COX, designated COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, mediates the synthesis of prostaglandins including PGE₂ and is required for constitutive physiological functions, such as maintaining gastrointestinal, kidney and reproductive functions (23). COX-1 was found to be over expressed in 30 of 44 breast tumor tissues compared to normal breast epithelium (24). COX-2 expression can be induced by cytokines, growth factors, oncogenes and tumor promoters (25), is reportedly up regulated in a some metastatic breast cancer cell lines (26) and leads to high levels of PGE₂ production by these cells. Given the development of idiopathic chronic suppurative peritonitis and bowel inflammation in COX-2 knockout mice (27), the involvement of COX-2 in inflammatory and immune responses appears likely but is as yet poorly understood. Similarly, the consequences of COX expression and PGE₂ production for anti-tumor immune responses remain poorly understood.
Breast cancer cells produce soluble factors that suppress lymphocyte proliferation. (Tasks 1, 3 & 6)

We sought to determine if other human breast cancer cell lines produced soluble T cell inhibitory factors. In addition to MCF-7 cells seven of eight breast cancer cell lines tested (BT-20, MCF-10, BT-474, MDA-MB 231, SUM52PE, SUM149PT and SUM190PT) also secreted soluble factors that were capable of inhibiting the proliferation of MN cells (Table 1). By contrast, CM from two cell lines, SUM185 cells and the human breast epithelial HBL-100 cells did not inhibit lymphocyte proliferation (Table 1) under the same experimental conditions. Thus, the production of soluble factors that inhibit lymphocyte proliferation is a common though not universal characteristic of human breast cancer cells.

<table>
<thead>
<tr>
<th>Human and mouse cell lines</th>
<th>% inhibition of PHA stimulated MN cells</th>
<th>PGE(_2) production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-100* (breast epithelial)</td>
<td>5%</td>
<td>100</td>
</tr>
<tr>
<td>BT-20</td>
<td>41%</td>
<td>0</td>
</tr>
<tr>
<td>MCF-10</td>
<td>99%</td>
<td>300</td>
</tr>
<tr>
<td>MCF-7</td>
<td>97%</td>
<td>40</td>
</tr>
<tr>
<td>BT-474</td>
<td>96%</td>
<td>40</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>90%</td>
<td>260</td>
</tr>
<tr>
<td>SUM52PE</td>
<td>66%</td>
<td>30</td>
</tr>
<tr>
<td>SUM149PT</td>
<td>56%</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>SUM185PE</td>
<td>4%</td>
<td>0</td>
</tr>
<tr>
<td>SUM190PT</td>
<td>51%</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>SCK (A/J mouse strain)</td>
<td>75%</td>
<td>65</td>
</tr>
<tr>
<td>T2994 (BALB/C mouse strain)</td>
<td>15%</td>
<td>40</td>
</tr>
<tr>
<td>NT5 (FVB mouse strain)</td>
<td>100%</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MT901 (BALB/C mouse strain)</td>
<td>100%</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

**Table 1A.** PGE\(_2\) levels and inhibition of PHA stimulated MN cell proliferation by CM from a normal human breast epithelial cell line (HBL-100) and human breast carcinoma cell lines (all others).

<table>
<thead>
<tr>
<th>Human Breast Cancer Cells</th>
<th>PHA</th>
<th>PMA/ionomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>97%</td>
<td>98%</td>
</tr>
<tr>
<td>MCF-10</td>
<td>99%</td>
<td>98%</td>
</tr>
<tr>
<td>BT-474</td>
<td>96%</td>
<td>93%</td>
</tr>
<tr>
<td>BT-20</td>
<td>41%</td>
<td>0%</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>90%</td>
<td>0%</td>
</tr>
<tr>
<td>T47D</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

**Table 1B.** The inhibitory effect of conditioned media (CM) from different tumor cell lines on the proliferation of mitogen stimulated mononuclear cells (MN). The percent inhibition was calculated as follows: % inhibition = 100% - (100% x cpm of mitogen stimulated MN cells incubated with sample CM at a dilution of 1:4/cpm of mitogen stimulated MN cells).
Adenoviral transfer of B7-1 to MCF-7 breast cancer cells (Tasks 3 and 4).

We have shown previously that cultured human melanoma cells do not express B7-1 and do not co-stimulate proliferation of T cells in response to PHA (40). However, when transduced with AdhB7-1 these cells acquire the ability to co-stimulate T cells. Similar to other human tumor cells all cultured human breast cancer cells examined in this study do not express endogenous B7-1 at levels detectable by flow cytometric analysis. To test whether adenovirally delivered B7-1 expression affect co-stimulatory activity to these breast cancer cells, we first transduced MCF-7 cells with AdhB7-1. Flow cytometric analysis demonstrated that >90% of MCF-7 cells transduced with AdhB7-1 expressed the B7-1 protein on their cell surface. We observed previously that many human melanoma cells adenovirally transduced to express B7-1 effectively stimulate proliferation of purified human T cells (40). To assess the co-stimulatory activity of B7-1 expressed by MCF-7 cells, we measured the proliferation of allogeneic T cells in co-culture with B7-1 expressing MCF-7 cells in comparison with B7-1 expressing WM9 melanoma cells. Purified human T cells were co-cultured with untransduced, B7-1 transduced, or lacZ transduced tumor cells. B7-1 expressing WM9 cells induced at least a 53-fold increase in T cell proliferation (Table 2) over that achieved by untransduced WM9 cells. This effect was not due to the presence of adenovirus as seen by the low stimulation index (SI) of WM9/lacZ cells (Table 2). In contrast to melanoma cells, MCF-7 cells transduced with AdhB7-1 did not stimulate T cell proliferation. To determine whether the lack of co-stimulation by MCF-7 cells was due to tumor-derived secreted factors, we added medium conditioned by MCF-7 cells (CM) to mononuclear (MN) cultures. MCF-7 CM blocked proliferation of MN preparations stimulated with either PHA (Fig. 1A) or PMA/ionomycin (Fig. 1B) indicating that MCF-7 CM exerts its effect independently of the stimulus used for T cell activation. Inhibition of lymphocyte proliferation was potent and dose-dependent with significant inhibition observed even at 1:4,000 dilution (Fig. 1A, B).

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>T cells</th>
<th>cpm</th>
<th>sd</th>
<th>SI</th>
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<tbody>
<tr>
<td>MCF-7</td>
<td>-</td>
<td>39</td>
<td>7</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>55</td>
<td>8</td>
<td>0.3</td>
</tr>
<tr>
<td>MCF-7/B7-1</td>
<td>-</td>
<td>48</td>
<td>6</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>551</td>
<td>169</td>
<td>0.9</td>
</tr>
<tr>
<td>MCF-7/lacZ</td>
<td>-</td>
<td>37</td>
<td>7</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>43</td>
<td>6</td>
<td>0.1</td>
</tr>
<tr>
<td>WM9</td>
<td>-</td>
<td>25</td>
<td>4</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1287</td>
<td>109</td>
<td>24</td>
</tr>
<tr>
<td>WM9/B7-1</td>
<td>-</td>
<td>32</td>
<td>6</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>68374</td>
<td>11736</td>
<td>1340</td>
</tr>
<tr>
<td>WM9/lacZ</td>
<td>-</td>
<td>291</td>
<td>55</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>300</td>
<td>236</td>
<td>0.2</td>
</tr>
<tr>
<td>T cells alone</td>
<td></td>
<td>51</td>
<td>6</td>
<td>/</td>
</tr>
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Table 2. Thymidine uptake and relative proliferation of human T cells cultured with B7-1 expressing MCF-7 cells or with B7-1 expressing WM9 cells. Purified T cells were co-cultured with untransduced, AdhB7-1 transduced or AdlacZ transduced tumor cells. Their proliferation is expressed relative that of T cells in the absence of tumor cells as indicated by SI. Thymidine incorporation (mean cpm) and
standard deviation (sd) were obtained from quadruplicates. B7-1 expressing MCF-7 cells failed to stimulate T cell proliferation.

![Graph A](image1.png)

**Figure 1.** Thymidine uptake (cpm) of MN cells stimulated with (A) PHA or (B) PMA/ionomycin after the addition of MCF-7 CM. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. MCF-7 CM inhibited the proliferation of stimulated MN cells.
Soluble TGFβ alone does not account for the inhibitory effect of MCF-7 CM on the proliferation of MN cells in response to PHA. (Task 5)

Breast carcinoma cells including MCF-7 have been described to express TGFβ which is known to inhibit T cell proliferation (29). Therefore, we sought to determine whether MCF-7-derived TGFβ was responsible for the inhibitory effect of MCF-7 CM on T cell proliferation. We determined that the MCF-7 cells used in the present study also secrete TGFβ at a rate of 1150 pg/1x10⁶ cells in a 24 h period. To assess whether TGFβ in MCF-7 CM contributes to the inhibition of lymphocyte proliferation, we evaluated the effect of MCF-7 CM on PHA-dependent MN cell proliferation in the presence of different concentrations of a neutralizing antibody that is reactive with all three known human TGFβ isoforms (Fig. 2). We have described specificity and activity of this antibody earlier (30,31). The TGFβ antibody was used in excess to block the biological effects of TGFβ found in MCF-7 CM. At the highest concentration tested here (100 µg/ml) the TGFβ antibody neutralizes 28 ng/ml of recombinant TGFβ1 as determined by TGFβ bioassay. MCF-7 derived CM contained only 34.5 pg total TGFβ (Fig. 2). At none of the concentrations tested did the neutralizing TGFβ antibody affect inhibition of MN proliferation by MCF-7 CM (Fig. 2). These results effectively exclude TGFβ as a cause of the inhibitory effect.

![Figure 2](image-url)

**Figure 2.** The effect of MCF-7 CM on the proliferation (thymidine uptake in cpm) of PHA stimulated MN cells after incubation with a pan-specific TGFβ neutralizing antibody (100 µg/ml to 2.5 ng/ml). Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. TGFβ did not account for the inhibitory effect of the MCF-7 CM.
PGE$_2$ production by breast carcinoma cells. (Task 3)
PGE$_2$, a known modulator of T cell proliferation (17,18) has been shown to be produced by some breast carcinomas (3). We measured PGE$_2$ production by non-tumorigenic breast epithelial cells and by the breast cancer cell lines used in this study. We found that eight of ten cell lines produced PGE$_2$ at levels detectable by ELISA assay (Table 1). Interestingly, MCF-7 cells produced relatively low levels of PGE$_2$ (40 pg/ml) when compared to SUM190PT or SUM149PT cells which produced in excess of 1000 pg/ml PGE$_2$. With the exception of HBL-100, all of the cell lines that produced PGE$_2$ also inhibited the proliferation of PHA stimulated MN cells although there was no correlation between the amount of secreted PGE$_2$ and the inhibitory capacity. In addition, BT-20 CM had a significant inhibitory effect although it did not produce detectable levels of PGE$_2$. Taken together, these results suggested that PGE$_2$ may play an important role in the inhibition of a proliferative response of MN cells and lymphocytes. Although commonly expressed by breast cancer cells in culture it may act in concert with other factors other factors to this process.

Indomethacin treatment of breast cancer cells restores lymphocyte proliferation. (Task 4)
We next attempted to inhibit PGE$_2$ production by breast cancer cells to probe the relative contribution of PGE$_2$ to inhibition of T cell proliferation by breast carcinoma-derived CM. COX is expressed in some human breast cancers (24, 26) First, we investigated whether the inhibition of COX activity by indomethacin restores lymphocyte proliferation. MCF-7 cells were treated with indomethacin at a concentration of 100 µg/ml for 24 hours. Indomethacin inhibited both the COX-1 and COX-2 enzymes and reduced the PGE$_2$ content in MCF-7 CM cells to less than 30 pg/ml (the lower limit of detection in this assay). Indomethacin pretreatment abrogated the inhibitory effect of MCF-7 CM on PMA/ionomycin stimulated proliferation of MN cells at a 1:4,000 dilution (Fig. 3). Indomethacin containing medium itself (not exposed to tumor cells) did not influence the proliferation of MN cells in response to PMA/ionomycin. However, the proliferation of MN cells was still inhibited when untreated MCF-7 CM or CM from indomethacin-treated MCF-7 cells that was more concentrated (1:40 dilution) was added to MN cells in the presence of mitogen. This is consistent with our observation that MCF-7 CM from indomethacin-treated cells still contained residual amounts of PGE$_2$ detectable by liquid chromatograph-mass spectroscopy (LC-MS).
Figure 3. Indomethacin treatment of breast cancer cells restores the proliferation of lymphocytes. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. Thymidine incorporation by MN cells stimulated with PMA/ionomycin in the presence of absence of CM from MCF-7 cells treated with indomethacin. Indomethacin treatment of MCF-7 cells partially alleviated the immunosuppressive effect of MCF-7 CM.

Removal of PGE\textsubscript{2} from breast cancer CM elicits B7-1-dependent stimulation of MN cells and purified T lymphocytes. (Task 4)

Next, we attempted to remove PGE\textsubscript{2} more effectively from MCF-7 CM. We used an immuno-affinity column that binds up to 10 ng PGE\textsubscript{2} (62-fold more than the amount detected in the CM). MCF-7 CM passed through this immuno-affinity column completely lost its inhibitory effect on MN proliferation at all dilutions tested (Fig. 4A). These results indicate that breast cancer derived PGE\textsubscript{2} even at relatively low concentration (40 pg/ml) blocks mitogenic lymphocyte responses.

The effect of PGE\textsubscript{2} on MN responses to mitogens has been reported before (11, 16), whereas the effect of PGE\textsubscript{2} on B7-1 dependent T cell proliferation has not been studied in great detail. To confirm that breast cancer-derived PGE\textsubscript{2} contributes to suppression of the T cell response irrespective of other cell types present in MN cell preparations we performed T cell co-culture assays using MDA-MB 231 breast carcinoma cells. These cells produced markedly higher amounts of PGE\textsubscript{2} than MCF-7 cells (see Table 1). MDA-MB 231 cells were transduced with AdhB7-1 to overexpress B7-1 and treated with either indomethacin or NS398, a COX-2 specific inhibitor. As a control, MDA-MB 231 cells and T cells were co-cultured in ethanol containing medium since ethanol served as a solvent for the COX inhibitors. Untreated B7-1 expressing MDA-MB 231 cells produced 312 pg/ml PGE\textsubscript{2} whereas pretreatment with either indomethacin or NS398 reduced production of PGE\textsubscript{2} to undetectable levels. Untreated or
untransduced MDA-MB 231 cells did not stimulate allogeneic T cells in the absence or presence of PHA (Fig. 4B). However, DNA synthesis by T cells was markedly stimulated upon co-culture with B7-1 expressing, indomethacin-pretreated tumor cells. In contrast, there was no thymidine incorporation by T cells co-cultured with ethanol treated or NS398 treated, B7-1 expressing MDA-MB 231 cells or with unmodified MDA-MB 231 cells in PHA containing medium (Fig. 4B). These results indicate that the inhibition of COX enzyme activity by indomethacin restores T cell proliferation in the presence of PHA.

A

![Graph A](image)

dilution factor of MCF-7 CM

B

![Graph B](image)
Figure 4. (above) Removal of PGE$_2$ from breast cancer CM elicits B7-1-dependent stimulation of MN cells and purified T lymphocytes. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. (A) Thymidine incorporation by MN cells (square), by MN cells stimulated with PMA/ionomycin (diamond), by PMA/ionomycin stimulated MN cells incubated with MCF-7 CM (circle) and by PMA/ionomycin stimulated MN cells incubated with PGE$_2$-depleted MCF-7 CM (triangle). MCF-7 CM inhibited the mitogenic response of MN cells. The inhibitory effect of MCF-7 CM was completely alleviated when PGE$_2$ was eliminated via PGE$_2$ affinity column. (B) Thymidine incorporation by T cells co-cultured with B7-1 expressing MDA-MB 231 cells after the treatment with indomethacin, NS 398 or ethanol. In the presence of PHA indomethacin treatment of B7-1 expressing MDA-MB 231 cells abrogated T cell proliferation.

Breast cancer derived PGE$_2$ inhibits B7-1-dependent T cell proliferation induced by melanoma cells. (Tasks 4 and 5) We sought to confirm the role of PGE$_2$ on immune responses in a different system in the absence of endogenous PGE$_2$ and B7-1. To this end, we turned to B7-1 transduced WM9 melanoma cells which produce no PGE$_2$ and stimulate T cell proliferation. When MCF-7 CM was added to T cells co-cultured with B7-1 expressing melanoma cells the proliferation of T cells was completely inhibited (Fig. 5, diamond). As shown in Fig. 5, the depletion of PGE$_2$ from MCF-7 CM using the immunoaffinity column restored DNA synthesis of T cells in the presence of PHA (circle), whereas the addition of exogenous PGE$_2$ to MCF-7 CM blocked thymidine uptake of stimulated T cells (triangle). These data indicate that PGE$_2$ secreted by tumor cells is essential for the inhibition of B7-1 induced T cell proliferation.
Figure 5. Breast cancer derived PGE₂ inhibits B7-1-dependent T cell proliferation induced by melanoma cells. Thymidine incorporation by T cells co-cultured with B7-1 expressing WM9 cells after the addition of MCF-7 CM (diamond), PGE₂ depleted MCF-7 CM (circle) and MCF-7 CM previously depleted of PGE₂ with exogenous PGE₂ (triangle). In the presence of PHA the proliferation of T cells co-cultured with B7-1 expressing WM9 cells and PHA was inhibited in the presence of MCF-7 CM or in the presence of MCF-7 CM that had been depleted from PGE₂ and substituted with exogenous PGE₂. The proliferation of T cells in response to B7-1 expressing WM9 cells and PHA was restored when PGE₂ depleted MCF-7 CM was added.

**Tumor cells transduced to produce PGE₂ block B7-1 dependent T cell proliferation (Task 3).**

To further test the idea that tumor cells block B7-1 dependent T cell mediated immune responses primarily through the production of PGE₂, we generated PGE₂ producing and B7-1 positive melanoma WM9 cells by cotransduction with AdCOX-1 and AdhB7-1. Proliferation assays using T cells co-cultured with WM9 cells modified to express B7-1 and to produce PGE₂ were performed. Allogeneic T cells were stimulated to proliferate by co-culture with B7-1 expressing WM9 cells in the absence or presence of PHA (Fig. 6). However, the proliferation of T cells was inhibited when incubated with WM9 cells transduced to express B7-1 and COX-1. There was a four fold increase of PGE₂ in the five days supernatant of T cells co-cultured with B7-1, COX-1 expressing WM9 cells (538 pg/ml) as compared to the supernatant from T cells co-cultured with B7-1 expressing WM9 cells. The allogeneic T cell response of B7-1 expressing WM9 cells was completely restored by the co-culture of T cells with WM9 cells transduced with a mixture of AdhB7-1 and AdBgl2. These results clearly indicate that the
expression of COX-1 and the resulting production of PGE\(_2\) by WM9 cells powerfully inhibits B7-1 induced T cell proliferation.

**Figure 6.** Melanoma cells transduced to produce PGE\(_2\) block B7-1 dependent T cells proliferation. Thymidine incorporation by T cells co-cultured with WM9 cells co-expressing COX-1 and B7-1. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. The proliferation of T cells in response to B7-1 expressing WM9 cells was inhibited by the co-expression of COX-1 on the tumor cells.

**Tumor cell membranes inhibits a mixed lymphocyte reaction** (Task 4)
We prepared lipid soluble extracts of the tumor condition media but were unable to demonstrate that this fraction contained any inhibitory activity. At this point we believe that this is a technical issue resulting from air oxidation of the lipid fraction during isolation by extraction. However, we have found that membrane containing fractions of tumor cell debris are able to inhibit T cell activation in a mixed lymphocyte reaction. This activity appears to be associated with a protein, not a lipid, constituent of the tumor cell. We plan to pursue this further in our on going studies.
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Stephen L. Eck, M.D., Ph.D.

Discussion

The present study demonstrates that expression of the co-stimulatory molecule B7-1 on human MCF-7 breast carcinoma cells fails to elicit proliferative responses of allogeneic T cells. This is in contrast to human melanoma cells which, upon adenoviral transfer of B7-1, efficiently stimulate the proliferation of T cells(28). In addition, murine melanoma cells expressing B7-1 are rejected by syngeneic, immunocompetent mice (28, 32).

One possible cause for the observed failure of B7-1 expressing breast cancer cells to stimulate T cell proliferation is the release of immunosuppressive factors by these tumor cells. In support of this hypothesis we observed that CM obtained from eight out of ten breast cancer cell lines inhibited the proliferation of MN cells in response to PHA. In addition, soluble factors contained in CM derived from these breast cancer cell lines also inhibited the proliferation of MN cells stimulated with PMA/ionomycin. Tumor-derived TGFβ has been implicated in the progression of mammary carcinoma growth and metastasis by immune suppression. Anti-TGFβ antibody treatment inhibited breast cancer tumorigenicity and enhanced the activity of NK cells in an animal model of breast carcinoma (29).

Although we found that MCF-7 cells secrete TGFβ, it appears unlikely that TGFβ is the principal source of the inhibition of T cell proliferation. First, titration experiments revealed that the inhibitory factor contained in MCF-7 CM was active at dilutions up to 1:4,000. TGFβ is produced by MCF-7 cells at a rate of approximately 1 ng/1x10⁶ cells in a 24 h period. A 1:4,000 dilution of the MCF-7 CM would reduce the TGFβ content to concentrations far below the biologically active range. Furthermore, we found that the addition of neutralizing anti-TGFβ antibody to undiluted MCF-7 supernatant did not affect inhibition of T cell proliferation by MCF-7 CM. The concentrations of antibody used were far in excess of those needed to neutralize the effects of TGFβ in a sensitive TGFβ-responsive luciferase reporter assay.

In contrast, PGE₂ was shown to be an important contributor to the T cell inhibitory effect of breast cancer CM. Several lines of evidence support this conclusion. Indomethacin treatment of MCF-7 cells reduced PGE₂ production and partially alleviated inhibition of MN cell proliferation. Indomethacin did not completely remove the inhibitory effect which is consistent with the presence of residual PGE₂ that could be detected by LC-MS. More conclusive evidence for the involvement of PGE₂ in T cell growth inhibition was obtained when we removed PGE₂ from CM using an affinity column that specifically binds PGE₂. The selective elimination of PGE₂ from MCF-7 CM removed its MN cell growth inhibitory activity completely. Thus, inhibition of MN cell proliferation is mediated, at least in large part, by PGE₂ produced by MCF-7 cells. Furthermore, PGE₂ was detected in CM from additional breast cancer cell lines that also inhibited MN cell proliferation. The amount of PGE₂ production did not linearly correlate with inhibition of the proliferation of stimulated MN cells. CM from the immortalized, non-tumorigenic human breast epithelial cell line HBL-100 produced significant levels of PGE₂ (more than MCF-7) but did not suppress MN cell proliferation. Similarly, the CM from two cell lines (SUM149PT and SUM190PT) that produced the most PGE₂ showed only moderate inhibition of MN cell proliferation. Based on these results it appears likely that other factors in the breast cancer CM likely contribute to the immunosuppressive effect. In support of this notion we found that BT-20 cells did not produce significant amounts of PGE₂ but inhibited PHA-dependent proliferation of MN cells by 41%. This suggests that other tumor-derived factors may induce immunosuppression as previously
reported (33, 34). Nevertheless, seven of nine breast cancer cell lines produced PGE\(_2\), and all inhibited the proliferative response of MN cells to PHA. Taken together these data suggest that PGE\(_2\) is a necessary but not always sufficient cofactor of tumor-derived immunosuppression, acting in concert with other factors.

The mechanism(s) by which PGE\(_2\) affects MN or T cell proliferation are as yet fully understood. Intracellularly, PGE\(_2\) was found to attenuate p59fyn phosphorylation and its kinase activity, thus suppressing T cell proliferation during burn and sepsis (35). Both suppression of lymphokine production (20,21) and down regulation of MHC class II expression on antigen presenting cells (17,18) by PGE\(_2\) have been demonstrated. PGE\(_2\) inhibits IL-2 production and enhances IL-4 production by T cell, thus shifting a T helper cell 1 response towards a T helper cell 2 response (36). Recently, small cell lung cancer-derived PGE\(_2\) was found to up-regulate IL-10 production by lymphocytes and to down-regulate IL-12 production by macrophages (37) whereas in our study pure T cell cultures were used to investigate the effect of breast cancer derived PGE\(_2\). Furthermore, the expression of CD40L on human memory T cells was blocked by PGE\(_2\) (38). These studies suggest that PGE\(_2\)-dependent inhibition of T cell-mediated anti-tumor responses simultaneously affects antigen presentation and shifts the local cytokine milieu to a state unfavorable to an effective immune response (37).

The significance of tumor-derived PGE\(_2\) to inhibition of cells of the innate immune system, e.g., natural killer cells was highlighted by recent reports demonstrating therapeutic efficacy in the prevention and treatment of breast cancer by non-steroidal anti-inflammatory drugs (NSAID) including indomethacin itself (14). Importantly, indomethacin reduces PGE\(_2\) production by inhibiting the enzymatic activity of the COX-1 which is the rate-limiting enzymes in the PGE\(_2\) biosynthetic pathway. Indomethacin has also demonstrated efficacy in the treatment of mouse mammary carcinoma cells. For example, mice injected with mammary adenocarcinoma C3-L5 cells received long-term indomethacin therapy on day 15 followed by two rounds of IL-2 administration for five days. Regression of primary tumors, reduction of lung metastases and prolonged survival were observed in the group receiving the combination therapy as opposed to the group receiving IL-2 treatment alone. Furthermore, the long-term intake of indomethacin in combination with IL-2 was shown to activate tumoricidal lymphocytes in situ (14). Here we show that a combination strategy of B7-1 transfer onto breast cancer cells along with indomethacin treatment activated the proliferation of T cells in vitro.

B7-1 immunotherapy has been reported to be effective in immunogenic tumors (39). Tumor cells are generally termed “immunogenic” when they express antigens that result in the rejection of tumor cells by syngeneic animals previously immunized with the irradiated parental tumor cells (39). Accordingly, non-immunogenic tumors are rejected when similarly tested. However, there is no consensus about the parameters that render tumor cells more or less immunogenic. Breast cancer cells are known as non-immunogenic tumors and thus escape immune surveillance. Here, B7-1 immunotherapy was not successful in vitro in stimulating T cell proliferation. The majority of non-immunogenic breast cancer cell lines produce PGE\(_2\). We were able to show that the production of PGE\(_2\) specifically inhibited B7-1 induced T cell proliferation. First, B7-1 expressing MDA-MB 231 cells did not secrete PGE\(_2\) at detectable levels after indomethacin treatment and stimulated T cells to proliferate when PHA was present. Second, the elimination of PGE\(_2\) from MCF-7 CM restored the response of T cells to B7-1 expressing melanoma cells only in the presence of PHA indicating that the inhibition by PGE\(_2\) can only
be overcome when a strong signal through the T cell receptor was provided. Finally, in the melanoma system the sole modification of co-expression of COX-1 on B7-1 expressing WM9 cells was sufficient to inhibit T cell proliferation. These results underline the potential of a combined modification of breast cancer cells consisting of B7-1 expression and reduction of PGE$_2$ in order to induce T cell proliferation.

In summary, this study provides evidence that PGE$_2$ derived from human breast cancer cells can contribute to inhibition of cellular immunity in vitro. Since levels of PGE$_2$ are elevated in at least some breast cancers (40, 41), the production of PGE$_2$ may contribute to the impaired anti-tumor immune response. Reversal of tumor-induced immunosuppression offers a potential approach to cancer therapy and may be particularly useful in combination with immunotherapy against breast cancer.

**Key Research Accomplishments**

- Prostaglandin E2 production is a common but not universal finding in breast cancers
- Prostaglandin E2 production results in inactivation of T cell responses but is likely modified by other factors
- Inhibition of prostaglandin E2 production can restore T cell responsiveness
- Expression of prostaglandin E2 is sufficient to block T cell responses.

**Reportable Outcomes**

1. A manuscript is being prepared for submission to Cancer Research.
2. Heike Nesbit was awarded a Ph.D based on this worked which was a significant portion of her Ph.D. thesis.
3. Our findings that proteins in the membrane of breast tumor can inhibit an immune response is the basis of an grant application to further pursue this finding. ("GA733-2 Alters MHC Class II Antigen Processing", IDEA Award application to US Army)

**Conclusions**

We have previously reported that adenoviral transfer of B7-1 to human tumor cells elicits allogeneic T cell responses despite the production of potentially immunosuppressive factors by the tumor cells. In the present study we show that B7-1 expression on human breast cancer MCF-7 cells failed to stimulate effective T cell responses in vitro under comparable experimental conditions. We demonstrate that the failure of B7-1 expressing breast cancer cells to induce T cell proliferation was due in part to soluble immunosuppressive agents produced by the tumor cells. In addition, we provide evidence that tumor-derived PGE$_2$, but not TGFβ, was essential for curtailing T cell proliferation in this experimental setting. However, in PGE2 negative cells, transfection and overexpression of cyclooxygenase was sufficient to inhibit T cell responses. Furthermore, inhibition of PGE$_2$ synthesis or removal of PGE$_2$ restores the T cell response. These data suggest that pharmacologic intervention with COX inhibitors may be a useful adjunctive therapy to breast cancer vaccines. Finally, certain membrane proteins in breast cancer cells appear to block T cell responses. Further characterization of this effect will be pursued in year 2 of this award.
References


Appendix  Curriculum Vitae of Principal Investigator

UNIVERSITY OF PENNSYLVANIA - SCHOOL OF MEDICINE
Curriculum Vitae

June 1999

Stephen L. Eck, M.D., Ph.D.

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The University of Pennsylvania
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Education:

1971-1975 B.A. Kalamazoo College (Chemistry)
1975-1977 M.S. Harvard University (Chemistry)
1977-1981 Ph.D. Harvard University (Chemistry)
1983-1987 M.D. University of Mississippi School of Medicine

Postgraduate Training and Fellowship Appointments:

1981-1982 Senior Scientist, Monsanto Company, St. Louis, MO.
1982-1987 Res. Associate, Dept. of Biochemistry, Univ. Miss. School of Medicine, Jackson, MS.
1987-1988 Intern in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1988-1989 Residency in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1989-1992 Hematology/Oncology Fellow, Univ. of Michigan Hospitals, Ann Arbor, MI

Military Service: None

Faculty Appointments:

1981-1982 Instructor, St. Louis Community College
1992-1993 Lecturer In Internal Medicine, Division of Hematology/Oncology, University of Michigan.
1993-1994 Instructor, Division of Hematology/Oncology, University of Pennsylvania.
1994-pres. Ann B. Young Assistant Professor of Cancer Research, Division of Hematology/Oncology, Department of Medicine, Univ. of Pennsylvania.

Hospital and Administrative Appointments:

1992 Admissions Committee, The University of Michigan School of Medicine.
1992-1993 Home Infusion Service, Experimental Therapeutics Grant Review Committee. The University of Michigan School of Medicine
1993-present, Director, Gene Therapy Program, The University of Pennsylvania Cancer Center

Specialty Certification:

1990 Board Certified, American Board of Internal Medicine
1996  Board Certified, Hematology
1993  Board Eligible, Medical Oncology

Licensure:

Awards, Honors and Membership in Honorary Societies:
1972-75  Heyl Fellowship In Science, Kalamazoo College
1975  Honors Thesis, Kalamazoo College
1992-95  Merck-American Fed. for Clinical Research, M.D./Ph.D. Postdoctoral Fellowship
1994-pres.  Anne B. Young Assistant Professor for Cancer Research, July 1, 1994.
1995-96  Measly Fellowship Award
1998  University of Pennsylvania Nominee for Rita Allen Award.

Memberships in Professional and Scientific Societies:
Local Societies:
  Philadelphia Cancer Research Association
  Pennsylvania Chapter of the American Chemical Society
National Societies:
  American Association for the Advancement of Science
  American Federation of Clinical Research
  American Association for Cancer Research
  American Chemical Society
  The Brain Tumor Society
  American Society of Gene Therapy

Peer Review Activities
NIH, NCI PO1 Review, Boston, MA 6/25-27/95
NCI RFA Review Committee, Ad hoc reviewer 6/11-13/96
NIH, Neurosciences 3 Study Section, Ad hoc reviewer 6/26-28/96
NIH, NCI PO1 Review, New York, 7/7-9/96
NIH, NCI, Medicine Branch, Ad hoc reviewer, Wash. D.C. 9/10-12/96
NIMH PO1 Review, Washington, DC. Ad hoc reviewer, 12/96
NIH, NCI Ad hoc Reviewer 6/30/97
State of Massachusetts Breast Cancer Program 10/18-19/97
Chairman, Scientific Review Committee, NCI sponsored North American Brain Tumor Consortium (NABTC) and New Approaches to Brain Tumor Therapy (NABTT) consortium multigroup glioma gene therapy clinical trial. 1997-8
External Reviewer NCI PO1, Massachusetts General Hospital 5/98
NIH, NCI PO1 Review, Los Angeles, 7/27-29/98
State of Massachusetts Breast Cancer Program 10/24-25/98
NCI, Subcommittee D “Clinical Research Studies” 11/30-12/1/98
US Army Ovarian Cancer Study Section 1/20/99-1/22/99
NCI, RAID Review 3/31/99-4/1/99
NIH, Career Development Award Review 6/21-22/99

Editorial Positions:
Scientific Advisor, Education Committee, Pennsylvania Biotechnology Association, State College, PA 1995
Cancer Gene Therapy, Editorial Board, Simon & Schuster Publisher 1996-.
Gene Therapy, Editorial Board, Stockton Press. 1999-
Ad hoc reviewer for:

Principal Investigator of Grants:
Ligand Mediated Gene Therapy, American Federation for Clinical Research. 7/92-6/95, $150,000 (3 yrs)
Hepatic Gene Transfer for the Treatment of Metastatic Colon Cancer, Penn Home Infusion Therapy. 7/94-6/95, $23,520 (1 yr)
Stimulation of Breast Cancer Immunity by B7 Expression, 1UO1 CA 65805,
   Project 1 NIH. 8/94-12/98, $280,359 (year 3)
Phase 1 Trial of HSVTK Gene Therapy for CNS Tumors, RO1 CA 67799, 8/1/95 -7/31/99, $155,192 (renewal pending review)
Recombinant Adenovirus Vaccine for Colon Cancer 10/1/96-9/30/00, National Cancer Institute, 1RO1CA71915-01 $939,324 (4 yrs)
The CO17-1A/GA733 Antigen in Colorectal Cancer Therapy, 1 PO1 CA 74294-01, Project 2 leader, $218,577 (year 1)
   Cancer Program, 1997-2000, $75,000/yr
Gene Based Therapies and Imaging of Malignant Gliomas (pending review) NCI $299,973, 3/1/00 –2/28/04
The Mechanism of mEGP: How Tumor Cells Inhibit APCs, RO1 NCIS200,000 2000-2004 (pending review).
GA733 Alters MHC Class II Antigen Processing, US Army Breast Cancer Program, 1999 (pending review)

Academic Committees at the University of Pennsylvania:
Clinical Trials Scientific Review and Monitoring Committee, University of Pennsylvania Cancer Center 1996-present
University of Penn. General Clinical Research Center Internal Review Committee 1996-97
Faculty Grievance Commission 1997-2000
Molecular Life Sciences Advisory Committee 1998-present
Vagelos Scholars Advisory Committee 1998-present
Short Term Experience in Research Advisory Committee 1999
Major Teaching and Clinical Responsibilities at the University of Pennsylvania (last 3 yrs):

1993-1999 Attending Physician, Oncology & Hematology Services, Hospitals of the University of Pennsylvania.
1994-1999 Attending Physician, Oncology & Hematology Services, Philadelphia Veterans Administration Hospital.
1996 Critical Care Nurse Practitioner Course, "Hematology in the Critical Care Setting"
1995-1999 Selected Topics in Chemistry (Chemistry 700)
1996-1999 The Molecular Basis of Gene Therapy, (CAMB 610)

- Medicine 101C, Differential Diagnosis
  1997-1999 Introduction to Gene Therapy (CAMB 610, Fall)
  Course Director
  1997 Wistar Cancer Biology Graduate Student Seminar
  1997, 1998 Cancer Biology and Genetics Course (Pathology, Fall)
  1998, 1999 Topics in Cancer Pharmacology (PHARM, Fall 1998, 1999)

Lectures by Invitation:


April 25, 1995 "Gene Therapy", Medical Grand Rounds, Doylestown Hospital, Doylestown, PA.


May 26, 1995 "Adenovirus-Mediated Cancer Gene Therapy", University of North Carolina, Department of Medicine, Division of Hematology and Oncology Research Seminar Series. Chapel Hill, NC.


October 1, 1995  "Treatment of Primary CNS Tumors with Adenovirus Mediated Gene Transfer," The GAAC Meeting on Gene Therapy, Secon, Germany.

January 18, 1996  "Adenoviral-Mediated Therapy of Brain Tumors", The Preuss Foundation Meeting on Gene Therapy for CNS Malignancies, The Salk Institute, La Jolla, CA.

February 1, 1996  "Cancer Gene Therapy", Cooper Medical Center Medical Grand Rounds, Camden, NJ.

April 17, 1996  "Gene Therapy", The Estelle Lasko Memorial Lecture, The Twenty-fourth Annual Chester County Cancer Conference, Chester County Hospital, West Chester, PA.

April 19, 1996  "Gene Therapy for Inherited and Acquired Diseases", Genetics in the Cause and Treatment of Malignancies Conference, Sacred Heart Hospital, Allentown, PA.

May 13-7, 1996  "Laboratory and Clinical Approaches to Cancer Gene Therapy" 1996 Short Course in Cancer Biology, University of Nebraska Medical Center, Omaha, NE.


July 11, 1996  "Cancer Gene Therapy" Shering-Plough Corporation, Kenilworth, NJ.


January 9, 1997  "Adenoviral Vectors for the Gene Therapy of Cancer", Wayne State University, Center for Molecular Medicine and Genetics, Detroit, MI.

March 7, 1997  Gene Therapy for Gliomas and Colon Cancer, University of South Carolina, Department of Microbiology, Charleston, SC.

April 25, 1997  "Colon Cancer Vaccines", Megabios Corporation, Burlingame, CA.


Aug. 7, 1997  "Gene Therapy of Malignant Gliomas" Cancer Section, Gordon Conference, Newport, RI.


Oct. 20, 1997  "Experimental Therapies for Malignant Gliomas", Pathology Grand Rounds, Suburban General Hospital, Norristown, PA.


March 28, 1998  "Phase I Trial of Gene Therapy for Primary Brain Tumors", Cerebral Vascular Biology 1998 Conference, Portland (Lincoln), OR.
June 29, 1998  "Phase I Trial of Gene Therapy in Primary Brain Tumors", American Society of Gene Therapy, Seattle, WA.


August 18, 1998 "New Developments in Cancer Gene Therapy", Grand Rounds, Chestnut Hill Hospital, Philadelphia, PA

May 16, 1999  "Enzyme-Prodrug Gene Therapy for Cancer", American Society of Clinical Oncology Meeting, Atlanta GA.

June 12, 1999  "mEGP Blocks Class II Antigen Presentation" American Society of Gene Therapy, Washington D.C.

June 18, 1999  "Imaging Cancer Gene Therapy with PET" 25th Annual Pendergrast Symposium, Department of Radiology, University of Pennsylvania, Philadelphia, PA

Bibliography:

Original Papers


18. H. K. E. Boxhorn, M. Jost, U. Rodeck, S. L. Eck. Human breast cancer cell lines inhibit the proliferation of human peripheral blood mononuclear cells by PGE2 and other immunosuppressive factors 1999 (submitted in revision)


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