Rapamycin and PF4 Induce Apoptosis by Upregulating Bax and Down-Regulating Survivin in MNU-Induced Breast Cancer

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Abstract

Background: To elucidate the role of rapamycin and PF4 on apoptosis regulation via Bax (pro-apoptosis), Bcl-2 (anti-apoptosis) and survivin activation on the growth in the 1-methyl-1-nitrosourea -induced invasive breast carcinoma model. Materials and Methods: Thirty five female Sprague Dawley rats at age 21-day old were divided into 4 groups; Group 1 (control, n=10), Group 2 (PF4, n=5), Group 3 (rapamycin, n=10) and Group 4 (rapamycin+PF4, n=10). MNU was administered intraperitionally, dosed at 70mg/kg body weight. The rats were treated when the tumors reached the size of 14.5±0.5mm and subsequently sacrificed after 5 days. Rapamycin and PF4 were administered as focal intralesional injections at the dose of 20 μg/lesion. The tumor tissue was then subjected to histopathological examinations for morphological appraisal and immunohistochemical assessment of the pro-apoptotic marker, Bax and anti-apoptotic markers, Bcl-2 and survivin. Results: The histopathological pattern of the untreated control cohort showed that the severity of the malignancy augments with mammary tumor growth. Tumors developing in untreated groups were more aggressive whilst those in treated groups demonstrated a transformation to a less aggressive subtype. Combined treatment resulted in a significant reduction of tumor size without phenotypic changes. Bax, the pro-apoptotic marker, was significantly expressed at higher levels in the rapamycin-treated and rapamycin+PF4-treated groups compared to controls (p<0.05). Consequently, survivin was also significantly downregulated in the rapamycin-treated and rapamycin+PF4-treated group and this was significantly different when compared to controls (p). Conclusions: In our rat model, it could be clearly shown that rapamycin specifically affects Bax and survivin signaling pathways in activation of apoptosis. We conclude that rapamycin plays a critical role in the induction of apoptosis in MNU-induced mammary carcinoma.

Keywords: Breast cancer - apoptosis - Bax - Bcl-2 - survivin - rapamycin

Asian Pac J Cancer Prev, 15 (9), 3939-3944

Introduction

Breast cancer is the commonest malignancy in women and the second leading cause of cancer deaths. The latest report from Ma et al, in 2012, an expected 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths are predictable to arise among U.S. women (Hassan et al., 2013). While the incidence and mortality of breast cancer rates over the past 20 years, have been rising speedily in economically less developed regions. Based on the report from GLOBOCAN in 2008, half of the new worldwide breast cancer cases (1.38 million) and 60 % of breast cancer deaths (458,000) occurred in developing countries (GLOBOCAN 2008 (IARC) 2008).

In Malaysia, breast cancer is the most common cancer in women. About 3,242 cases of breast cancer were diagnosed in women in 2007, making up 18.1% of all reported cancer cases and 32.1% of all women-related cancer (National Cancer Registry Report 2007) which affects all Malaysian principal ethnicities (Yip et al., 2006).

Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is a strong evidence that tumor growth is not just a result of uncontrolled proliferation, but also of reduced apoptosis (Hahm, 1998; Hengartner, 2000). The balance between cellular proliferation and apoptosis is crucial in determining the overall growth or regression of the tumor in response to cancer therapies (Signoreet al., 2013; Wan et al., 2014).

Apoptosis or programmed cell death is an active, energy-dependent process of cell death, which occurs during normal morphological development, in response to certain physiological stimuli, and secondary to cell injury.

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Thirty five females (35) Sprague Dawley (SD) rats were obtained from the Animal Research and Services Centre of Science University of Malaysia (ARACS). The ethical clearance for using experimental animals was obtained from the USM’s Animal Ethics Committee [PPSG/07 (A) /044/(2010) (56)]. The rats were housed and maintained at the Animal House Unit. Caging and rat handling were performed in accordance with good laboratory practice criteria set by the Animal House. The rats were caged in groups of three and were fed with a standard laboratory diet and water (Jaafar et al., 2009; Thompson et al., 1995). The SD rats were divided into 4 groups and each group was given different interventions: a control (untreated) group 1 [n=10], rapamycin-treated group 2 [n=10], Platelet Factor 4-treated group 3 (PF4) [n=5] and combined rapamycin and PF4-treated group 4 (rapamycin+PF4) [n=10].

MNU, rapamycin & PF4 preparation and tumor induction
MNU was provided by Sigma Aldrich. The crystallized form of MNU was dissolved in freshly-prepared 0.9% normal saline prior to tumor induction. The MNU was injected intraperitoneally at a dose of 70 mg/kg body weight into 21-day-old rats (Jaafar et al., 2009). The rats were weighed and palpated for mammary tumor lesions weekly. The onset of tumors was monitored by daily visual inspection and palpation of the mammary regions. The increase in tumor size was monitored by measuring the subcutaneous mass using vernier caliper. Rapamycin was dissolved in absolute ethanol and diluted in mixtures of 10% polyethylene glycol (PEG) –400, 8% Ethanol: 10% Tween-80 to a final concentration of 20 µg/0.2ml, while PF4 was dissolved in physiological saline to the same final concentration of rapamycin. From 30 days post-MNU administration until study termination, rats were palpated twice weekly and measured using a digital vernier caliper for tumor size quantification. Normal mammary glands were obtained from virgin female Sprague Dawley rats (n=5) whose age were approximately around 13 weeks old—the average age of tumor-bearing animals at excision.

Experimental design
Malignant mammary gland lesions were monitored for 90 days post-MNU injection. All rats were randomly assigned to four groups after lesions reached a mean tumor diameter of 14.5±0.5 mm. The rats in group 1 (n=10) served as control cohort. Physiological normal saline was injected by a single intralesional injection for the vehicle into each lesion (n=10) at size 14.5±0.5 mm in the control group. Meanwhile, the rats in group 2 (n=10), group 3 (n=5) and group 4 (n=10) were treated with rapamycin, PF4 and rapamycin+PF4, respectively. Rapamycin was dissolved in absolute ethanol and diluted in mixtures of 10% PEG-400, 8% Ethanol: 10% Tween-80 of 20 µg/0.2ml, while PF4 was dissolved in physiological saline to the same final concentration of rapamycin (R. Namba et al., 2006). Each solution was freshly prepared prior to injections. Rats were anesthetized intraperitoneally with a mixture of ketamine-HCl and xylazine (100 and 10 mg/kg, respectively) immediately before injecting allotted interventions. Both groups received a focal intralesional injection at the dose of 20 µg/lesion twice daily for

Materials and Methods
Animal procedures
Thirty five females (35) Sprague Dawley (SD) rats

consecutive days until lesions consistently decreased in 5 days. In synergistic tumor suppression arm, the rats in group 4 (n=10) received a focal intraslesional injection of rapamycin followed by PF4 on consecutive days at the dose of 20 µg/lesion for each injection. The injections were again given on a daily basis until lesions reach their final sizes at day 5 post injection. In the negative control [group 5 (n=5)], all rats received no treatment and they represent the normal physiology of rats’ mammary pad. All rats were subsequently sacrificed by euthanization via exposure to carbon dioxide in a closed chamber.

Tumor sample collection
Breast tumor specimens were collected when the tumor reached the size (14.5±0.5mm) at which the rats were sacrificed and grouped accordingly into control and intervention groups. Rats were sacrificed using gaseous CO2 inhalation, provided by the ARACS. At necropsy, the rats were skinned and the dissected skins with the tumors intact were photographed to record tumor location and size. All palpable tumors were carefully excised and fixed in 10% formalin for Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) stainings.

Immunohistochemistry analysis
The IHC staining was performed to demonstrate the expression of each apoptotic marker separately: monoclonal Bax (diluted at 1:200 (µl); Santa Cruz Biotechnology), polyclonal Bcl-2 (diluted at 1:100 (µl); Santa Cruz Biotechnology) and monoclonal survivin(diluted at 1:50 (µl); Santa Cruz Biotechnology) and monoclonal Bax (diluted at 1:200 (µl); Santa Cruz Biotechnology). A standard-labelled streptavidin biotin (Dako, Glostrup, Denmark) method was used on formalin-fixed paraffin-embedded tissue sections. The tissue blocks were trimmed and sectioned with microtome (Leica, Wetzlar, Germany) to obtain 3-5-µm thick sections which were later deparaffinised in xylene and dehydrated. Slides were then pretreated with Tris EDTA buffer (10mM, pH 9.0) for 14 min and heated in a microwave oven. Subsequently, all the sections were treated for 10 min with peroxidase blocking reagent (DAKO Glostrup, Denmark) to quench the endogenous peroxidase activity, and then incubated with primary antibodies, followed by rinsing with Tris buffered saline (pH 7.2). The sections were incubated for 30 min with optimally diluted biotinylated secondary antibody and for 30 min with horseradish peroxidase before it is ready for use. For visualization, the slides were immersed in diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) substrate for 5 min, followed by washing in distilled water. The slides were then counterstained with Harris hematoxylin, dehydrated and mounted. To assess the specificity of the reactions, gastric adenocarcinoma, tonsillar tissues and colon CA were used as positive controls for Bax, Bcl-2 and survivin expressions, respectively. Negative controls (incubation without the primary antibody) were also used for this purpose.

Immunohistochemistry Scoring System (ISS)
The light microscope (Nikon, Japan) was used to examine the immunohistochemistry stained breast cancer slides. We first scored the expression and immunostaining of Bax, Bcl-2 and survivin according to the procedures as recommended by Assanuma et al. (2005). Bax, Bcl-2 and survivin immunoreactivities were evaluated semiquantitatively according to the percentage of cells showing distinct diffuse cytoplasmic immunohistochemical reaction. Cytoplasmic immunoreactivities were assessed in at least five high-power fields at x400 magnification and assigned to one of the following categories: 0=<5%; 1=5% to 20%; 2=>20%. Since tumor cells showed heterogeneous staining, the dominant pattern was used for scoring. However, we further grouped the scores obtained into two categories.

Staining intensity was scored on a positive scale (positivity), ranging from 0 to 2 where, 0 was equivalent to no staining, 1+ weakly stained, and 2+ moderately to strongly stained.

Statistical analysis
Data was descriptively presented in mean (SD) / median (IQR) and frequency (percentage). The differences in terms of immunohistochemical expression across all experimental groups were determined by the Kruskal–Wallis test followed by Mann-Whitney test with Bonferroni correction for multiple comparisons. All statistical analyses were carried out using Statistical Product and Service Solutions (SPSS) version 20 (IBM, New York USA).

Results

Type of tumor in intervention groups
The histopathology of the untreated control cohort showed that the severity of the malignancy worsens as the mammary tumor growth. Tumors developed in untreated groups behaved more aggressively [Infiltrating ductal carcinoma, Not otherwise specified (IDC, NOS) and papillary carcinoma] whilst tumor in treated groups transformed to a less aggressive (cribriform carcinoma) subtype. Combined treatment resulted in significant reduction of tumor size without phenotypic tumor changes (Table 1).

Histology of MNU-induced mammary carcinoma
The majority of the carcinoma induced were the cribriform type in all experimental groups. The cribriform carcinoma displayed epithelial clusters surrounded by intense desmoplastic reaction and lymphocytic infiltration (Figure 1A). The other phenotype was papillary carcinoma that displayed numerous papillary projection with thin fibrovascular core (Figure 1B). IDC, NOS infiltrated the breast tissue diffusely and in cords and clusters (Figure 1C). The IDC, NOS carcinoma cells have
large, pleomorphic nuclei with prominent nucleoli and moderate cytoplasm. Mitotic figures were easily seen. The surrounding stroma showed desmoplasia (Figure 1D).

**Figure 1. Representative Histology Staining Analysis of Normal Mammary Gland and Invasive Tumor in MNU-Induced Breast Carcinoma.** Cribriform carcinoma was from control untreated group displaying the epithelial clusters are surrounded by intense desmoplastic reaction and lymphocytic infiltration A). Papillary carcinoma was from the rapamycin-treated group when the tumor regressed displaying numerous papillary projections with thin fibrovascular core B). Invasive Ductal Carcinoma (IDC, NOS pattern, was from . Papillary carcinoma was from the . The Expression of Bax, Bcl-2 and Survivin Amongst the Intervention Groups

**Figure 2. Immunohistochemical Expressions of Representative Markers on Tumor Specimens.** A) Cribriform carcinoma positive for Bax. B) Negative staining of Bax in cribriform carcinoma. C) Papillary carcinoma positive for Bcl-2 D) positive staining of survivin in cribriform carcinoma. (Original magnification x200)

**Table 1. The Expression of Bax, Bcl-2 and Survivin Amongst the Intervention Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases (n)</th>
<th>Cytoplasmic localization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>p-value</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85.5 (11.3)</td>
<td>50 (23.8)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>85.5 (11.3)</td>
<td>50 (23.8)</td>
</tr>
<tr>
<td>PF4</td>
<td>85.5 (11.3)</td>
<td>50 (23.8)</td>
</tr>
</tbody>
</table>

**Table 2. The Expression of Bax, Bcl-2 and Survivin between Intervention and Control Groups**

**Table 3. The Expression of Bax, Bcl-2 and Survivin Amongst the Intervention Groups**

**Table 4. Intensity of Bax, Bcl-2 and Survivin Immunostaining Expression in Control and Intervention Groups**

**Expression of Bax, Bcl-2 and survivin between intervention and control groups (Table 2)**

There were significant increases in Bax expression in both rapamycin and rapamycin+PF4 groups compared to control (p=0.001 and 0.049, respectively) (Table 2). Bax expression was also significantly higher in all PF4-treated groups (p=0.007 and p=0.003, respectively). On the other hand, Bcl-2 showed similar expression in both groups (p>0.05).

**Expression of Bax, Bcl-2 and survivin amongst the intervention groups (Table 3)**

Survivin expression was significantly reduced in all groups. There was a significant median difference in terms of survivin expression score between PF4 and rapamycin-treated groups (p=0.003) (Table 2). However, Bcl-2 and Bax expression was not significantly different between both groups (p value>0.05) (Table 3).

The addition of PF4 to rapamycin causes a significant increase in Bax expression compared to rapamycin group (rapamycin+PF4 vs rapamycin: 80.5 vs 62, p value
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Survivin was found to be highly expressed in almost all malignancies, but was rarely detected in normal differentiated adult tissues. Our result shows that survivin was highly expressed in treated groups. In contrast, Jha et al., (2012) theorized that survivin inhibits apoptosis, promotes cell proliferation and enhances angiogenesis, cementing its key role in cancer progression. In neuroblastoma cancer model, the expression of survivin was increased when induced by rapamycin (Samkari et al., 2012). However the relationship between both proteins is one of antagonistic. Bax promotes apoptosis whilst survivin inhibits apoptosis (Huang et al., 2012).

Nevertheless, different survivin isoforms may have different effects on apoptosis. For example, (Verdecia et al., 2000) reported that while survivin-ΔEx3 retained its anti-apoptotic function in transfected renal cancer cells, survivin-2B had reduced capacity for inhibiting apoptosis. On the other hand, obligatory expression of survivin-2B was found to inhibit cell growth and sensitize leukemic cells to doxorubicin-induced apoptosis (Zhu et al., 2004).

From these findings, the authors recommended that the 2a form of survivin may also be pro-apoptotic or at least be able to attenuate the anti-apoptotic effects of survivin from other isoforms.

In conclusion, our study provides compelling experimental evidence that the Bcl-2 family, pro-apoptotic protein Bax and survivin play a critical role in mediating the mechanism of cell death induced by rapamycin and PF4. Our data support the hypothesis that rapamycin and PF4 up-regulates pro-apoptotic programmed cell death in cells that are unable to undergo Bax/Bcl-2-mediated apoptotic cell death and that apoptosis activity can be increased by attenuating Bax expression in breast cancer cells. This can be further enhanced by the direct promotion of apoptosis using the rapamycin+PF4 alone or in combined. This is obviously a novel addition to the existing literature since for the first time rapamycin was shown to exert its anti-tumor effects on another pathway that is independent of mTOR in breast cancer model. These strategies, which are highly focusing on and targeting the apoptotic pathways deserve further investigation in advanced animal models of breast cancer.
References


