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Original article

Pentoxifylline regulates the cellular adhesion and its allied receptors to extracellular matrix components in breast cancer cells

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ABSTRACT

Pentoxifylline (PTX) is a methylxanthine derivative that improves blood flow by decreasing its viscosity. Being an inhibitor of platelet aggregation, it can thus reduce the adhesiveness of cancer cells prolonging their circulation time. This delay in forming secondary tumours makes them more prone to immunological surveillance. Recently, we have evaluated its anti-metastatic efficacy against breast cancer, using MDA-MB-231 model system. In view of this, we had ascertained the effect of PTX on adhesion of MDA-MB-231 cells to extracellular matrix components (ECM) and its allied receptors such as the integrins. PTX affected adhesion of breast cancer cells to matrigel, collagen type IV, fibronectin and laminin in a dose dependent manner. Further, PTX showed a differential effect on integrin expression profile. The experimental metastasis model using NOD-SCID mice showed lesser tumour island formation when treated with PTX compared to the control. These findings further substantiate the anti-adhesive potential of PTX in breast cancer and warrant further insights into the functional regulation.

1. Introduction

> "The nontoxic curative compound remains undiscovered but not undreamt".
>
> James F. Holland

Cancer is a major health concern in the global scenario, accounting for the second most leading cause of deaths among all ages and sexes [1]. The majority of cancer related deaths are due to the dissemination of tumour cells from the primary to distant target sites, a process referred to as metastasis. It is indeed a very complex phenomenon and thus targeting metastasis holds a great promise for anticancer therapy. During the process of metastasis, tumour cells need to invade their surroundings and surpass these barriers. The ability to alter cellular migration and adhesion to the extracellular matrix (ECM) components helps the tumour cells to bypass these obstructions, paving a passage into circulation for distant localisation. Cellular adhesion is an important biological phenomenon required for the functioning of living organisms. The cells transmit signals from their environment via different surface receptors that act as the relaying switches. One of the crucial members among this family are the integrins that mediate both cell–cell as well as cell to ECM interactions. A typical integrin is a heterodimer made up of α and β subunits linked non-covalently. A total of 18 α and 8 β subunits have been identified till date that forms a total of 24 αβ distinct combinations [2]. The normal functioning of an organism requires the regulated coordination between the cells as well as their extracellular milieu. These interactions play a very important role in diverse aspects of cellular behaviour such as cellular adhesion, proliferation, apoptosis and angiogenesis.

Binding of ECM molecules/ligands to the integrins leads to clustering and subsequently promotes the localised aggregation of signalling moieties culminating in downstream intracellular signalling. Conversely, cytoplasmic signals modulates the integrin functioning and thus regulate cellular dynamics. Hence, integrins orchestrate both outside–inside and inside–outside signalling events. Extracellular ligation of integrins triggers a large variety of signal transduction events such as proliferation, invasion, migration, survival or apoptosis angiogenesis, immunity and homeostasis [3–6].

Numerous studies have documented marked differences in surface expression and distribution of integrins in malignant breast tumours compared with pre-neoplastic tumours of the same type. Integrins such as αv, α3, α5, β1, and αvβ3 are being involved in the process of cancer progression [7]. A large number of reports are suggestive of the correlation between integrin expression and breast cancer metastasis. Integrin α3β1 is

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associated with the process of tumourigenesis and invasion [8] while αvβ3, αvβ5, α5β1 are associated with breast cancer progression [3]. Expression of α5β1 is highly up regulated in drug resistant breast carcinoma cells and associated with the ability to regulate invasion via MMP-2 [9]. Moreover, αvβ3 is associated with Mitogen Activated Protein Kinase (MAPK) signalling affecting proliferation [10] while β1 is an indicator for survival in breast cancer [11]. However, α2β1 has been shown to play a key role of metastasis suppressor in a spontaneous model of breast carcinoma [12].

Pentoxifylline (1-[5-oxohexyl]-3,7-dimethyl-xanthine], oxpentifylline, PTX) is a methylxanthine derivative that elevates c-AMP by inhibiting the phosphodiesterase activity. It is being used clinically for the treatment of intermittent claudication and peripheral vascular diseases [13]. PTX has been shown to inhibit the integrin-mediated adherence of IL-2 activated human peripheral blood lymphocytes to human endothelial cells, matrix components and tumour cells [14]. It also inhibited the integrin-mediated adhesion and activation of human T lymphocytes [15]. Further, we have earlier shown in our laboratory that PTX modulates integrin expression in B16F10 mouse melanoma cells [16]. Based on these observations, we propose to investigate the effect of PTX on the adhesion of human breast MDA-MB-231 cells to ECM components as well as its allied receptors, integrins. Further, we have also demonstrated the effect of PTX *in vivo* using experimental metastasis model. These studies surely scores PTX to be a promising agent for targeting metastasis in breast cancer.

2. Material and methods

2.1. Reagents and antibodies

Pentoxifylline, matrigel, collagen type IV, fibronectin, laminin, vitronectin, MTT, beta-tubulin, bovine serum albumin (BSA) and paraformaldehyde were purchased from Sigma-Aldrich (India). Antibodies against integrins such as αv, α2, α3, α5, β1, β3 and β5 were purchased from Santa Cruz (CA, USA). Fluorescein isothiocyanate (FITC) labeled secondary antibodies were acquired from Invitrogen (India). All the other chemicals/reagents used were either of analytical grade or highest purity that were commercially available.

2.2. Cell culture

MDA-MB-231 and MCF-7 human breast cancer cell lines were purchased from NCCS (Pune, India). The cells were maintained in 10% Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% heat inactivated foetal bovine serum (FBS, GIBCO). Primocin was added at a concentration of 100 μg/mL. The cells were maintained at 37 °C in 5% CO_2 humidified atmosphere as described earlier [17].

2.3. Adhesion assay

Adhesion of breast cancer cells to different to ECM components was proceeded as per the protocol described earlier [17,18]. Briefly, 96 well plates were pre-coated with matrigel (10 μg/mL), collagen type IV (10 μg/mL), fibronectin (2.5 μg/mL), laminin (5 μg/mL) and vitronectin (2.5 μg/mL). The plates were then kept at 4 °C for polymerisation overnight. Subsequently, the plates were washed with PBS and then treated with 1% BSA. MDA-MB-231 cells treated with sub-toxic doses of PTX (0 mM, 1 mM, 2.5 mM and 5 mM) were then harvested using saline EDTA. Cells were then seeded at a density of 3×10^4 per well suspended in 0.1% BSA containing plain DMEM. A percentage relative adhesion was then calculated for PTX treated cells at different time points viz. 15, 30, 45, 60 and 90 min. Differences in adhesion to ECM components was also compared between MDA-MB-231 and MCF-7 cells using the above mentioned substrates at different time points *viz.* 15, 30, 45, 60 and 90 min respectively.

2.4. Flow cytometry

Surface expression of integrins *viz.* αv, α2, α3, α5, β1, β3 and β5 was compared between MDA-MB-231 as well as MCF-7 cell lines. The effect of PTX at sub-toxic doses was then evaluated on MDA-MB-231 cells. Briefly, cells were harvested using saline EDTA and then fixed using 4% paraformaldehyde. Approximately 1×10^6 cells were treated with 1 μg of the respective integrin (αv, α2, α3, α5, β1, β3 and β5) antibody. Cells were mixed thoroughly and kept at 4 °C for an hour. Subsequently, cells were washed using fluorescence activated cell sorting (FACS) buffer twice and then incubated with FITC labelled secondary antibody for 1 h at 4 °C. Cells were washed twice using FACS buffer and then finally suspended in it. Acquisition was done on FACS Calibur and the results were analysed using Cell Quest software as done earlier [19].

2.5. Western blotting

Western blotting was carried out as per the procedure followed earlier [20]. Cells treated with sub-toxic doses of PTX were harvested using saline EDTA and then lysed using modified Radio-Immuno Precipitation Assay (RIPA) buffer. Fifty micrograms protein were then loaded on an 8% SDS-PAGE gel and then run at constant current of 25 mA. After, the electrophoresis the protein was electro-blotted onto PVDF membranes overnight at a constant voltage of 20 V. Membranes were later blocked using 5% BSA for 1 h. Subsequently, the membranes were incubated with primary (αv, α2, α3, α5, β1, β3 and β5) and secondary (HRP labelled) antibodies for 1 h each. Washings were done using Tris-Buffered Saline-Tween 20 (TBST) after an each successive step. β-tubulin was used as a loading control. The membranes were finally processed for signal detection using Pierce Femto Chemiluminesence system.

2.6. In vivo experimental metastasis

The animal experiments were performed after the approval from the ethical clearance committee at ACTREC. Briefly, 1×10^6 cells were injected intravenously (IV) into the tail vein of 6–8 weeks old female NOD-SCID mouse. Three groups ($n = 5$) were being formed:

- PBS only;
- PTX 40 mg/kg;
- PTX 60 mg/kg.

PTX was injected intraperitoneally (i.p) from day 1 to day 9 continuously. Animals were sacrificed when found to be moribund. The lungs were being excised and then fixed in buffered formalin solution. The tissues were then sectioned and stained using Haemotoxylin-Eosin as done earlier [18]. Images were then captured using an upright microscope from Zeiss (Germany) at 10 × magnification.

2.7. Chick Chorioallontic Membrane (CAM) assay

Fertilized eggs from White Leghorn hen were placed in an incubator at 37 °C under approximately 70% humid conditions. The eggs were then kept at 37 °C for 5 days prior to PTX treatment. PTX (400 μg) was added through a small window made using a sterile scalpel. The eggs were then resealed using parafilm and incubated