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Anti-tumor activity of staurosporine in the tumor microenvironment of cervical cancer: An *in vitro* study

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ABSTRACT

Aim: The fundamental events for cancer progression and metastases include loss of cell adhesion, cell proliferation, anchorage-independent cell growth (evading anoikis), cell migration and cell invasion. All these events leading to cancer progression happen in a favorable nurturing tumor microenvironment. This study was designed to explore the anti-tumor activity of staurosporine (a nonspecific protein kinase inhibitor) in the tumor microenvironment of cervical cancer.
Main methods: The anti-tumor activity of staurosporine was investigated by cell adhesion assay, colony formation assay, apoptosis assay and quantitative real-time polymerase chain reaction (PCR) in cervical cancer cell lines.
Key findings: The cell adhesion assay showed that staurosporine induces adhesion of cervical cancer cells to the extracellular matrix (ECM) protein fibronectin. The soft agar colony formation assay showed that staurosporine inhibits both the number and size of colony formation in a dose dependent manner and also induces adherent tendency in the cancer cells. Staurosporine also induces prominent apoptosis in single cell suspensions compared to adherent cells. Stroma cell induced transcription of matrix metalloprotease 1 (MMP1) and matrix metalloprotease 2 (MMP2) in cervical cancer cells was inhibited by staurosporine.
Significance: Our results indicate that staurosporine induces anti-tumor response in the cervical tumor microenvironment by inhibiting the fundamental events for cancer progression and metastases. The present study represents an attractive area for further research and opens up new avenues towards the understanding of cervical cancer therapeutics.

1. Background

Metastases are the primary cause of death in cancer patients. Cancer progression is a multistep process involving its initiation and progression [26]. Processes involved in tumor initiation and progression include loss of cell adhesion, anchorage-independent cell growth, and hydrolysis of the ECM leading to cell migration and invasion. A growing body of evidence indicates that neoplastic cells often show a loss of cell–cell and/or cell–matrix adhesion which allows these cells to escape from their site of origin, degrade the ECM, and acquire motile and invasion phenotypes leading to metastases [4,34]. These abnormal neoplastic cells evade the programmed cell death (anoikis) induced by inappropriate cell–cell and cell–ECM attachment leading to metastases [15]. There are a number of studies demonstrating the key involvement of MMPs in tumor invasion and metastases [14,16,28]. Protein kinases are also involved in these processes to support the survival and metastases of cancer cells [21,27]. Protein kinase C (PKC) is an important member of the protein kinase family regulating cell growth, proliferation, differentiation, apoptosis, and mobility [6,37]. An enhanced level of PKC in tumors has been correlated with promotion of the invasiveness and metastases [18]. It has been shown that significantly higher expression of PKC-α in lung cancer and its cytomembrane transportation play critical roles in regulating the progression and metastasis of lung cancer cells [30,41]. Staurosporine is an alkaloid isolated from the bacterium *Streptomyces staurosporeus* in 1977 [35]. Although staurosporine was described as an adenosine triphosphate (ATP) competitor and a potent PKC inhibitor [20], it has also been reported that staurosporine is a nonspecific inhibitor of a diverse array of different kinases [29]. Staurosporine blocks the transfer of phosphodiester bonds from DNA to active site tyrosine residues of topoisomerase II leading to inhibition of its enzyme activity [23]. Moreover, staurosporine induces apoptosis in a variety of cancer cells [10,43] including cervical cancer [3]. Staurosporine has been well studied in the context of apoptosis in many cancers; however, little information on the role of staurosporine in the inhibition of tumor promoting events in cervical carcinogenesis

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is available. This study was designed to investigate the effect of staurosporine on different hallmarks of cervical cancer with reference to its tumor microenvironment. In this study, using different concentrations of staurosporine we addressed its effect on different cancer promoting events in the tumor microenvironment of cervical cancer such as loss of cell adhesion, anchorage-independent cell growth and stroma cell induced expression of MMPs.

2. Materials and methods

2.1. Cervical cancer cell lines and normal cervical tissues

Cervical cancer cell lines (HeLa, SiHa and C-33A) were kind gifts from Dr. VVS Murty, Columbia University, New York, USA and have been previously characterized [17,32]. Normal cervical tissues (NC65 and NC66) taken from the joint of the ectocervix and endocervix were collected from patients undergoing hysterectomy at Sir Sunderlal Hospital, Banaras Hindu University, Varanasi, India, after written informed consent of the patients as per the approved protocol by the institutional ethical committee of the Institute of Medical Sciences, Banaras Hindu University. Both the NC tissues were treated with collagenase (150 units/ml for 5 h) to make single cell suspensions and cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) (HIMEDIA, India). Cell lines and cultured normal cervical cells were maintained in DMEM supplemented with 10% fetal bovine serum (GIBCO, USA), streptomycin, and penicillin at 37 °C in a humidified CO_2 incubator (Thermo Fisher Scientific, UK) containing 5% CO_2. Conditioned media of NC65 and NC66 were prepared by harvesting the spent media of 50–60% confluent cells followed by brief centrifugation to remove cell debris.

2.2. Cell adhesion assay

Cell adhesion assays were performed using a Vybrant Cell Adhesion Assay Kit (Molecular Probes) in fibronectin (7 μg/cm^2) coated 96 well culture plates as per the recommended protocol. Briefly, cells were harvested, washed with phosphate buffered saline (PBS), re-suspended in incomplete media with 5 μM Calcein AM dye and incubated for 30 min at 37 °C. Cells were washed and re-suspended in complete media with or without staurosporine, followed by seeding of the cells in fibronectin coated 96 well plates in triplicate with cell density of 5×10^4 cells/well. Cells were incubated for 15 min in optimum culture conditions for cell adhesion. Non-adherent cells were washed with PBS and adherent cells were quantified by fluorescence measurement by a fluorescence microplate reader (BioTek, USA) at 494 ± 20 nm wavelength. For all the sets of experiments, cells used were in similar growth conditions (semi-confluent). All the experiments were performed in triplicate and repeated three times independently. Results are presented as percent cell adhesion using the formula: (relative fluorescence unit of adherent cells × 100) / relative fluorescence unit of total cells seeded.

2.3. Soft agar colony formation assay

The anchorage-independent growth of cervical cancer cell lines, HeLa and SiHa, was estimated by the soft-agar colony formation assay as described previously [1]. A top agar layer with 0.36% of agar in 2 ml complete DMEM was plated over a comparable hard bottom layer of 0.75% agar in 2 ml complete DMEM per well of a 6 well culture plate. Single cell suspensions of 2×10^4 cells were incubated in the top layer with or without staurosporine for ten days resulting in colony formation. Colonies were photographed, and the number and size of the colonies were quantified by the NIS-Elements Analysis D 4.20.00 lnk software.

2.4. Apoptosis assay

For the apoptosis assay in adherent cells, HeLa and SiHa monolayer cells were exposed to different concentrations of staurosporine (4 nM, 16 nM, 200 nM) for 4 h at standard culture conditions. For the apoptosis assay in cell suspensions, cells were harvested and incubated with above concentrations of staurosporine in single cell suspensions in a sterile glass culture vial for 4 h at standard culture conditions. Cells were shaken after each half an hour during incubation to keep the cells in suspension. After incubation, cells were harvested and washed with PBS. Apoptosis assays were performed using a Vybrant™ apoptosis assay kit (Invitrogen, USA) according to manufacturer's protocol. Briefly, cells were incubated in an Alexa Fluor 488 labeled anti-annexin V antibody and Propidium Iodide for 15 min followed by dilution in annexin V binding buffer. Results were analyzed by the CellQuest Pro software of a fluorescence activated cell sorter (FACS) (BD Biosciences, USA).

2.5. Immunoblotting

Whole cell proteins from both adherent and cell suspensions were isolated by lysing the staurosporine treated cervical cancer cell line SiHa in RIPA buffer [100 mM NaCl, 50 mM Tris–Cl (pH 7.4), 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF 1% NP-40, 0.1% SDS] containing a protease inhibitor cocktail (Sigma, USA) on ice. Protein concentration was estimated by the Bradford assay. An equal amount of protein (50 μg) was separated on 10% SDS-PAGE and electro-blotted to the PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in TBST buffer (Tris–Cl, NaCl, Tween-20) for 1 h at room temperature and probed with an rabbit anti-poly (ADP-ribose) polymerase (anti-PARP) primary antibody (1/1000 dilution; recognizing 116 kDa full length and 89 kDa cleaved fragment; CST, USA) in 5% non-fat milk in TBST for overnight at 4 °C. After washing with TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1/2000 dilution; Bangalore Genie, India) in 5% non-fat milk in TBST. After washing twice with TBST, the blot was developed with ECL (Thermo, USA) and imaged. The membrane was stripped and re-probed with an anti-GAPDH antibody (Imgenex, India) as a loading control. The immunoblot experiment was repeated twice.

2.6. Reverse transcriptase (RT) PCR

Total RNA was extracted immediately after harvesting control and treated cell lines using TRIzol (Invitrogen, USA), according to the manufacturer's protocol followed by DNaseI (Fermentas, USA) treatment. RNA was quantified by NanoDrop (Thermo Scientific, USA) and stored at −80 °C. This RNA was used for first strand cDNA synthesis using a high capacity cDNA reverse transcription kit (ABI, USA) according to the manufacturer's protocol. First strand cDNA samples were used for expression profiling of MMP1 (Gene ID: 4312), MMP2 (Gene ID: 4313) and internal control β-actin (Gene ID: 60) by semi-quantitative PCR using respective primers of MMP1 (forward — 5′-TTCGGGGAGAAGTGATGTTC-3′; reverse — 5′-GCTGTAGATGTCCTTGGGGTA-3′), MMP2 (forward — 5′-AGAATACCATCGAGACCATGC-3′; reverse — 5′-TGATCATGATGTCTGCCTCTC-3′) and internal control β-actin (forward — 5′-AAATCTGGCACCACACCTTC-3′; reverse — 5′-AGCACAGCCTGGATAGCAAC-3′).

To validate the semi-quantitative PCR results, quantitative real-time PCR was performed with a 2× SYBR Green PCR master mix (ABI, USA) according to the manufacturer's protocol using MMP1, MMP2 and β-actin (as an internal control) expression primers. Briefly, 12.5 μl SYBR Green PCR master mix, 4 pM of each primer, and 50 ng of cDNA were used to determine the mRNA expression levels of MMP1 and MMP2 by the Real-Time PCR System (Applied Biosystems). PCR was performed thrice in duplicate with all the samples for MMP1, MMP2 and the internal control. Relative gene expression of MMPs was analyzed with the comparative C_T method of quantification [38].