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Original article

Autophagy as a potential therapeutic target during epithelial to mesenchymal transition in renal cell carcinoma: An *in vitro* study

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ABSTRACT

Cancer progression toward invasive and metastatic disease is aided by reactivation of epithelial–mesenchymal transition (EMT), involving transdifferentiation of epithelial cells into mesenchymal phenotype. This leads to increased migratory and stem cell-like features in the cells. These EMT cells are more resistant to chemotherapy and it is hypothesized that the phenomenon of autophagy induces resistance, providing a survival strategy for cells.

In the present study, we induced EMT-like phenotype in renal carcinoma cells and identified corresponding higher autophagy flux in these cells. The EMT transformed cells may be a representative of the resistant cancer stem cell(CSC)-like phenotype. Autophagy was identified as a potential mechanism of cell survival in these cells thus implying that autophagy inhibition can lead to enhanced cell death. We also observed that tumor cells especially EMT transformed cells, have been 'primed' to undergo autophagy by mTOR inhibition. We observed that combined use of autophagy inhibitor and temsirolimus (TEM) improved antitumor activity against RCC in EMT transformed metastatic cells. One of the approaches for inhibiting autophagy was the use of lysosomotropic anti-malarial drug, chloroquine (CQ) and we explored the therapeutic potential of combination of CQ and the mTOR inhibitor, TEM. EMT transformed cells showed increased cell cytotoxicity when autophagy was impaired by addition CQ with TEM. This led us to conclude that inhibition of autophagy with the current therapeutic regimen could be useful in targeting the EMT transformed cells along with the bulk tumor cells in RCC.

1. Introduction

Renal cell carcinoma (RCC) is the most common and aggressive renal neoplasia. RCC is potentially curable by surgical excision if diagnosed early; however, it becomes virtually incurable on metastasis to distant sites. Amongst the various subtypes of RCC, clear cell renal cell carcinoma (CCRCC) is the most prevalent form and constitutes 70–80% of all renal cancers [1].

Epithelial to mesenchymal transition (EMT) is a crucial process regulating the initial steps of metastatic progression. Therefore, key regulatory mechanisms and mediators of EMT may provide the new paradigms to develop novel therapeutics with broad clinical applicability. In RCC, it has been demonstrated that there is acquisition of EMT-like characteristics during the malignant transformation of renal epithelial cells [2,3]. A recent study have considered and evaluated expression of EMT linked genes as a prognostic factor in the RCC [4]. EMT may lead to cancer cell invasion, metastasis, and drug resistance [5]. It has been reported earlier that anti-cancer therapies, including cytotoxic chemotherapy and pathway inhibitors can induce autophagy in most cancer cell lines [6]. Besides metabolic or cell intrinsic stresses, therapy-induced autophagy can limit the antitumor efficacy of a number of therapies [7]. Although autophagy can exert negative effects on tumor formation and progression, it has also been observed to support tumor survival and progression in many cases [8]. There exists a complex relation between autophagy, EMT and metastasis. Cells undergoing EMT require autophagy activation for survival during the metastatic spreading, on the other hand, autophagy acts as oncosuppressive signal to inhibit the early metastasis. During EMT, cancer cells go through morphological reprogramming and reorganisation of the cytoskeleton and promote autophagy for viability of potentially metastatic cancer cells [9,10]. The activation of autophagic machinery has also been shown to cause reversion of the EMT phenotype [11–15]. Various evidences suggest that autophagy can control EMT through selective degradation of EMT proteins [16,17]. In view of the dual role of autophagy in tumor

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progression, it is important to test its role in context dependent manner. It has been reported that the serine- threonine kinase, mTOR facilitates cell proliferation in presence of nutrients and growth factors while suppressing autophagy [18]. Pharmacologic inhibition of mTOR has been shown to supress tumor growth in both preclinical models and in patients with RCC. mTOR inhibitors (temsirolimus and evorilimus) have demonstrated a significant clinical activity in patients of advanced RCC [19–21] however the improvement in overall survival is not statistically significant [22,23]. The duration of response is often limited and after sometime tumor cells become refractory to treatment and resistance develops in majority of patients [24]. Since mTOR inhibitors are known to induce autophagy therefore autophagy can be considered as a mechanism for tumor cells to escape therapy induced cell death and hence compromise efficacy of mTOR inhibitors in RCC.

In the present study, we identified autophagy as a potential mechanism of cell survival in metastatic RCC cells *in vitro* thus implying that autophagy inhibition can lead to enhanced cell death. We also observed that tumor cells especially EMT transformed cells, have been 'primed' to undergo autophagy by mTOR inhibition. It was our hypothesis that combined use of autophagy inhibitor and temsirolimus (TEM) would result in improved antitumor activity against RCC in EMT transformed metastatic cells. One of the approaches for inhibiting autophagy is the use of lysosomotropic anti-malarial drug, chloroquine (CQ) so we explore the therapeutic potential of combination of CQ and the mTOR inhibitor, TEM. EMT transformed cells showed increased cell death when autophagy was impaired by addition CQ with TEM. This led us to conclude that modulation of autophagy with the current therapeutic regimen could be useful in targeting the EMT transformed cells along with the bulk tumor cells in RCC.

2. Material and methods

2.1. Cell culture

In-*vitro* studies were done with A498 cell line procured from National Centre for Cell Sciences (NCCS), Pune India. The cells were further cultured in Dulbecco's modified eagle's medium-high glucose (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and with antibiotics (penicillin- streptomycin,1U/ml) at 37 °C at 5% CO_2 in humidified incubator.

2.2. EMT induction in cell line

The A498cells were seeded in 6-well culture plates cells and treated with TGF- β (5 ng/ml) for 48 h. TGF-β treated cells were considered as EMT induced or EMT $^{+ve}$ while the control cells where no TGF-β treatment was given were considered as EMT^{-ve}. This protocol for EMT induction is as reported in previous literature [22,23].

2.3. Acridine orange (AO) staining for autophagy detection

A498 cells (4×10^3/well) were treated with the mTOR inhibitor, temsirolimus (TEM) for indicated time points. After treatment, cells were stained with AO (1 μg/ml) for 15 min in dark. After incubation cells were washed twice with PBS and autophagosome formation was visualized by using Fluorescent microscope (Olympus 1×51) under blue filter.

2.4. Western immunoblot

The expression of autophagy markers, LC3II and Beclin were observed by Western immunoblot. Briefly cells were treated with 10 ng/ml TGF-β, for 48 h, harvested by scraping and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1.0% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 1.0 mM ethylenediaminetetraacetic acid, 0.1% [w/v] sodium dodecyl sulfate [SDS]. Protein concentrations were determined using the BCA Kit (Thermo Scientific,Grand Island, NY). Extracts were analyzed by SDS–polyacrylamide gel electrophoresis, transferred to PVDF membrane followed by immunoblotting with appropriate antibodies. The membrane was washed and finally developed using ECL plus detection kit (BIORAD). Band expression was detected on Chemidoc gel documentation system (Bio-Rad).

2.5. MTT assay

Cell viability in EMT induced cells treated with TEM and CQ was assessed by MTT assay. MTT measures the reduction of 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. Briefly, Briefly, cells were seeded in 96- well plates and allowed to grow overnight. Cells were treated with either TEM (10 μM)/CQ (50 μM) or combination of TEM + CQ in fresh medium for 24 h. MTT solution (2.5 mg/ml) was added as per protocol and cells were lysed by addition of 100 μl DMSO per well. The purple color conversion of MTT by live cells was measured at a wavelength of 565 nm (Tecan, M7500 pro).

2.6. Annexin V-Propidium iodide assay

Apoptosis was determined using Annexin V apoptosis detection kit (immunostep) according to manufacturer's instructions. Briefly, cells were washed with PBS, and resuspended in 200 μl of binding buffer. These were incubated with 5 μl of Annexin V-FITC (10 min) in the dark and were washed with binding buffer and counter stained with 5 μg/ml PI. These were immediately analysed on Becton Dickinson FACS Aria-II flowcytometer and further analysed by using FACS DIVA (BD, USA) software.

2.7. Sub G1 population analysis

After incubation with the drugs, cells were harvested and washed twice with PBS, fixed in ethanol (70%) and stained with RNase/PI (Invitrogen) according to manufacturer's instruction. The sub-diploid DNA content was assessed in the fraction of nuclei by histogram analysis using FACS ARIA (BD, USA). Proportion of apoptotic cells was determined by with FACS DIVA software.

2.8. Cell death detection ELISA

Apoptosis induction in the A498 cell line was analyzed by using CDD ELISA kit (Roche, Germany) according to the manufacturers' instructions. This is a quantitative sandwich ELISA based assay using respective mouse monoclonal antibodies against DNA and histones. The apoptotic index was calculated as follows:

Apoptotic Index (AI) = (O.D. treated/O.D. untreated)

2.9. Analysis of pro and anti-apoptotic factors

The expression of Bax, Bcl_2, was performed by Flow cytometric analysis by using corresponding primary antibodies. After treatment cells were trypsinized and fixed with 4% PFA, washing was done with PBS and permeabilized with 0.1% Triton X-100. Cells were then washed with PBS and blocking was done with 2% BSA for