



SAC mediated modulation of HepG2 cell survivability –an in vitro study

KEYWORDS

SAC, Caspase3, apoptosis

Sujan Chatterjee

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Debadrita Pal

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Ankita Malakar

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Udipta Chakraborti

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Dipanwita Sengupta

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Avik Sarkar

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Soumosish Paul

Molecular Biology and Tissue Culture Laboratory, Post Graduate Department of Zoology, Vidyasagar College, Kolkata-700006, India.

Kaustav Dutta Chowdhury

Cyto-genetics Laboratory, Department of Zoology, Rammohan College, 102/1, Raja Rammohan Sarani, Kolkata- 700 009, India.

Gobinda Chandra Sadhukhan

UGC-Academic Staff College, Jadavpur University, Kolkata-700032, India

ABSTRACT

Cancer is a group of diseases having a common characteristic of abnormal cell growth with a potential to invade other parts of the body. Available cancer therapeutics mostly includes chemotherapy, radiation, and/or surgery. Thinking of side effects during and after these treatments the therapy with herbal drugs is considered as a possible alternative to fight the cancer. In our study HepG2 cells, a suitable model for liver hepatocellular carcinoma, was treated with SAC, an organosulfur present in garlic. Data suggested effective suppression of HepG2 cell survivability by 48hr treatment of 10mM SAC. Further analysis recommended the important role of caspase3 mediated apoptosis in the SAC mediated curbing of hepatocarcinoma during in vitro study.

Introduction:

Hepatocellular carcinoma (HCC) is one of the main type of liver cancer and the third most common cause of cancer mortality worldwide [Hatziaepostolou et al., (2011)]. In eastern Asia, China alone accounts for more than 50% of the world's cases (age-standardized incidence rate; men:0.0352%,women: 0.0133 %) [El-Serag et al., (2007)]. The major etiological risk factor for HCC is chronic hepatitis and viral (HBV and HCV) infection [El-Serag et al., (2007)]. Other risk factors for HCC include alcoholic liver disease and possibly nonalcoholic fatty liver disease. These risk factors lead to the formation and progression of cirrhosis, which is present in about 80–90% of HCC patients. The 5-year cumulative risk of developing HCC in patients with cirrhosis ranges between 5% and 30% depending on etiology (highest in HCV), region or ethnicity (highest in Asians), and stage of cirrhosis (highest in decompensated disease) [El-Serag et al., (2007); Fattovich G. et al., (2004)].

Although the molecular mechanisms leading to HCC have not been well characterized. As a result currently followed cancer therapeutic strategies, particularly in human hepatocellular carcinoma (HCC), have limited treatment options with generally poor prognosis [Minguez et al., (2009)].

Widely practiced remedies for hepatocellular carcinoma

includes surgery [Nakagawa et al., (2012)], chemotherapy, radiotherapy, resection and radiofrequency ablation (RFA) as well as liver transplantation [Sengupta, D. et al., (2014)]. But cumulatively they all gives little hope to the patients. These treatment procedures are costly and comes with many side effects [Marin, J.J.G. et al., (2008)]. Considering the situation herbal drug therapy presently included in the treatment schedule because they are easily biodegradable and are quickly absorbed within the cell [Bonifácio B.V. et al., (2014)]. They are characteristically tissue targeted and tissue protective in nature [Bonifácio B.V. et al., (2014)] and have a microdose effect [Bonifácio B.V. et al., (2014)]. As a result bystander death is limited [Thomson, M. et al., (2003)].

S-allyl cysteine (SAC) is garlic derived water soluble organosulfur compound that effectively [Cohen, L.A., (1999)] suppress tumor growth in leukemia, melanoma, epidermoid carcinoma, hepatoma, oral carcinoma, glioblastoma, prostate carcinoma, and gastric carcinoma [Sengupta, D. et al., (2015)]. Our experimental analysis explored the efficacy of SAC against this survivability of HepG2 cells, a well known model for liver cancer study.

Methods

Cell culture and treatments

HepG2 cell line was maintained in supplemented DMEM (Invitrogen Life Science Technologies) medium containing 10% fetal bovine serum (Invitrogen Life Science Technologies), 1% PEN-STREP (Sigma Aldrich) and 0.1% Fungizone (Sigma Aldrich). Cell culture was nurtured at 37°C in humidified 5% CO₂ incubator. Cells at subconfluent stage were exposed to 0.1, 1, 10 and 100mM SAC for 12, 24, 36, 48, 60 and 72hr of experimental period.

Wst assay

For the cell survivability assay 10⁴-10⁵ HepG2 cells were incubated in a 96 well microtiter plate 18hr. Then adhered cells were treated for 12-72hr with different doses of SAC estimated. Finally 10µl WST-1 (Roche Life Science) reagent was added after the stipulated period of drug exposure cells were incubated for 0.5-4hr at 37°C in 5% CO₂ incubator. Absorbance of formed formazan dye was measured with an ELISA reader at 420-480nm against the reference value of 650nm. Absorbance values before and after SAC treatment were directly correlated to the residual metabolically active cell in culture and representative value of control group was considered as 100% survivability. Calculated Data were presented in percentages of cell survivability with respect to untreated HepG2 cells.

Caspase3 activity estimation

For Caspase3 activity analysis HepG2 cells were trypsinized and were washed with PBS buffer PH 7.4., 1 x 10⁵ cells were resuspended in 50µl of chilled cell lysis buffer and incubated on ice for 10min. Then cellular lysate was centrifuged for 1min at 10000g. Cytosolic supernatant are considered as enzyme source and for activity analysis 100µg protein was diluted to 50µl cell lysis buffer. To which 50µl of 10 mM DTT containing 2X reaction buffer was added and finally was incubated at 37°C for 1 to 2hr in presence of 5µl 4 mM DEVD-pNA with a 200µM final concentration. Change in absorbance was determined at 405nm in UV-VIS spectrophotometer (UV-1240Pharma Spec, Shimadzu).

Flow cytometry analysis

Trypsinized cells were washed with serum-containing media. 1x10⁵ cells were suspended in 500µl of 1X binding buffer. Then 5µl of AnnexinV-FITC and 5µl of propidium iodide (PI 50mg/ml) was added to cell suspension and incubated at room temperature for 5min in the dark. AnnexinV-FITC binding was analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2). Early apoptosis was defined as AnnexinV-positive and PI-negative cells. Late apoptosis was defined as AnnexinV positive and PI-positive cells. Data was represented in % of apoptotic cells after compensating the values against PI fluorescence.

Statistical Significance

Calculation of means and SEM was performed following the standard procedure. Statistical significances were estimated in GraphPad Instat software (Graph Pad, La Jolla, CA, USA). Values were regarded as significant when p<0.05.

Result

Estimation of the concentration of SAC required for the regulation of HepG2 cell survivability

To analyze the effect of SAC, WST-1 assay was performed. WST-1 is a tetrazolium salt which is converted into a coloured dye by mitochondrial dehydrogenase enzyme (NA-

DPH). Generated formazan dye is released resulting alteration of the colour of media. This colour change is directly proportional to the net metabolic activity of the cell indicating cell viability or survivability. Sub-confluent HepG2 cells were incubated with 100mM, 10mM, 1mM and 0.1mM concentrations of SAC for 24hr. Data suggested effective reduction in cell survivability (less than 80%) only after an incubation with 10mM SAC. Values were further reduced in 100mM SAC treated group (Figure 1).

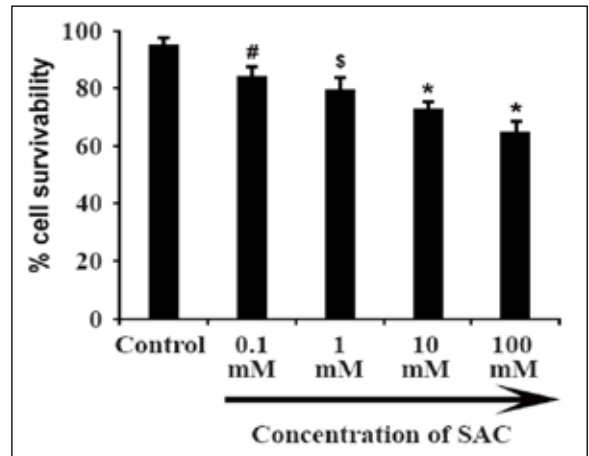


Figure 1: Determination of cell survivability after 0.1mM, 1mM, 10mM and 100mM SAC treatment for 24hr. Data were mean±SD of four independent experiments. #p<0.05, \$p<0.02 and *p<0.01 vs untreated group of HepG2 cell.

Determination of optimum period for the SAC treatment against HepG2 survivability

Cell survivability assay was also performed for the determination of effective period of SAC exposure. For this analysis sub-confluent HepG2 cells were treated with 10mM SAC for 12hr, 24hr, 36hr, 48hr, 60hr and 72hr. Data suggested that 48hr of 10mM SAC treatment efficiently reduced cell-survivability below 40%. Values were further reduced with the increase in the hour of treatment (in 60hr and 72hr) of HepG2 cells with SAC (Figure 2).

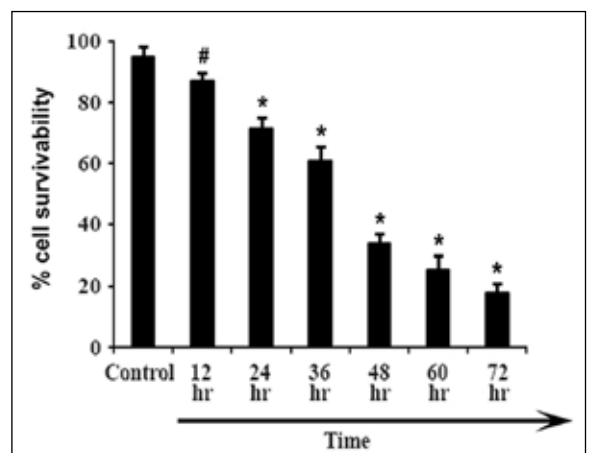


Figure 2: Estimation of cell survivability after 12hr, 24hr, 36hr, 48hr, 60hr and 72hr of 10mM SAC treatment. Values were mean±SD of four independent experiments. #p<0.05 and *p<0.01 vs untreated group of HepG2 cell.

Induction of apoptosis after SAC treatment in HepG2 cells

Generally in control living cell, phospholipids of the cell membrane are asymmetrically distributed along the inner and outer leaflets of membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet, while phosphatidylserine (PS) is located on the inner surface of lipid bilayer. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of plasma membrane. Exposure of PS is indirectly estimated by determining annexinV-FITC fluorescence positive cells. Flowcytometric estimation demonstrated 47.93% HepG2 cells were annexinV-FITC positive after 48hr of 10mM SAC treatment .Against the control value of only 3.3% (Figure 3) .

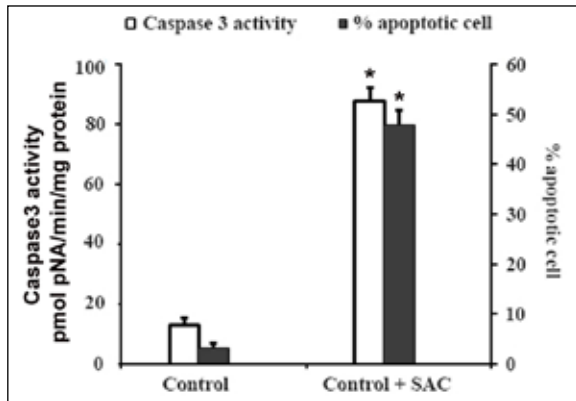


Figure 3: Flow cytometric PS exposure and spectrophotometric caspase3 activity were estimated in control and drug treated group. Values were represented in % apoptotic cells and pmol pNA/min/mg protein, respectively. Data were mean \pm SD of four independent experiments. * $p < 0.01$ vs untreated group of HepG2 cell.

Caspase3 activation after SAC treatment in HepG2 cells

Apoptosis is a biochemical event which is executed by the high level of effector caspase3 activity. Active caspases3 mediated cleavage of DEVD-pNA was determined following spectrophotometric assay kit before and after drug treatment. Data suggested 6.7fold enhancement of pNA concentration indicating effective activation of caspase3 after 48hr of 10mM SAC treatment. Moreover combinatorial analysis suggested an equiproportional change in caspase3 activity with PS exposure representing caspase3 mediated induction in HepG2 cell death after specified SAC treatment .

Discussion

Hepatocellular carcinoma is the 5th most common cancer in the world and causes death of the common people at a continuous rate [Sengupta,D. et.al., (2014)]. Since majority of primary liver carcinoma arises in hepatocytes [Fielding,L., (2006)]; therefore HepG2 cell line, the well known hepatocellular carcinoma cell line, is widely used in the understanding of molecular signaling associated with liver cancer cell survivability. Commensurating the previous studies here we found reduced cell survivability after SAC treatment. According to our data treatment with 10mM SAC was able to reduce HepG2 cell survivability below 80%. Further analysis was performed with 10mM SAC for a period from 12hr to 72hr with an interval of 12hr. Data suggested that 48 hour is sufficient to reduce cell survivability below 40% in treated group of cells. Although enhancement in treatment period reduced more and more cell survivability as observed in our study. Analysis also suggested that induction in apoptosis might be the probable reason of fall in cell survivability after SAC treatment. Flowcytometric analysis with annexinV-FITC confirmed increased PS exposure (treated group 47.3% with respect to 3.3% in control) and loss of lipid asymmetry in the HepG2 cell surface of drug treated group. Occurrence of apoptosis in a cell generally requires a activation of caspases [Li,J. et.al., (2008)] and status of the signalling pathway is identified by the estimation of effector caspase3 activity. According to our result, 10mM SAC treatment for 48hr increased caspase3 activity to at about 6.7 fold with respect to untreated group of HepG2 cell. Co-presentation of the data indicated direct co-relation between apoptosis induction and caspase3 activity during SAC treatment. Therefore SAC stimulated caspase3 activity resulting induction of apoptotic mechanism and finally reduction in HepG2 cell number after the extent of proposed therapy .

Conclusion

SAC mediated induction of HepG2 cell death suggested the potential role of the drug in the amelioration of hepatocarcinoma. Thus, it may be considered and included in the in vivo experimental analysis to introduce an alternative tissue targeted therapy against liver carcinoma.

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