Original Article

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Effect of Celergen, a marine derivative, on *in vitro* hepatocarcinogenesis

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ABSTRACT: The aim of this study was to test for a potential anticarcinogenic effect of Celergen, a marine derivative devoid of traceable amounts of inorganic arsenic, on cell proliferation, cell cycle progression and apoptosis in the HepG2 human liver cancer cell line. Celergen significantly inhibited the proliferation of cancer cells in a dose-dependent manner while limiting the cell cycle progression at the G1 phase and significantly inducing apoptosis. Further examination showed that Celergen enhanced expression of the *p21*$^{CIP1/WAF1}$, *GADD153* genes and downregulated the *c-myc* gene. These results suggest that Celergen exerts promising chemopreventive properties to be further investigated.

Keywords: Antimutagenic effect, cell cycle, apoptosis, celergen, marine compound

1. Introduction

Marine derivatives, with their immense diversity and selection driven along millions of years of evolution may offer promising options for new drug discovery (*1-5*). In particular, several marine bioactive compounds are under study for their potential antitumor effect (*6-9*). Although the detailed mechanisms involved are still a matter of study, antioxidative and antimutagenic properties have been advocated for (*10-12*). We have recently shown that Celergen, a popular GMP-controlled marine bioceutical, could significantly inhibit ultraviolet (UV)-induced matrix-metalloproteinases (MMP) transcription and exert an antioxidant effect protecting skin fibroblasts (*13*). On the other hand, most recently the same food supplement has been vaguely suggested to be implicated in a case report in which the user showed increased levels of α-fetoprotein (*14*). Although this report suffers some limitations and a possible major methodological flaw, the issue deserved further clarification, given that several herbal supplements, once believed as safe or even claimed to be "liver protectors" have been recently shown to be potentially hepatotoxic or even mutagenic (*15-18*). Thus, the aim of the present study was to test the novel biomarine derivative Celergen on *in vitro* liver carcinogenesis.

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2. Materials and Methods

2.1. *Chemicals*

Celergen was obtained from Swisscap company (100 mg composition: DNA extract from fish milt 46 mg, fish collagen hydrolysate plus fish elastin 35 mg, whole fish protein hydrolysate 6 mg, lutein-coenzyme Q10-selenium 11 mg). As a pre-requisite, samples were blindly sent to an official Good Manufacturing Practice and Good Laboratory Practice registered toxicology laboratory which found no traceable amounts of heavy metals including organic and inorganic arsenic, having set a threshold of > 5 ppm (Redox Lab, Monza, Italy, report n. 2013001054/LAB). 7,12-Dimethylbenz[a] anthracene (DMBA) and 12-Q-tetradecanoylphorbo-13 acetate (TPA) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). 4-Nitroquinoline I-oxide (4NQO) was obtained from Nacalai Tesque Co. (Kyoto, Japan).

2.2. *In vitro analysis of cell growth*

Human liver cancer cell line HepG2 were cultured in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. HepG2 cells were then seeded at a density of 4×10^4 cells/2 mL medium in 35-mm diameter

dishes. Celergen were dissolved in tetrahydrofuran (THF) including 0.025% butylhydroxytoluene (BHT, as an antioxidant) and diluted to its final concentration expressed as μg/mL in each culture dish. An equivalent volume of vehicle (THF + BHT) was added to control dishes and it showed no measurable effect on HepG2 cells. Celergen was added at graded concentrations 1 day following the inoculation. At the 72 h observation, the number of viable cells was counted using a trypan blue dye exclusion methodology.

2.3. *Flow cytometry*

Cells were plated at a density of 1×10^6 cells/10 mL medium in 100 mm diameter dishes. Celergen was added 1 day after the inoculation. Cells were harvested from culture dishes by trypsinization and centrifugation. After a wash with phosphate buffered solution (PBS) (-), cells were suspended in a 0.1% Triton X-l00 solution. After the suspension was filtered through 50 μm nylon mesh, 0.1% RNase A and 50 pg/mL propidium iodide were added to stain DNA. The DNA content in stained nuclei was analyzed by a flow cytometer (FACS Calibur™, Becton-Dickinson, Franklin Lakes, NJ, USA). The percentage distribution of cells in each cell cycle phase was determined using ModFit LT™ software (Becton-Dickinson) based on DNA histograms. Five separate experiments were used each time to determine the final values.

2.4. *Isolation of total RNA*

HepG2 cells were plated at a density of 1×10^6 cells/10 mL medium in 100 mm diameter dishes. Celergen was added 24 h after the inoculation. Total RNA was isolated from the cells at pre-fixed times in the absence or presence of Celergen using RNeasyR Kit (QIAGEN GmbH, Hilden, Germany). Briefly, total RNA was adsorbed onto a silica membrane following cell lysis. Membranes were subsequently washed and RNA was eluted with 50/11 of RNase-free water. Final RNA concentrations were assessed by spectrophotometry.

2.5. *Real-time quantitative RT-PCR*

Total RNA was isolated from HepG2 cells as described above. Total RNA (5 pg) was transcribed to cDNA in a 20 μL reaction volume, with Superscript II Reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA), using oligo (dT) 12-18 primers. The reaction mixture was incubated at 42°C for 50 min, then at 70°C for 15 min. An equivalent volume of cDNA solution was used for the quantification of specific cDNA by real-time quantitative RT-PCR. The primer sequences used were as follows: for the $p21^{CIPIIWAFI}$ gene (318 bp), 5'-ATTAGCAGCGGAACAAGGAGTCAGA CAT3' and 5'-CTGTGAAAGACACAGAACAGT ACAGGGT-3', for *GADD153* (309 bp), 5'GAAA CGGAAACAGAGTGGTCATTCCCC-3' and 5'-GTGGGATTGAGGGTCACATCATTGGCA-3', for c-*myc* (209 bp), 5'-GGCAAAAGGTCAGAGTCTGG-3' and 5'-GTGCATTTTCGGTTGTTGC-3', for *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, 181 bp), 5'-CAACTACATGGTTTACATGTTC-3' and 5'-GCCAGTGGACTCCACGAC-3'. Real-time quantitative RT-PCR was performed with the LightCycler™ system (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green I Kit as instructed by the manufacturer. *GAPDH*, $p21^{CIPIIWAFI}$ and *GADD153* were amplified with a precycling hold at 95°C for 10min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 10 s. *c-myc* was amplified with a precycling hold at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 63°C for 5 s and extension at 72°C for 10 s. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Quantification data were analyzed using LightCycler analysis software. The expression levels of $p21^{CIPIIWAF1}$, *GADD153*, and *c-myc* were normalized to the level of GAPDH mRNA of the same sample.

2.6. *Statistical analysis*

For statistical analysis "One Way" variance analysis was employed and significance between the experimental and control groups was determined by Bonferroni's method. A difference of $p < 0.05$ was considered significant. Results were expressed as mean ± S.D.

3. Results

3.1. *HepG2 cell proliferation: in vitro effects of Celergen*

Treatment of HepG2 cells with Celergen brought about a significant inhibition of human liver cancer cell proliferation in a dose-dependent manner. Three days after the incubation with 25 μM, 50 μM, 100 μM and 200 μM Celergen, HepG2 cell growth decreased to 63, 52, 38 and 27% of the control, respectively. The concentration of Celergen with 50% growth inhibition was calculated as 50 μM (Figure 1).

3.2. *Effect of Celergen on G1 arrest and apoptosis in HepG2 cells*

The DNA content of HepG2 cells was calculated by flow cytometry analysis to identify whether the growth inhibitory effect of Celergen was determined by specific actions on cell cycle-related events. From DNA histograms it appeared that Celergen enabled a not significant trend increase of the ratio of G1 cells, but most significantly it increased pre-G1 apoptotic cells at

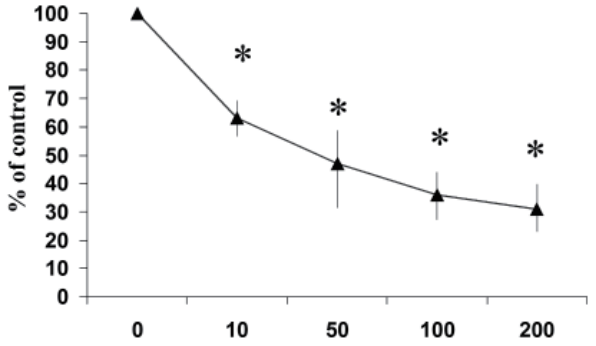

Figure 1. Growth inhibitory effect of Celergen on HepG2 cells. Cells were cultured for 72 h with or without Celergen at concentrations ranging from 25 to 200 μM. On day 3, the number of viable cells was determined. Data are expressed as means ± S.D. of three separate experiments.

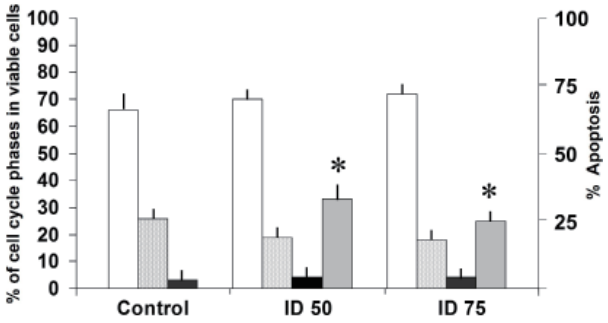

Figure 2. Effect of Celergen on cell cycle and apoptosis in HepG2 cells. HepG2 cells were treated with Celergen at ID 50 or ID 75 for 72 h and stained with propidium iodide to assess DNA content. The percentage of cells in each phase of the cell cycle was assessed by flow cytometric analysis. Data are expressed as means ± S.D. of five separate experiments. Left vertical line indicates the ratio of each phase in viable cells. Right vertical line indicates the percentage of apoptotic cells. White bars: Gl; dotted bars; S; black bars: G2/M and grey bars: apoptosis. * $p < 0.01$ compared with control.

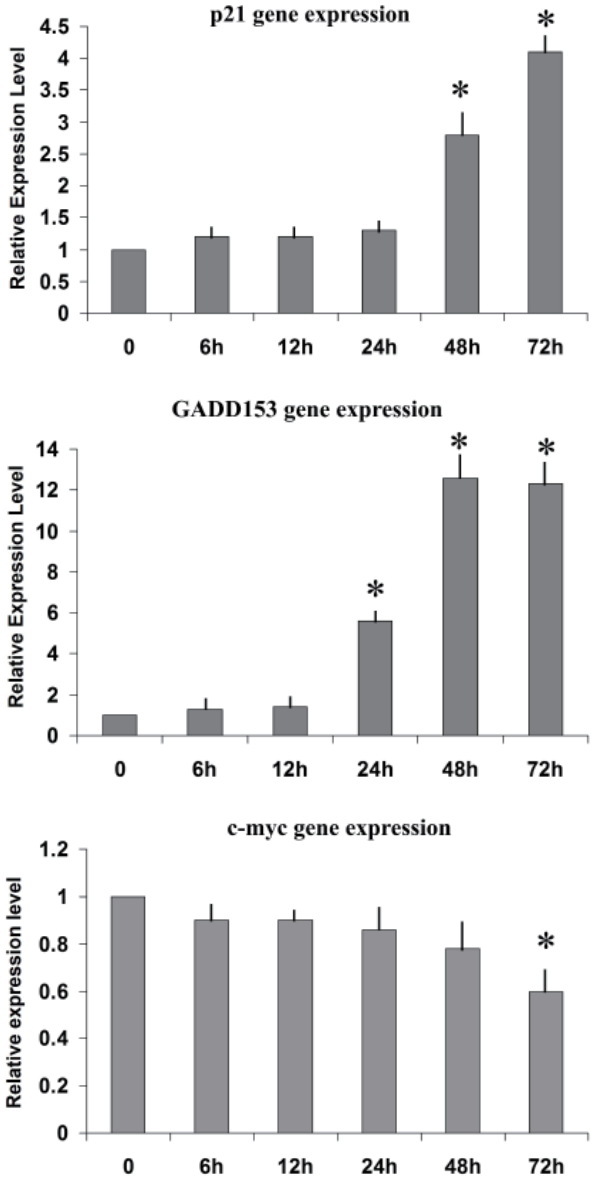

Figure 3. Effect of Celergen on $p21^{CIP1/WAF1}$, *GADD153*, and *c-myc* mRNA expression. Real-time quantitative RT-PCR was performed with 5 μg of total RNA from HepG2 cells treated with 50 μM Celergen or vehicle alone (control) for the test periods indicated. Values are means ± S.D. of five separate tests. All values were normalized to the GAPDH expression level. * $p < 0.05$ compared with control.

the 72h observation ($p < 0.005$, Figure 2). On the other hand, the effect of Celergen on the cell cycle on days 1 and 2 was not significant (not shown)..

3.3. *Gene expression of $p21^{CIP1/WAF1}$, GADD153 and c-myc expression: effect of Celergen regulation*

The effects of Celergen on the expression of cell cycle and apoptosis-related genes were examined using real-time quantitative RT-PCR. Significant induction of $p21^{CIPIIWAFI}$ and GADD153 was clearly observed at 48 h following treatment with Celergen and it was maintained throughout the observation period (72 h) while a significant late downregulation of c-myc occurred as well ($p < 0.01$, Figure 3).

4. Discussion

Celergen is a popular marine derivative containing DNA, collagen elastin and protein extracts and we have been recently shown that it exhibits a strong skin fibroblast protecting effect against UV irradiation (*13*).

In the present study, Celergen was shown to significantly inhibit cell growth *via* induction of G1 arrest of the cell cycle and apoptosis in HepG2 cells. To explore the action mechanism of cell cycle inhibition by Celergen, we assessed the expression of cell cycle and apoptosis related genes and found that the expression of $p21^{CIP1/WAF1}$ and GADD153 was significantly induced by Celergen. It is known that $p21^{CIP1/WAF1}$ is significantly induced by DNA damage and regulates the Gl and G2/M checkpoints (*19,20*) and that the induction of p21*CIP1/WAF1* usually follows a

p53 dependent mechanism (*21*). Moreover, *GADD153* and *c-myc* are known to be involved in apoptosis progression. GADD153 is a member of the CCAAT/enhancer-binding protein family of transcription factors and its expression is markedly enhanced by various cellular stresses (*22,23*). On the other hand, *c-myc* is a proto-oncogene and is implicated in various processes including cell growth, proliferation and cell death (*24*). In particular, the overexpression of c-myc represents a genetic abnormality frequently found in hepatocellular carcinoma (HCC) (*25*). Indeed, the relevance of c-myc expression in HCC has been confirmed in transgenic mice studies (*26*) and in clinics where overexpression of this gene is detected in most HCC patients while correlating also with poor prognosis (*27*). In our study, the induction of p21*CIP1/WAF1* and GADD153 occurred significantly at 48 h after treatment with Celergen and subsequently, an increase in G1 phase and apoptotic pre-G1 cells was observed at 72 h. However, c-myc showed a late but significant downregulation. Since p53 is known to be functional in HepG2 cells, p21*CIP1/WAF1* is likely to have been induced by a mechanism which depends on p53 and may be implicated in G1 arrest exerted by Celergen. Nonetheless, the induction of apoptosis by Celergen may be caused by GADD153 and has to be considered as well.

In summary, our data suggest that Celergen inhibits the proliferation of HepG2 cells and induces G1 arrest and apoptosis. The induction was associated with enhanced expression of $p21^{CIP1/WAF1}$, GADD153 but suppression of c-myc. These results suggest that such marine compounds may have anticarcinogenic properties while more finely-detailed proteomic studies are awaited. This data is in contrast with the albeit isolated clinical report of Chua *et al*. (*14*) suggesting an arsenic-related hepatocarcinogenesis effect of the same compound. However, that study bears the main methodological bias in that the authors measured the whole arsenic content, while the literature has clearly demonstrated that only the inorganic component is the one with the noxious effect (*28*) and the only one deserving monitoring from major epidemiological studies (*29,30*). On the contrary, the organic one is regarded as safe (*31*) and its concentration may also vary (*32*) as probably occurred in their study. For instance, seafood and seaweeds generally contain almost completely nontoxic organic arsenic such as arsenosugars (*33*). Thus, their suggestion cannot be supported and their observed abnormality of α-fetoprotein observed in their case report has to be correlated to other factors (episodic aflatoxin exposure, either environmental or food-borne *etc*.) which have not been analyzed in their report together with the lack of a separate measurement of organic and inorganic arsenic content. From the above, the study of Chua *et al*. has to be disregarded similarly because of what happened with a similar case of a different seafood compound after review by a number of experts in the field (*34-36*). While our study needs further research and *in vivo* follow up, our general conclusion is that marine derivatives, if properly farmed, collected and processed maintain a promising potential as a safe source of beneficial treatment.

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