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A mechanistic study of proliferation induced by *Angelica sinensis* in a normal gastric epithelial cell line

Yi N. Ye[a], Edgar S.L. Liu[b], Vivian Y. Shin[b], Marcel W.L. Koo[b], Yang Li[b], Er Q. Wei[a], Hirofumi Matsui[c], Chi H. Cho[b,*]

[a]*Department of Pharmacology, The University of Zhejiang, Hangzhou, China*
[b]*Department of Pharmacology, The University of Hong Kong, Faculty of Medicine, 5 Sassoon Road, Hong Kong, China*
[c]*Institute of Clinical Medicine, University of Tsukuba, Tsukuba Science City, Ibaraki 305-0006 Japan*

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Abstract

It has been reported that an extract from *Angelica sinensis* mainly consisting of polysaccharides (95%) prevented ethanol- or indomethacin-induced gastric mucosal damage (Cho CH *et al*. Planta Med 2000;66:348–51). However, it is not known whether *Angelica sinensis* has a direct stimulatory effect on the healing of gastric mucosal lesions. To study the hypothesis that *Angelica sinensis* has a direct mucosal healing effect in rats and in isolated gastric epithelial cells, we assessed the wound repair in both animals and normal cell culture (RGM-1), as well as [^{3}H]thymidine incorporation, ornithine decarboxylase (ODC) activity, and ODC protein and c-Myc protein expression after different treatments in RGM-1 cells. We found that *Angelica sinensis* crude extract (ASCE) dose-dependently enhanced gastric ulcer healing in rats and promoted wound repair in RGM-1 cells. It also significantly stimulated [^{3}H]thymidine incorporation and ODC activity in RGM-1 cells in a concentration-dependent manner. ODC and c-Myc protein expression was also increased as a result of this process. DL-α-difluoromethyl-ornithine repressed the [^{3}H]thymidine incorporation and ODC activity induced by ASCE. Pretreatment with c-Myc antisense oligodeoxynucleotides blocked the stimulatory action of ASCE on [^{3}H]thymidine incorporation and ODC protein expression. These data suggest that ASCE has a direct mucosal healing effect on gastric epithelial cells, while ODC and c-Myc are closely associated with this effect.

Keywords: Angelica sinensis; Polysaccharide; Gastric epithelial cells; Proliferation; c-Myc; ODC

1. Introduction

Epithelial cells line the gastrointestinal mucosa and form an important barrier to a wide array of noxious substances in the lumen. Superficial wounds to the mucosa promote the process of mucosal restitution, which involves epithelial cell proliferation and migration into the defect [1,2]. Depending on the types of cells, certain growth factors and cytokines are required to facilitate these processes. The proliferative response of gastric mucosal cells to growth factors might be of special importance in maintaining gastric mucosal integrity and accelerating ulcer healing [3–6].

The polyamines, including spermidine, spermine, and their precursor putrescine, have been under active investigation for nearly three decades [7]. There is a great deal of evidence that they play an important role in gastrointestinal mucosal growth [8]. Intracellular polyamine levels are highly dependent on the activity of ODC, the initial rate-determining enzyme in polyamine biosynthesis, and can be almost completely depleted by DFMO, a specific and irreversible inhibitor of ODC [9]. Although it has been repeatedly demonstrated that, in most cells, polyamine depletion leads to inhibition of cell proliferation, the actual mechanism by which this is brought about is not thoroughly understood. However, there are results supporting the mechanistic link between polyamines and transcription of growth-related c-*myc*, c-*fos*, and H_2A genes [10]. On the other hand, c-*myc* and c-*fos* oncogenes have also been shown to be transcriptional factors for ODC [11,12].

The root of *Angelica sinensis* is also known as "Danggui" in Chinese medicine, and is used to treat gynecological

* Corresponding author. Tel.: +852 2819 9252; fax: +852 2817 0859.
E-mail address: chcho@hkusua.hku.hk (C.H. Cho).

Abbreviations: ASCE, *Angelica sinensis* crude extract; AS-ODNs, antisense oligodeoxynucleotides; DFMO, DL-α-difluoromethyl-ornithine; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide; and ODC, ornithine decarboxylase.

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diseases. Recently, great advances have been made in chemical and pharmacological studies of the drug. Different components have been identified and demonstrated to increase myocardial blood flow and produce radioprotection [13–15]. It has also been reported that a crude extract from *Angelica sinensis* mainly consisting of polysaccharides (95%) prevented ethanol- or indomethacin-induced gastric mucosal damage [16]. However, it is not known whether *Angelica sinensis* has a direct stimulatory effect on the healing of gastric mucosal lesions. In the present study, we aimed to investigate whether ASCE could affect gastric ulcer healing and cell growth in RGM-1 cells, a cell line derived from normal Wistar rat gastric epithelium [17], and its possible mechanisms of action possibly through the c-Myc and ODC cascade.

2. Materials and methods

2.1. Chemicals and drugs

Chemicals and drugs were purchased from Sigma Chemical Co. unless otherwise stated. ASCE was dissolved in culture medium.

2.2. Extract of polysaccharides from Angelica sinensis

The roots of *Angelica sinensis* (Oliv.) Diels, Danggui, were purchased from Minxian County, Gansu Province, China. Polysaccharides were isolated according to the method described by Cho *et al*. [16], with some modifications. All extracts were finally pooled and mixed with a concentrated ethanol solution (final concentration 75% v/v) to precipitate the polysaccharide-enriched fraction. High-performance anion-exchange chromatography was employed to concentrate the polysaccharide-rich fraction [18]. Gel filtration chromatography (gel filtration column, Biosep SEC-S3000, Phenomenex; mobile phase 0.15 mol/L sodium chloride solution; detector wavelength 220 nm) in conjunction with the phenol–sulfuric acid method was used to determine the molecular size of polysaccharide [19,20]. The amounts of uronic acids and proteins were also determined [21,22]. The total *Angelica sinensis* polysaccharide-enriched fraction (ASCE) was subsequently used for assay.

2.3. Gastric ulcer induction in rats

Male Sprague–Dawley rats (weighing 180–200 g) were used in the experiment. They were deprived of food but had free access to tap water 24 hr before ulcer induction. Gastric kissing ulcers were produced by a luminal application of acetic acid solution as described by Tsukimi and Okabe [23], with modifications. Thereafter, animals were fed a standard diet and given tap water. One day after ulcer induction, rats were given ASCE intragastrically at different doses (25 or 50 mg/kg) twice daily for three days. Animals receiving distilled water were treated as vehicle control. All animals were killed under anesthesia, and a person unaware of the treatment measured the ulcer size in the stomach. Mucosa was scraped and stored at −70° until determination for ODC activity.

2.4. Cell culture

RGM-1 cells, a cell line derived from normal Wistar rat gastric epithelium [17], were maintained in a 1:1 Dulbecco's modified Eagle's medium F-12 nutrient mixture medium (DMEM/F-12) (Life Technologies, Inc.) containing 20% (v/v) heat-inactivated FBS (Life Technologies, Inc.), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a water-saturated atmosphere of 5% CO_2 at 37°. Passages 7–15 were used in the experiments. There were no significant changes in biological function and characterization from passages 7–15.

2.5. Estimation of cell viability

Cell viability was estimated using MTT [24]. RGM-1 cells (1.5×10^4/100 μL medium) were seeded into a 96-well plate and incubated for 24 hr for attachment. They were then incubated with DFMO at 37° for 5 hr. At the end of incubation, the medium was aspirated. The remaining cells were further incubated with 0.25 mg/mL of MTT for 3 hr. MTT was extracted with isopropanol in 0.04 M hydrochloric acid, and the color change in the extract was measured at 595 nm.

2.6. Cellular wound restoration model

The model was based on the method described by Watanabe *et al*. [25], with modifications. A small circular wound of 2-mm diameter was created. It was then further cultured in a DMEM/F-12 medium supplemented with 0.1% FBS and with different concentrations of ASCE (0, 0.4, or 2 μg/mL). The healing of the wound was assessed by measuring the wound size, i.e. cell-free area for up to 24 hr after wounding. The wound area was captured by a digital image processor connected to a microscope (Nikon) and assessed with an image-analyzing programme (Leica).

2.7. Determination of cell proliferation

Cell proliferation was assessed as DNA synthesis. To evaluate DNA synthesis in cells, the incorporation of [^{3}H]thymidine into DNA was determined. Briefly, RGM-1 cells were seeded into a 24-well plate and cultured for 24 hr in the presence of 20% (v/v) FBS. They were then washed twice with Hanks' balanced salt solution (Life Technologies, Inc.), followed by incubation with 1 mL/well of the medium containing various substances for different times. In the next step, 0.5 μCi of [^{3}H]thymidine was added to

each well, and the cells were further incubated for 5 hr. Incorporation of [^{3}H]thymidine into cells was measured with a liquid scintillation counter (LS-6500; Beckman Instruments, Inc.).

2.8. ODC activity

ODC activity was assessed by measuring the amount of $^{14}CO_2$ liberated from DL-[1-^{14}C]ornithine [26]. Gastric mucosa was prepared for ODC activity measurement as previously described [27]. Cultured RGM-1 cells were scraped from the 6-well plates and placed in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol. Then, the cells were sonicated for 20 sec under an ice-cold condition and centrifuged at 17,968 *g* at 4° for 20 min. Again, the supernatants were used for the assay of ODC activity. A 300-μL aliquot of the supernatant was incubated in a test tube in the presence of 2.5 mM L-[1-^{14}C]ornithine for 15 min at 37°. The $^{14}CO_2$ liberated from the decarboxylation of ornithine was trapped by a piece of filter paper impregnated with 20 μL of 2.0 M sodium hydroxide. The paper was placed inside a well suspended from the stopper and above the reaction mixture. The reaction was terminated by the addition of 0.3 mL of 10% (w/v) trichloroacetic acid. The radioactivity of $^{14}CO_2$ trapped in the filter paper was measured with a liquid scintillation counter (LS-6500; Beckman Instruments, Inc.). The protein content of the supernatant was determined with the Bio-Rad protein assay kit using BSA as a standard. The enzyme activity was expressed as pmol $^{14}CO_2$ liberated per mg of protein per hr.

2.9. Western blot analysis

The cells were harvested at 4° with RIPA buffer (50 mmol/L of Tris–HCl, pH 7.5, 150 mmol/L of sodium chloride, 0.1% [w/v] SDS, 0.5% [w/v] α-cholate, 2 mmol/L of EDTA, 1% [v/v] Triton X-100, 10% [w/v] glycerol) containing 1 mmol/L of phenylmethylsulfonyl fluoride and 10 μg/mL of aprotinin. The cell suspension was sonicated on ice for 20 sec and centrifuged at 17,968 *g* at 4° for 20 min. The supernatant (25 μg of total protein) was denatured and separated by electrophoresis on a 7.5% [w/v] SDS–polyacrylamide gel followed by transference to nitrocellulose membrane (Bio-Rad). The membranes were probed with a rabbit polyclonal antibody against rat c-Myc (Cat # sc-788, Santa Cruz Biotechnology, Inc.) or a mouse monoclonal antibody against rat ODC (Cat # MS-464-P, Fremont). They were then developed with the use of an ECL chemiluminescence system (Amersham, USA) and exposed to x-ray film. Protein determinations were made with a Bio-Rad protein assay kit with BSA as a standard. Quantitation was carried out by video densitometry (Bio-Rad, Gel Doc 1000).

2.10. c-Myc Antisense oligonucleotides

c-Myc Antisense for rat was synthesized by Biognostik. The oligonucleotide is a reverse complement of a target sequence as reported by Hayashi *et al*. [28]. RGM-1 cells were pretreated with c-Myc antisense oligonucleotides for 24 hr in the presence of 10% (v/v) FBS before adding ASCE.

2.11. Statistical analysis

Results were expressed as means ± SEM. Statistical analysis was performed with ANOVA and unpaired Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of ASCE on gastric ulcer healing in rats

The ulcer size in the control animals four days after ulcer induction was 72.00 ± 2.43 mm^2. ASCE treatment given one day after ulcer formation dose-dependently reduced the ulcer size to 67.87 ± 7.60 and 54.00 ± 4.64 mm^2 for the doses of 25 and 50 mg/kg, respectively, which was significantly different from the control in the higher dose ($P < 0.01$). Such a dose of ASCE also increased the ODC activity in the gastric mucosa by about 30% when compared to the vehicle control.

Considering that a 200-g rat contains about 20 mL of blood in the body and assuming that the bioavailability of ASCE was lower than 100%, the drug concentration in the blood circulation would be below 500 μg/mL for a 50-mg/kg dose. As the bioavailability of the drug was largely unknown and the amount of drug actually accessible to the gastric epithelial cells was unclear, we selected concentrations of ASCE from 0.4 to 10 μg/mL, which were lower than the predicted circulated concentrations *in vivo* in the subsequent isolated cell experiment. These concentrations of the drug would likely be achieved in the gastric mucosa.

3.2. Effect of ASCE on wound repair in RGM-1 cell culture

ASCE (0.4 or 2 μg/mL) dose-dependently decreased the denuded area at 6, 12, and 24 hr after wounding. At 12 hr, the wound recovered 41 ± 3.8% of the initial wounded area and 74 ± 4.4% at 24 hr in the control group without ASCE treatment. Wound repair was accelerated significantly by 2 μg/mL of ASCE (72 ± 4.9% and 92 ± 5.1% at 12 and 24 hr, respectively after wounding, $P < 0.05$).

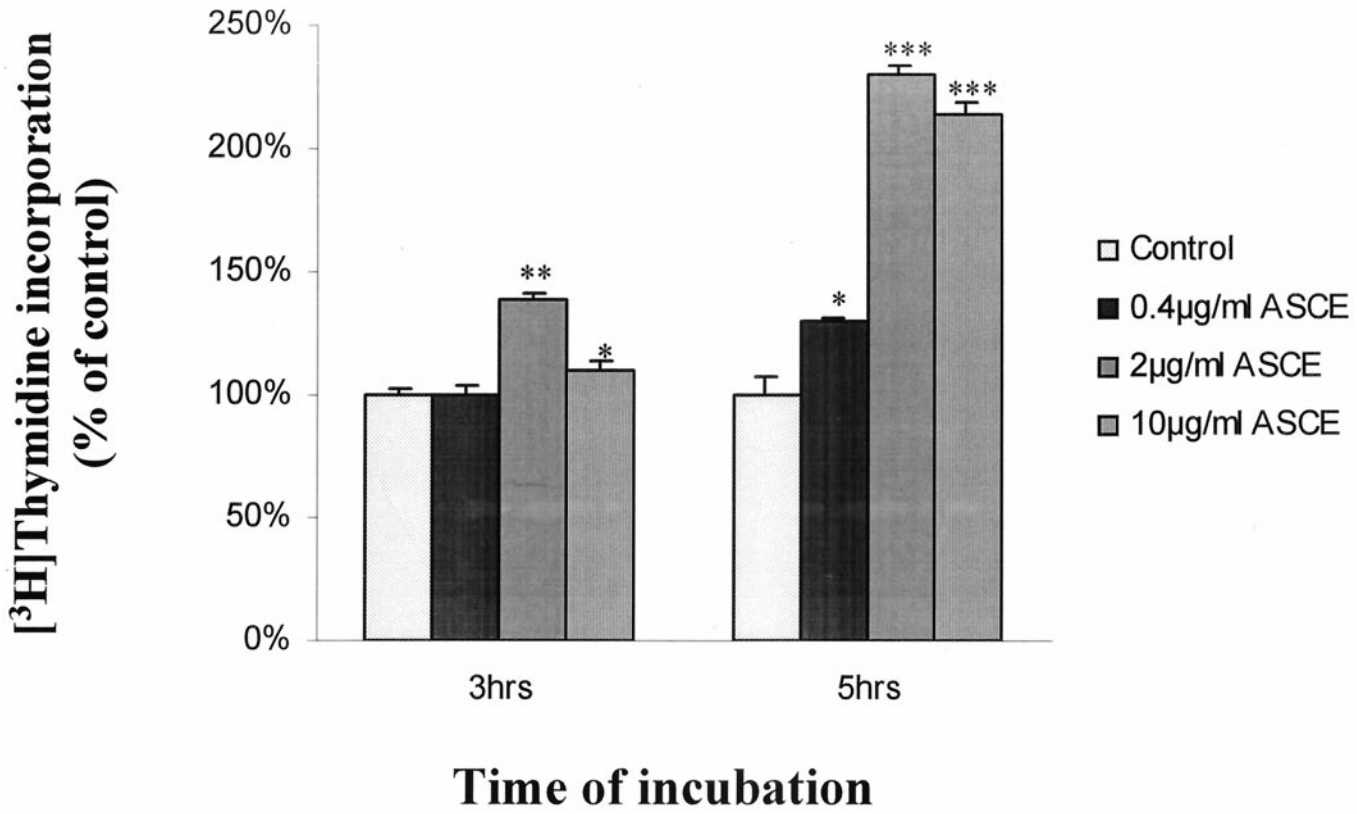

Fig. 1. Effect of ASCE on RGM-1 cell proliferation. The cells were incubated with or without ASCE for 3 and 5 hr. DNA synthesis was determined by [^{3}H]thymidine incorporation. Columns and vertical bars represent the means ± SEM of 6 samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to the control group.

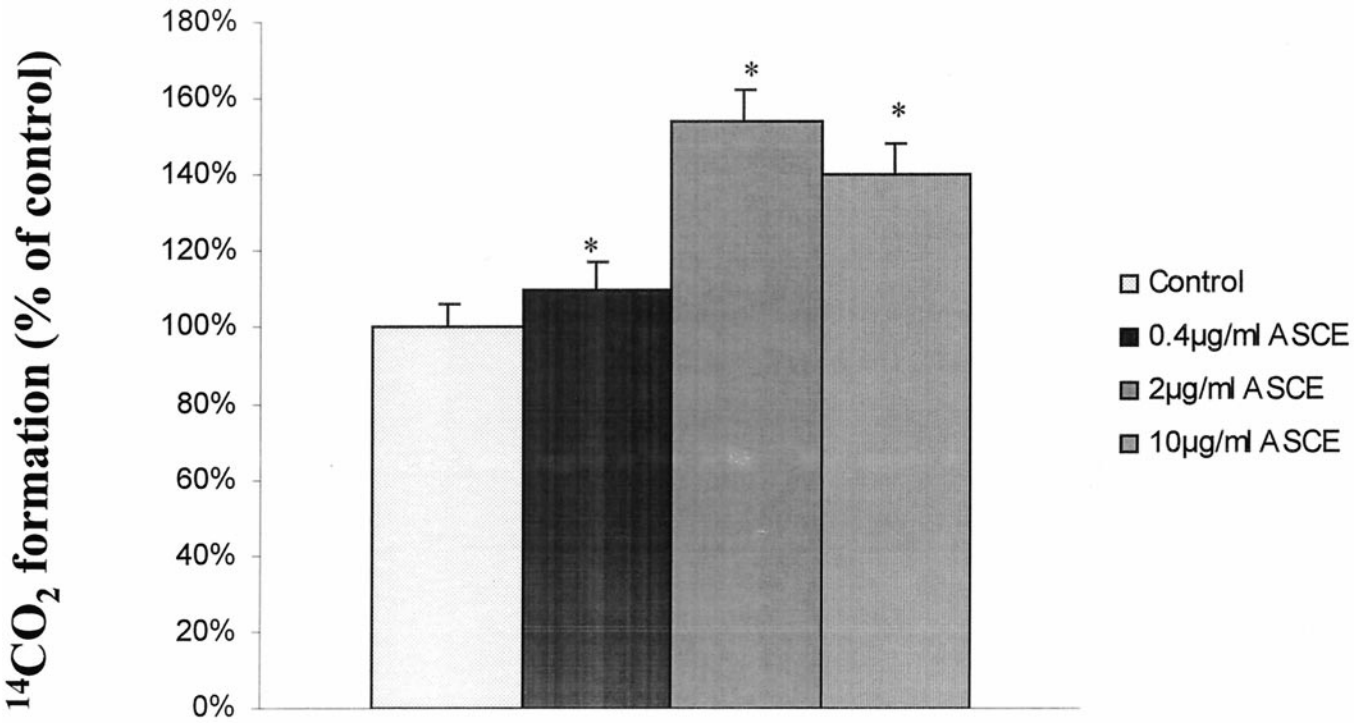

Fig. 2. Effect of ASCE on ODC activity in RGM-1 cells. The cells were incubated with or without ASCE for 5 hr. ODC activity was determined by the amount of $^{14}CO_2$ liberated. Columns and vertical bars represent the means ± SEM of 6 samples. *$P < 0.05$ when compared to the control group.

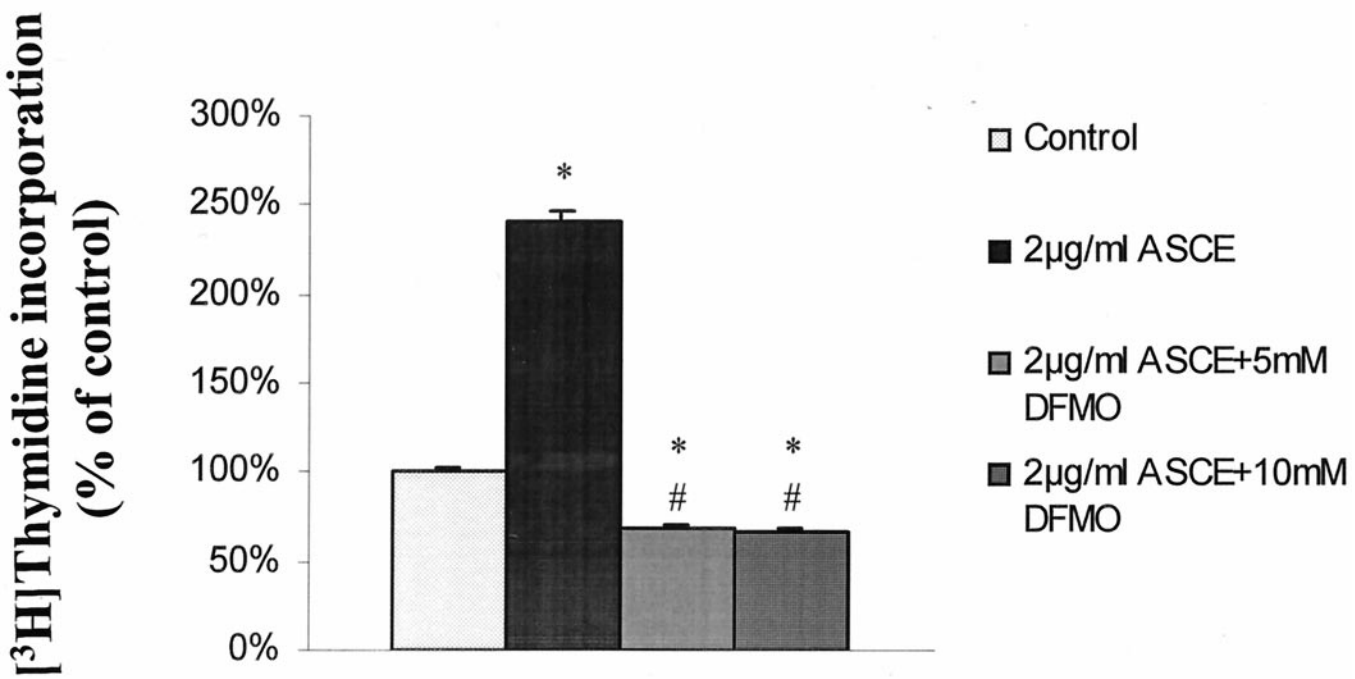

Fig. 3. [^{3}H]Thymidine incorporation into RGM-1 cells co-treated with ASCE and DFMO for 5 hr. Columns and vertical bars represent the means ± SEM of 6 samples. $^*P < 0.01$ when compared to the control group, $^{\#}P < 0.01$ when compared to the 2 μg/mL ASCE-treated group.

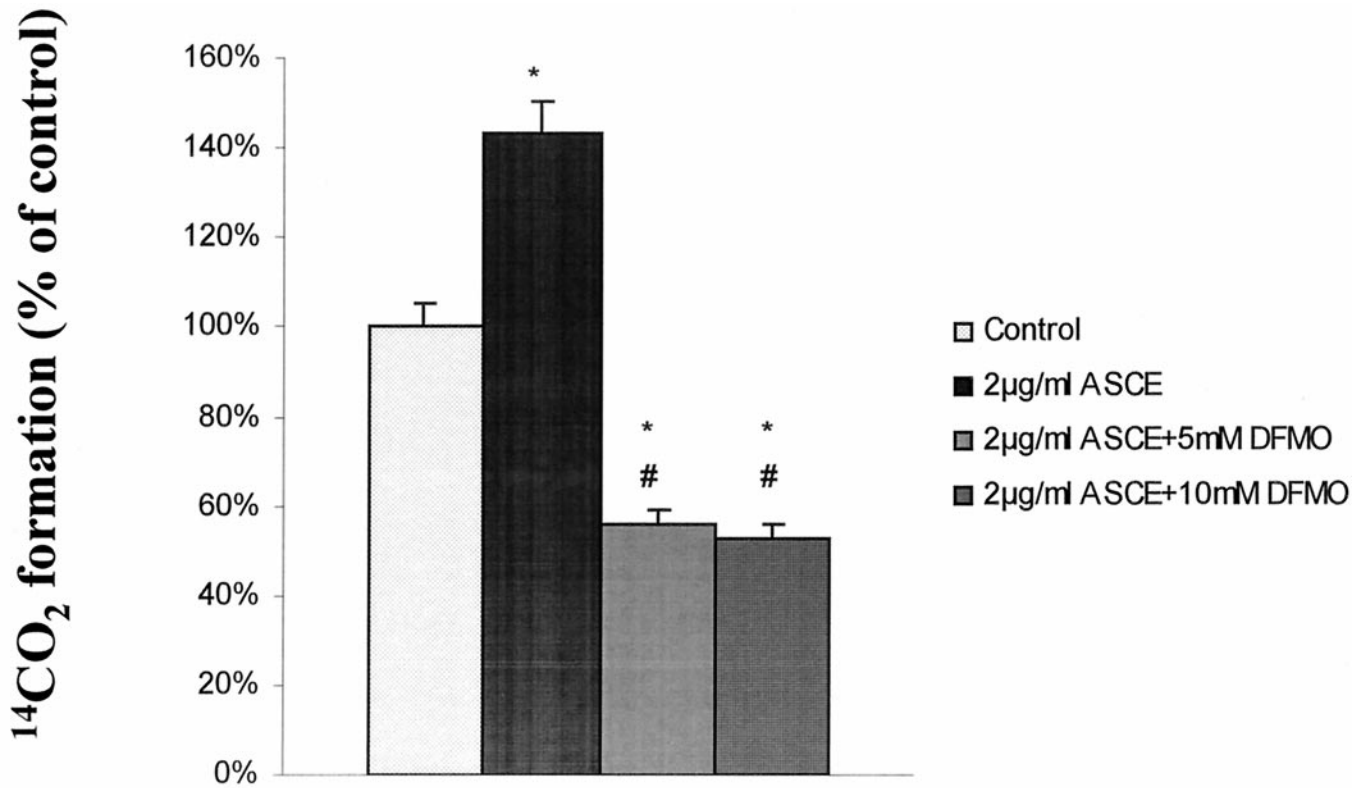

Fig. 4. ODC activity in RGM-1 cells co-treated with ASCE and DFMO for 5 hr. ODC activity was determined by the amount of $^{14}CO_2$ liberated. Columns and vertical bars represent the means ± SEM of 6 samples. $^*P < 0.05$ when compared to the control group, $^{\#}P < 0.05$ when compared to the 2 μg/mL ASCE-treated group.

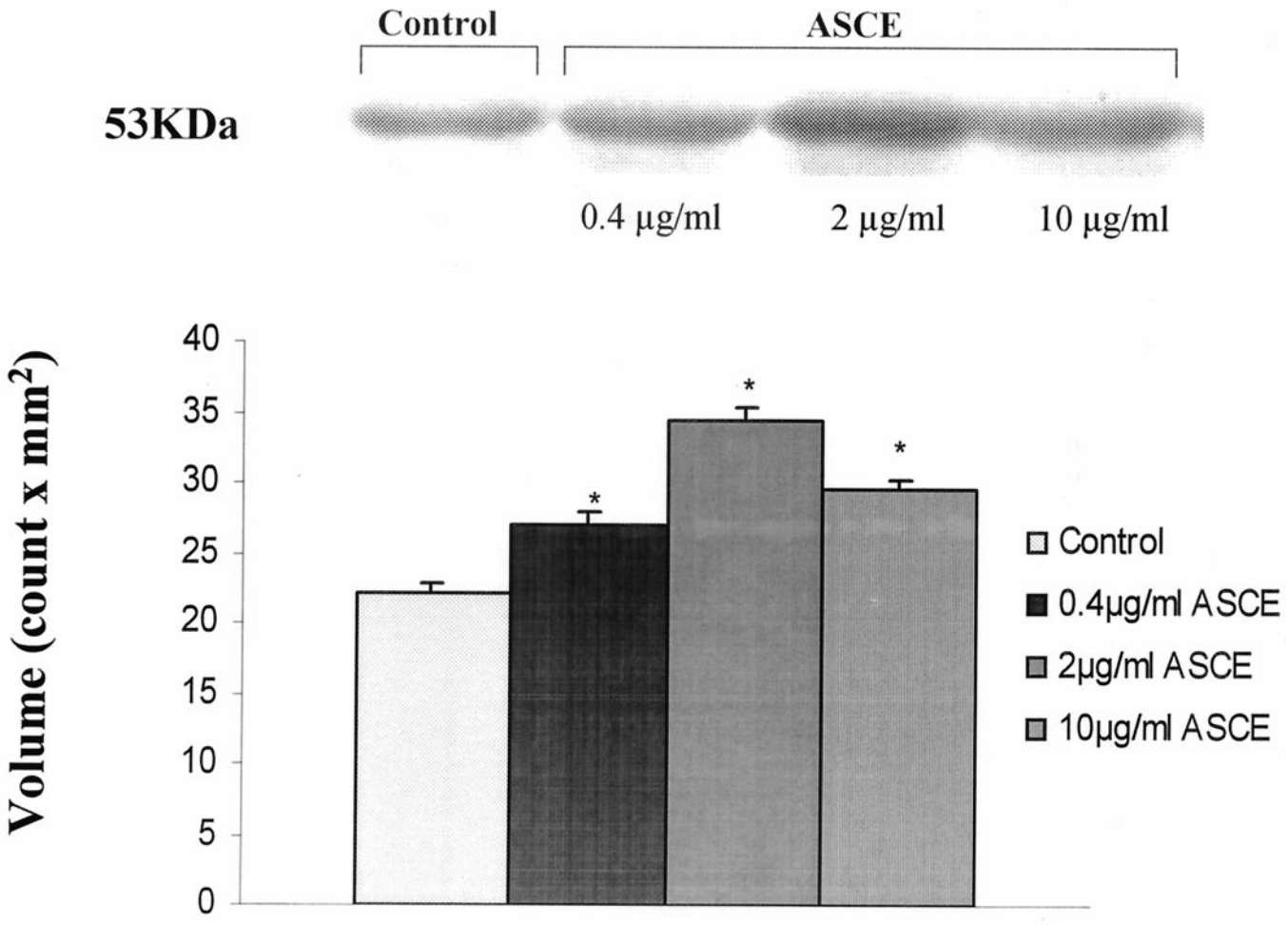

Fig. 5. Western blot analysis for ODC protein expression in RGM-1 cells incubated with different concentrations of ASCE for 5 hr. The photographic films were scanned with a computerized densitometer, and quantitation was carried out by a Bio-Rad image analysis system. Columns and vertical bars represent the means ± SEM of 6 samples. $*P < 0.05$ when compared to the control group.

3.3. Effect of ASCE on [^{3}H]thymidine incorporation in RGM-1 cells

ASCE increased [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 1). Time-course analysis showed a significant stimulatory effect of ASCE at 3 hr (2–10 μg/mL) and 5 hr (0.4–10 μg/mL) after incubation, with the DNA synthesis peaking at the concentration of 2 μg/mL of ASCE.

3.4. Effect of ASCE on ODC activity in RGM-1 cells

It has been reported that ODC is a housekeeping enzyme involved in polyamine synthesis and is necessary for cell proliferation [29]. To determine whether ODC is involved in ASCE-induced cell proliferation in RGM-1 cells, we measured ODC activity. As shown in Fig. 2, ODC activity in RGM-1 cells was significantly increased after treatment with different concentrations of ASCE (0.4, 2, and 10 μg/mL) for 5 hr. The maximal effect appeared at the dose of 2 μg/mL of ASCE.

3.5. [^{3}H]Thymidine incorporation and ODC activity in RGM-1 cells co-treated with ASCE and DFMO

Cell viability was unaffected by DFMO at the concentrations (5 and 10 mM) used in this study. DFMO at 5-mM concentration produced maximum repressive effects on ASCE-stimulated [^{3}H]thymidine incorporation (Fig. 3) and ODC activity (Fig. 4) in RGM-1 cells.

3.6. Effect of ASCE on ODC protein expression in RGM-1 cells

In order to understand whether ASCE stimulates cell proliferation through the activation of ODC activity or ODC protein expression, we measured the ODC protein level by Western blot analysis. ASCE significantly increased ODC protein expression after incubation for 5 hr, with a peak occurring at the concentration of 2 μg/mL (Fig. 5). This is consistent with the result of ODC activity (Fig. 2).

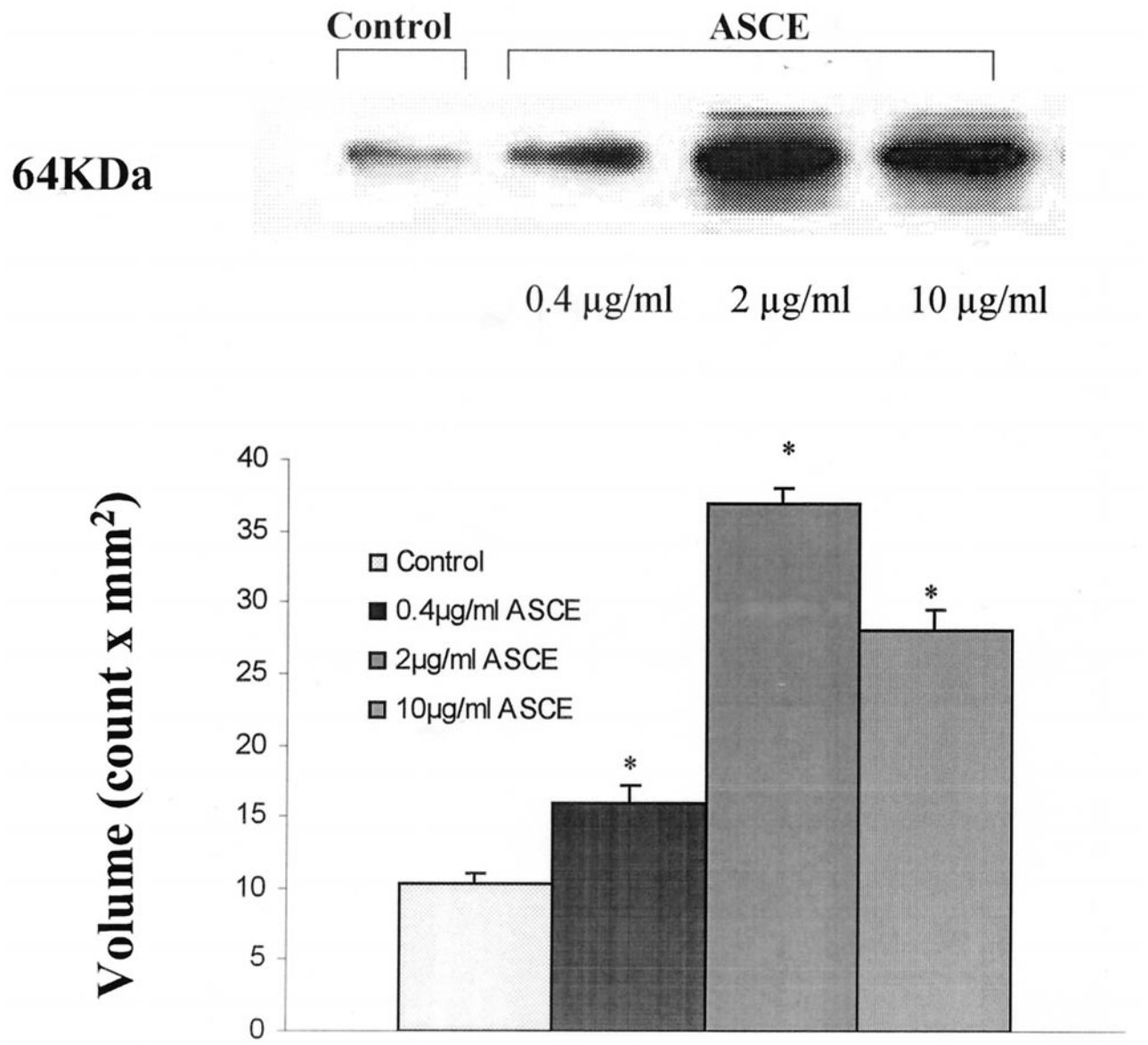

Fig. 6. Western blot analysis for c-Myc protein expression in RGM-1 cells treated with different concentrations of ASCE for 5 hr. The photographic films were scanned with a computerized densitometer, and quantitation was carried out by a Bio-Rad image analysis system. Columns and vertical bars represent means ± SEM of 6 samples. *$P < 0.01$ when compared to the control group.

3.7. Effect of ASCE on c-Myc protein expression in RGM-1 cells

In the immediate early response genes, c-*myc* gene expression is reported not to be restricted to a brief period at the G_0–G_1 transition, but to be continuously expressed in proliferating cells in a cell-cycle-independent manner [29]. Therefore, as a marker of cell proliferation, c-*myc* gene expression was examined. Western blot analysis demonstrated that c-Myc protein expression in RGM-1 cells was greatly increased by ASCE incubation in a dose-dependent fashion (0.4–10 μg/mL). The effect was maximal at the concentration of 2 μg/mL (Fig. 6).

3.8. Effect of c-Myc AS-ODNs on [^{3}H]thymidine incorporation and ODC protein expression

In view of the action of ASCE on c-Myc protein expression, the proliferative effect of ASCE was investigated in the presence of c-Myc AS-ODNs (Fig. 7). The stimulating effect of 2 μg/mL of ASCE on DNA synthesis was significantly inhibited by the presence of 2 μM c-Myc AS-ODNs when compared to both 2 μg/mL of ASCE alone and the scrambled ODN groups (Fig. 7). Pretreatment of cells with 2 μM c-Myc AS-ODNs, but not the scrambled ODNs, significantly blocked the ODC protein expression stimulated by 2 μg/mL of ASCE (Fig. 8).

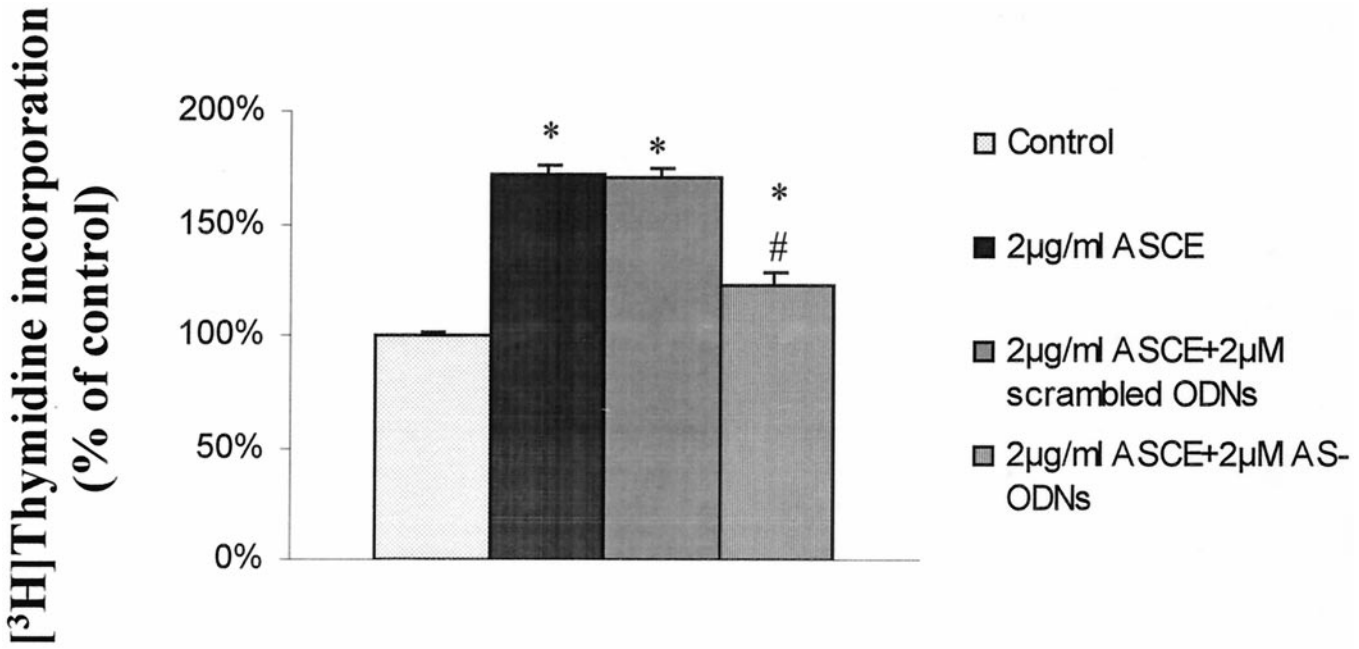

Fig. 7. [^{3}H]Thymidine incorporation into RGM-1 cells co-treated with c-Myc AS-ODNs and ASCE for 5 hr. Columns and vertical bars represent the means ± SEM of 6 samples. $^*P < 0.05$ when compared to the control group, $^{\#}P < 0.05$ when compared to the 2 μg/mL ASCE-treated group.

4. Discussion

ODC, a pyridoxal phosphate-dependent enzyme, is the first rate-limiting enzyme for the biosynthesis of polyamines, including spermine, spermidine, and putrescine, which are essential for the enhancement of DNA replication. It is present in very small amounts in quiescent cells, and its activity can be increased severalfold within a few hours of exposure to trophic stimuli, which include hormones, drugs, tissue regeneration, and growth factors [30–33]. In contrast, cigarette smoking decreased ODC activity and delayed gastric ulcer healing, which was reversed by a polyamine [27]. In the current study, it was observed that ASCE not only significantly increased gastric ulcer healing in rats, but also enhanced wound repair in cultured epithelial cells. The latter finding was fully supported by the increase in [^{3}H]thymidine incorporation (Fig. 1), ODC activity (Fig. 2), and ODC protein expression (Fig. 5) in RGM-1 cells. DFMO, a specific and irreversible inhibitor of ODC [34], repressed the [^{3}H]thymidine incorporation (Fig. 3) and ODC activity (Fig. 4) induced by ASCE. Moreover, pretreatment with c-Myc antisense blocked ODC protein expression (Fig. 8). These results suggested that ASCE induces proliferation of RGM-1 cells at the ODC protein level. The involvement of c-Myc in ODC protein expression was also reported in a recent mechanistic study wherein insulin-induced stimulation of hepatic ODC mRNA expression was accompanied by a concomitant increase in the expression of c-Myc and Max mRNAs [35]. Heterodimers of c-Myc/Max then bind cooperatively to the two adjacent, canonical E-boxes (CACGTG) located in the rat *ODC* gene and induce ODC mRNA expression [36]. All these findings imply that c-Myc could be upstream of ODC activation in the signal transduction pathway.

c-Myc is a transcription factor containing basic, helix–loop–helix, and leucine zipper structural motifs [37,38]. The transcriptional activity depends on its ability to form heterodimers with partners such as Max, and this ability can be prevented by Mad or Max [39–41]. Max is a basic helix–loop–helix/leucine zipper protein that plays a central role in the transcriptional control of myc oncoproteins. Myc–Max heterodimers stimulate transcription, whereas Max homodimers, or heterodimers between Max and members of the Mad family of basic/helix–loop–helix/leucine zipper proteins, repress transcription [42]. The c-Myc–Max heterodimer generates a functional DNA-binding domain that recognizes CACGTG DNA sequences [37,38]. Interaction with Max has been shown to be necessary for most of the physiological effects of c-Myc [43,44].

c-Myc has a central and necessary role in the proliferation of normal cells. Following mitogenic stimulation of quiescent cells, Myc is rapidly induced and remains elevated, suggesting that it is required for continuous cell growth [45]. In the present study, an increase in c-*myc* gene expression was observed 5 hr after exposure to ASCE (Fig. 6). More importantly, the present study also suggested a causal link between the ASCE-induced increase in c-*myc* gene expression and the ASCE-induced increase in cell proliferation, as pretreatment with an AS-ODN directed against c-Myc mRNA blocked the proliferative effects of ASCE (Fig. 7).

AS-ODNs have been used successfully to inhibit the expression of a number of gene products, including the c-*myc* oncogene [46]. Such studies have greatly contributed to the understanding of the cellular functions of these gene

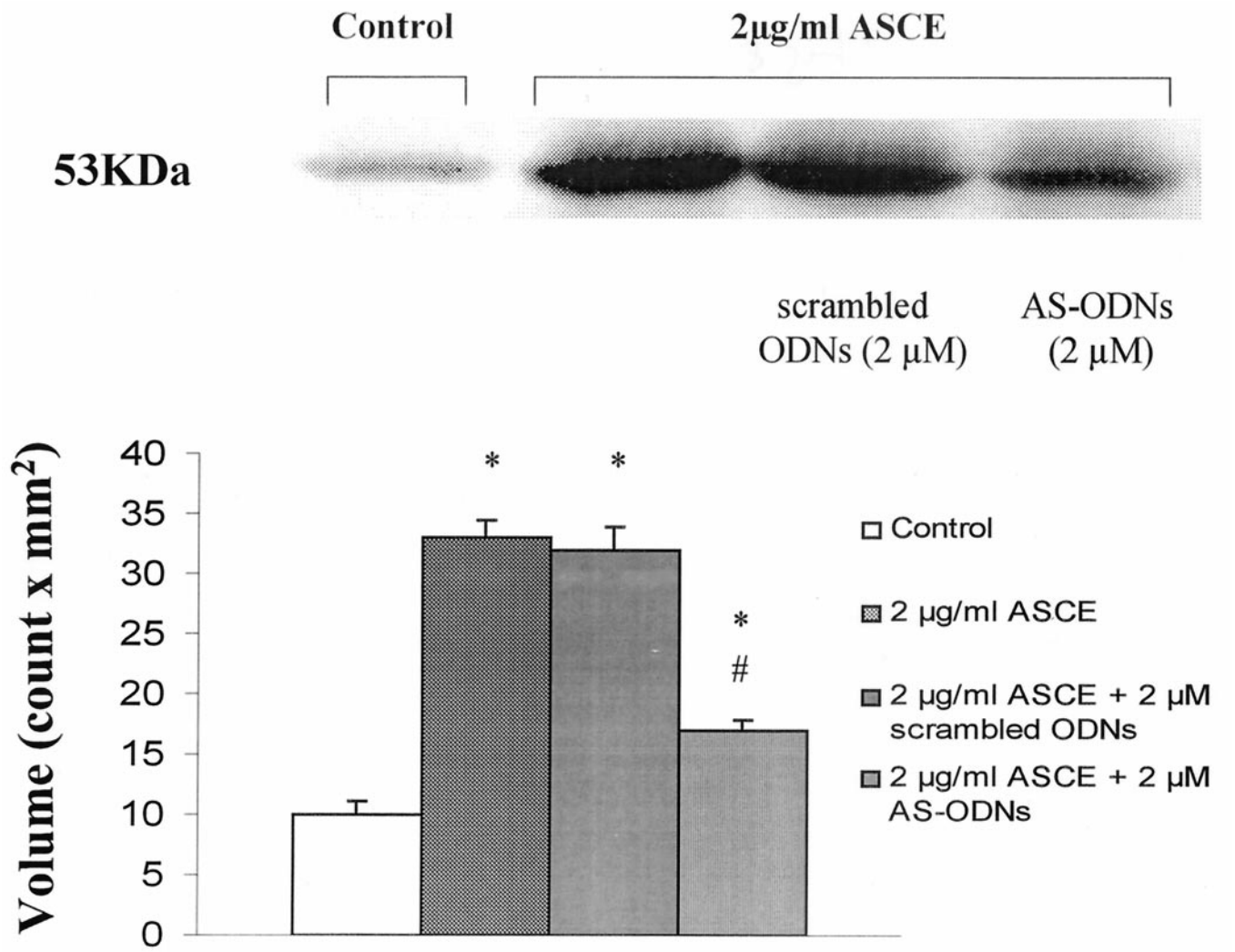

Fig. 8. Western blot analysis for ODC protein expression in RGM-1 cells co-treated with c-Myc AS-ODNs and ASCE for 5 hr. The photographic films were scanned with a computerized densitometer, and quantitation was carried out by a Bio-Rad image analysis system. $^{*}P < 0.001$ when compared to the control group, $^{\#}P < 0.001$ when compared to the 2 μg/mL ASCE-treated group.

products, and in the case of c-Myc, compelling evidence has indicated its critical role in cell proliferation. The proliferation of hematopoietic cells and their progression from the G to S phase of the cell cycle can also be blocked by c-Myc AS-ODNs [47,48]. AS-ODNs also successfully blocked the proliferative action of ASCE on RGM-1 cells, as pretreatment with c-Myc antisense inhibited the ASCE-induced increase in [^{3}H]thymidine incorporation in this cell line (Fig. 7). It would be interesting to investigate whether ASCE can produce a similar effect on c-Myc in cancer cells before the drug can be applied as an anti-ulcer drug in the stomach.

In summary, this study demonstrates that ASCE accelerates wound repair by stimulation of cell proliferation in gastric epithelial cells. Furthermore, it also shows that ODC and c-Myc are involved in this process. ASCE may act through the c-*myc* gene, which activates ODC mRNA and protein expression and stimulates cell proliferation.

Acknowledgments

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