Original Article

Alpha lipoic acid selectively inhibits proliferation and adhesion to fibronectin of v-H-*ras*-transformed 3Y1 cells

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(Received 1 May, 2011; Accepted 8 August, 2011)

Here, we focused on the effects of racemic α-lipoic acid on proliferation and adhesion properties of 3Y1 rat fibroblasts and the v-H-*ras*-transformed derivative, HR-3Y1-2 cells. Racemic α-lipoic acid inhibited proliferation of HR-3Y1-2 but not 3Y1 cells at 0.3 and 1.0 mM. R-(+)-α-lipoic acid also inhibited proliferation of HR-3Y1-2 cells equivalent to that of racemic α-lipoic acid. In addition, racemic α-lipoic acid decreased intracellular reactive oxygen species levels in HR-3Y1 cells but not 3Y1 cells. Next, we evaluated the effects of racemic α-lipoic acid on cell adhesion to fibronectin. The results indicated that racemic α-lipoic acid decreased adhesive ability of HR-3Y1-2 cells to fibronectin-coated plates. As blocking antibody experiment revealed that β1-integrin plays a key role in cell adhesion in this experimental system, the effects of racemic α-lipoic acid on the expression of β1-integrin were examined. The results indicated that racemic α-lipoic acid selectively down-regulated the expression of cell surface β1-integrin expression in HR-3Y1-2 cells. Intriguingly, exogenous hydrogen peroxide up-regulated cell surface β1-integrin expression in 3Y1 cells. Taken together, these data suggest that reduction of intracellular reactive oxygen species levels by α-lipoic acid could be an effective means of ameliorating abnormal growth and adhesive properties in v-H-*ras* transformed cells.

***Key Words*: reactive oxygen species, β1-integrin, abnormal growth, 3Y1 cells**

Ras proteins are oncogene products capable of inducing cell transformation and are associated with many types of human cancer. Indeed, *ras* mutation is recognized in a broad range of human cancers.[1] Wild-type Ras proteins play a central role in the regulation of normal cell proliferation, whereas activation mutation of Ras confers properties of cancer cells, such as deregulated proliferation.[2,3] Recent data attribute this abnormal proliferation of cells with activation mutation of *ras* to over-activation of NADPH oxidase (Nox1) and resulting reactive oxygen species (ROS) production.[4–7] The H-*ras* oncogene induces constitutive expression of Nox1 through the Raf-MEK-MAPK pathway and Nox1-generated ROS have an essential mediating role in *ras* oncogene transformation phenotypes, including augmented cell growth, altered cell morphology, anchorage-independent growth, and tumorigenesis.[8] The HR-3Y1-2 cell line established by Kimura *et al.*[9] is derived from 3Y1 rat fibroblasts transformed with the v-H-*ras* oncogene. Anchorage-independent proliferation and changes in cellular morphology are known features of 3Y1 cells transformed with v-H-*ras*,[10] and our previous study showed that HR-3Y1-2 cells are capable of proliferating faster than the parental cell line 3Y1.[11] Therefore, these cell lines are useful for evaluation of the effects of various treatments on the malignant characteristics associated with Ras activation. In addition, ROS production could control the integrin-related cancer migration, which means that the adhesive ability to extracellular matrix is maintained in the normal state under the control of cellular redox conditions.[12] Interfering with tumor cell attachment with integrin-binding peptides has been shown to be an effective strategy for antimetastatic therapy.[13]

Alpha-lipoic acid (6,8-dithio octanoic acid; ALA) is a naturally occurring antioxidative compound and is essential in humans, functioning as a coenzyme in various biological processes. ALA is widely distributed as lipoyllysine in vegetables and animal tissues.[14] Due to its potent antioxidant activity *in vitro* and *in vivo*, it is utilized as a preventive agent in diabetes mellitus, hypertension, and hepatic disorders.[15–17] Therefore, ALA is a promising agent for amelioration of activated *ras*-derived malignant characteristics by elimination of ROS. To evaluate this hypothesis, we evaluated the effects of ALA on proliferation and cell adhesion of 3Y1 and HR-3Y1-2 cells.

Materials and Methods

Chemicals. Racemic or *R*-(+)-α-Lipoic acid (*rac*-ALA or *R*-(+)-ALA) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO).

Cell culture. 3Y1 (also called 3Y1-B clone 1-6) and HR-3Y1-2 cells were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum containing 100 units/mL of penicillin G and 100 μg/mL of streptomycin. Cells were subcultured twice a week, and in actual *in vitro* experiments, the cells were seeded at 3.5×10^3 cells/cm^2 in 90-mm dishes or 24-well multiwell culture plates.

Cell cycle analysis. For cell cycle analysis, cells were treated with or without 1.0 mM *rac*-ALA for 24 h and the percentage of each cell cycle population was evaluated by flow cytometric analysis. Briefly, cells were fixed in 70% methanol for 1 h and 50% ethanol at 4°C overnight. Then, the nuclei were treated with 10 μg/ml propidium iodide and 10 μg/ml RNase. After staining, cell cycle analysis was performed using a COULTER Epics XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA) with MultiCycle software (San Diego, CA).

Intracellular redox state. At the end of the culture period, cells were washed with ice-cold PBS and stained with 50 μM DCFH-DA for 30 min at 37°C. After staining, cells were subjected to flow cytometric analysis (EPICS XL; Beckman Coulter).

Cell adhesion to fibronectin. Cells at growth phase were

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doi: 10.3164/jcbn.11-67

treated with serum-free DMEM for 60 min at 37°C. The cells pretreated with or without *rac*-ALA were then recovered and seeded at 2.0×10^5 cells/well in fibronectin (Fn)-coated 24-well plates (BD Biosciences, San Jose, CA). After 60 min, floating cells were removed from the well and adherent cells were counted. In an antibody blocking experiment, cells were coincubated with 50 μg/mL of purified hamster anti-rat CD29 (Invitrogen, Carlsbad, CA) or hamster IgM (Beckman Coulter) as an isotype control.

Western blotting analysis. Whole cell β1-integrin and β-actin were detected by Western blotting analysis. At the end of the culture period, cells were lysed in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 2% Triton X-100, 2 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, and 1/50 vol. protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL). Lysates containing 10 μg of protein were separated by electrophoresis on 10% SDS-polyacrylamide gels, and transferred onto PVDF Hybond-P membranes (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Blocking was performed using 3% defatted milk in Tris-buffered saline with 0.1% Tween-20 (TTBS), and antibodies were diluted in Can Get Signal solutions 1 and 2 (Toyobo, Tokyo, Japan). Anti-β1-integrin (N-20) and horseradish peroxidase-conjugated donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were washed with TTBS after each antibody binding reaction. Detection of each protein was performed using an ECL Plus kit (Amersham-Pharmacia).

Cell surface β1-integrin expression. At the end of the culture period, cells were washed with cold PBS, then incubated with 2 μg/mL of hamster anti-mouse CD29 FITC-conjugate (clone HM beta 1-1, AbD Serotec, Kidlington, UK) for 30 min at 4°C. Cells were washed and resuspend into 2% FBS, 2 mM

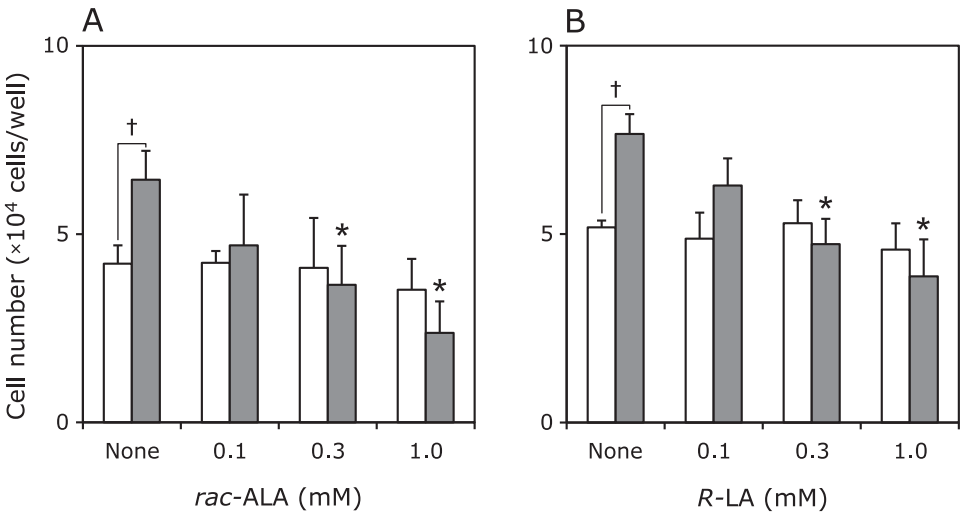

Fig. 1. Effects of ALA on proliferation of 3Y1 or HR-3Y1-2 cells. Cultures were treated with 0, 0.1, 0.3, 1.0 mM *rac*-ALA (A) or *R*-(+)-ALA (B) for 24 h. Results are means ± SD of 3 samples. Values marked with a dagger mark or asterisk are significantly different from the data in 3Y1 control or control value in each cell line at Values marked with a dagger mark or asterisk are significantly different from the data in 3Y1 control or control value in each cell line at $p<0.05$, respectively. Open column shows 3Y1 cells and shaded column shows HR-3Y1-2 cells and "None" means that cells were not treated with ALA.

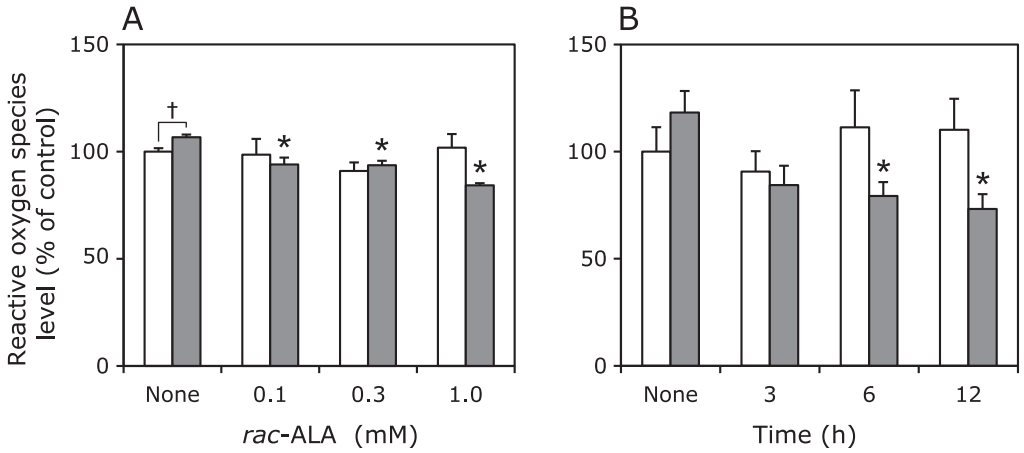

Fig. 2. Intracellular ROS levels in 3Y1 and HR-3Y1-2 cells. Cells were treated with 0, 0.1, 0.3, 1.0 mM *rac*-ALA for 12 h (A), 0, 1.0 mM *rac*-ALA for 0, 3, 6, 12 h (B). The production of ROS was measured using the oxidation-sensitive fluorescent probe 2'7'-dichlorodihydrofluorescin diacetate. Results are means ± SD of 3 independent experiments. Values marked with a dagger mark or asterisk are significantly different from the data in 3Y1 control or control value in each cell line at $p<0.05$, respectively. Open column shows 3Y1 cells and shaded column shows HR-3Y1-2 cells and "None" means that cells were not treated with *rac*-ALA.

Table 1. Effect of racemic alpha-lipoic acid on the cell cycle progression of 3Y1 cells and HR-3Y1-2 cells

		rac-ALA (mM)			
		None	0.1	0.3	1
3Y1 Distribution (%)	G1	50.6 ± 0.6	51.3 ± 2.6	54.3 ± 2.2	54.6 ± 2.1
	S	39.7 ± 1.7	38.9 ± 4.0	34.8 ± 4.7	35.7 ± 0.5
	G2/M	9.5 ± 2.3	9.6 ± 2.1	10.7 ± 2.6	9.5 ± 2.0
HR-3Y1-2 Distribution (%)	G1	33.9 ± 0.2	35.3 ± 0.4	38.9 ± 0.8*	45.0 ± 0.7*
	S	54.0 ± 2.7	54.5 ± 0.4	49.9 ± 3.0	45.4 ± 3.4*
	G2/M	12.0 ± 2.6	10.0 ± 0.7	11.0 ± 3.4	9.5 ± 2.8

Data are means ± SD for 3 samples. Values with asterisk mark(s) are significantly different from the None in each cell cycle phase.

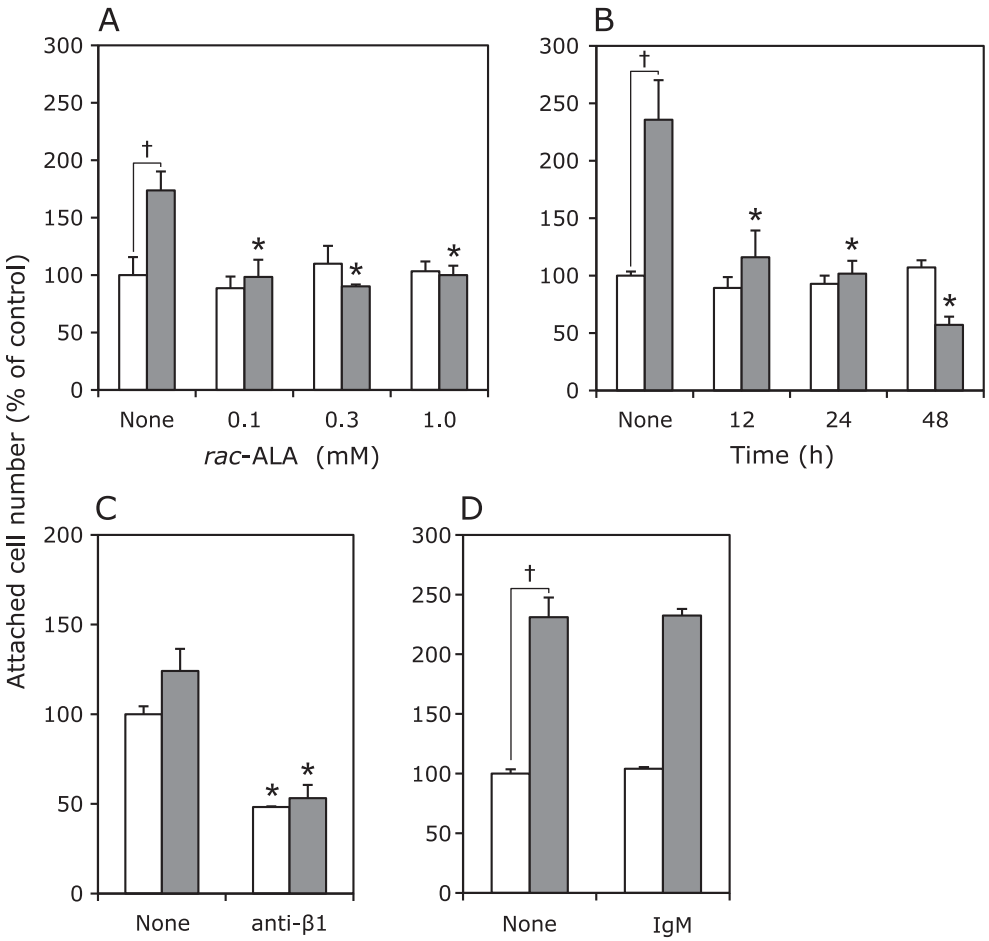

Fig. 3. Adhesion of 3Y1 and HR-3Y1-2 cells to fibronectin. Cells were treated with 0, 0.1, 0.3, 1.0 mM *rac*-ALA for 48 h (A) or 0, 1.0 mM *rac*-ALA for 0, 12, 24, 48 h (B) and then incubated in fibronectin-coated plates at 37°C for 60 min. In the antibody blocking experiment, cells were incubated with anti-β1-integrin antibody (50 μg/mL) (C) or hamster IgM (50 μg/mL) as an isotype control (D). Floating cells were removed and adherent cells were counted. Results are means ± SD of 3 samples. Values marked with a dagger mark or asterisk are significantly different from the data in 3Y1 control or control value in each cell line at $p<0.05$, respectively. Open column shows 3Y1 cells and shaded column shows HR-3Y1-2 cells and "None" means that cells were not treated with *rac*-ALA.

EDTA/PBS and subjected flow cytometric analysis (EPICS XL). Cells were stained with 10 μg/ml propidium iodide to eliminate dead cell from analysis.

Statistical analysis. Statistical analysis was done with 4 Steps Statcel2 software (OMS Publishing, Saitama, Japan). Data were analyzed by the Tukey-Kramer test, and differences at $p<0.05$ were considered significant.

Results

Growth inhibition and cell cycle anaysis. First, we evaluated the effects of *rac*-ALA on the growth of 3Y1 and HR-3Y1-2 cells. The results indicated that 0.3 and 1.0 mM *rac*-ALA significantly prevented the growth of HR-3Y1-2 but not 3Y1 cells at 24 h. (Fig. 1A). To know whether two enantiometric forms of ALA has different effect on the proliferation of HR-3Y1-2 cells,

doi: 10.3164/jcbn.11-67

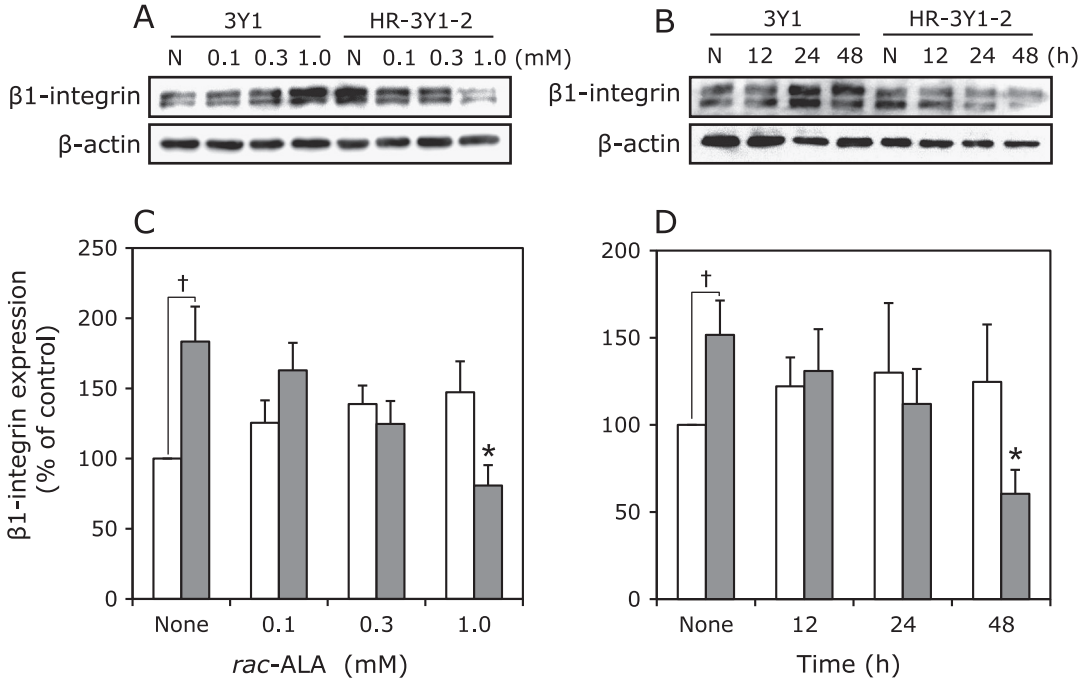

Fig. 4. β1-Integrin protein expression in 3Y1 and HR-3Y1-2 cells. Cells were treated with 0, 0.1, 0.3, 1.0 mM *rac*-ALA for 48 h (A, C) or 0, 1.0 mM *rac*-ALA for 0, 12, 24, 48 h (B, D). β1-Integrin protein was detected by western blotting and representative image data were shown in A and B, density of each band was quantified as a ratio of β1-integrin/β-actin and shown in C and D. The methods are described in detail in Materials and Methods. Results in C and D are means ± SD of 3 independent experiments. Values marked with a dagger mark or asterisk are significantly different from the data in 3Y1 control or control value in each cell line at $p<0.05$, respectively. Open column shows 3Y1 cells and shaded column shows HR-3Y1-2 cells and "None" and "N" mean that cells were not treated with *rac*-ALA.

cells were treated with 0, 0.1, 0.3 and 1.0 mM *R*-(+)-ALA. As a result, *R*-(+)-ALA also inhibited proliferation of HR-3Y1-2 equivalent to that of *rac*-ALA. To determine if *rac*-ALA regulated cell cycle progression, cells were treated with 0 to 1.0 mM *rac*-ALA and subjected to cell cycle analysis. Results showed that the G1 phase cell population was increased with decrease of S phase distribution at 0.3 and 1.0 mM *rac*-ALA treatment in HR-3Y1-2 cells (Table 1), whereas *rac*-ALA did not affect the cell cycle progression on the 3Y1 cells.

Intracellular ROS level. To evaluate the relationship between ROS accumulation and HR-3Y1-2-selective inhibition of proliferation, cells were stained with DCFH-DA to determine intracellular ROS levels in 3Y1 and HR-3Y1-2 cells. Intracellular ROS levels were slightly higher in HR-3Y1-2 cells than in 3Y1 cells. The results indicated that *rac*-ALA at 0.1, 0.3 and 1.0 mM treatment for 12 h caused a substantial decrease in intracellular ROS level at 12 h HR-3Y1-2 cells (Fig. 2 A and B). In contrast, *rac*-ALA induced no changes in 3Y1 cells.

Cell adhesion to fibronectin. As *rac*-ALA selectively inhibited the proliferation of HR-3Y1-2 cells and cellular ROS level, we next evaluated the effects of *rac*-ALA on integrin-related cell adhesion. As shown in Fig. 3, HR-3Y1-2 cells had greater adhesive ability to fibronectin-coated plates. HR-3Y1-2 cells pretreated with 0.1, 0.3 and 1.0 mM *rac*-ALA for 48 h (Fig. 3A) or 1.0 mM for 12, 24 and 48 h (Fig. 3B) showed reduced adhesion to fibronectin-coated plates. On the other hand, *rac*-ALA did not affect the adhesive ability of 3Y1 cells (Fig. 3 A and B). β1-Integrin blocking antibody inhibited the adhesion of 3Y1 and HR-3Y1-2 cells by 51% and 57%, respectively (Fig. 3C). Hamster IgM used as an isotype control did not affect adhesive ability of both cell lines (Fig. 3D). This result indicated that adhesion of these cell lines to fibronectin is due to β1-integrin and fibronectin interaction.

Whole cell β1-integrin expression. As shown in Fig. 3, β1-integrin plays a pivotal role in adhesion to fibronectin in these cell lines. We next examined whether *rac*-ALA could downregulate β1-integrin expression in HR-3Y1-2 cells. Fig. 4 A and B show representative blotting pictures of β1-integrin expression and intensity of bands on a blot was quantified and shown in Fig. 4 C and D. Basically, untreated HR-3Y1-2 cells expressed higher levels of β1-integrin than 3Y1 cells. *rac*-ALA at 1.0 mM but not at 0.1 and 0.3 mM significantly downregulated the expression of β1-integrin at 48 h in HR-3Y1-2 cells, whereas its expression was unaffected by *rac*-ALA treatment in 3Y1 cells.

Cell surface β1-integrin expression. Next, cell surface β1-integrin expression was also examined. HR-3Y1-2 cells treated with 0.1, 0.3 and 1.0 mM *rac*-ALA for 48 h (Fig. 5A) or 1.0 mM for 12, 24 and 48 h (Fig. 5B) showed reduced expression of cell surface β1-integrin. To reveal whether exogenous oxidative stress evokes HR-3Y1-2 like phenotype in 3Y1 cells, cell surface β1-integrin expression in 3Y1 cells treated with hydrogen peroxide. Result showed exogenous hydrogen peroxide dose-dependently unregulated cell surface β1-integrin expression in 3Y1 cells and cells treated with 100 μM hydrogen peroxide for 48 h expressed comparable level of cell surface β1-integrin with HR-3Y1-2 cells (Fig. 5C).

Discussion

Ras is known to play a pivotal role in regulating cell proliferation by activation of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways,[2,3] and mutations leading to activation of these cascades result in abnormal growth. There is accumulating evidence that activation mutation of H-*ras* triggers the activation of Nox1 and the resultant ROS production plays important roles in the malignant characteristics of *ras*-transformed

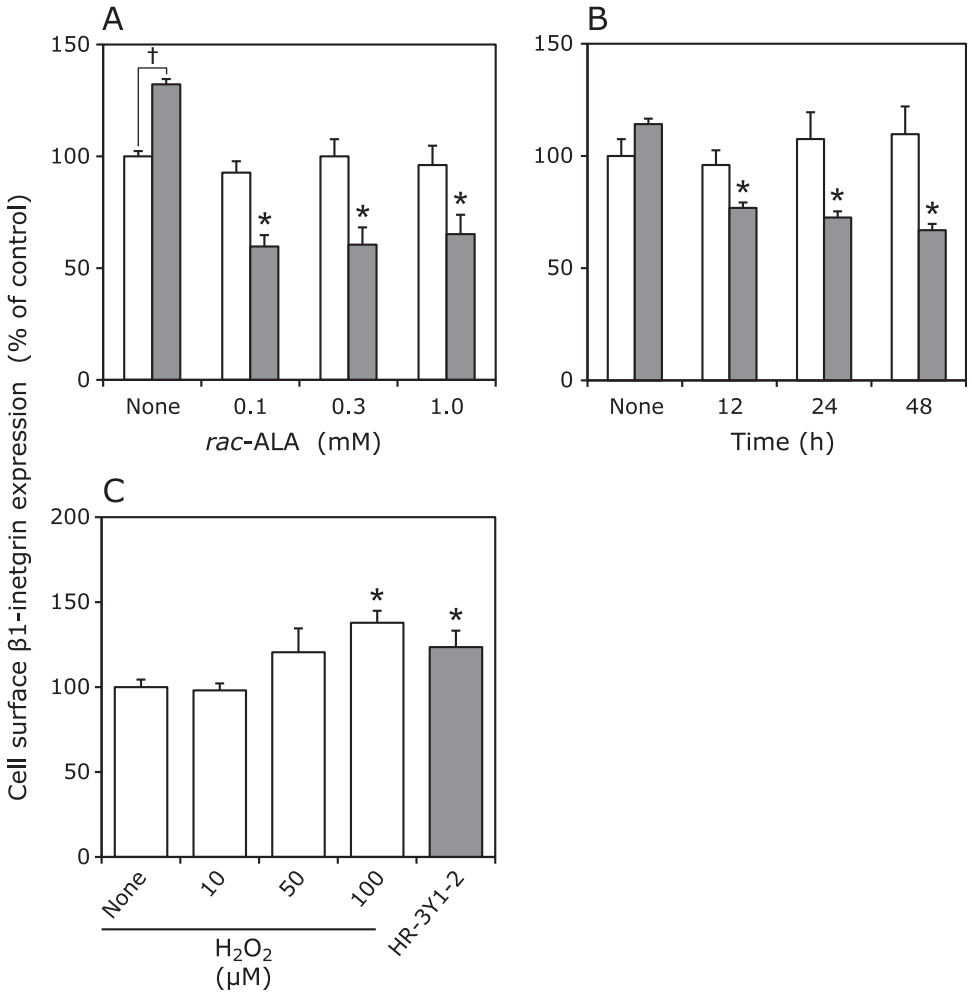

Fig. 5. Cell surface β1-integrin protein expression in 3Y1 and HR-3Y1-2 cells. Cells were treated with 0, 0.1, 0.3, 1.0 mM *rac*-ALA for 48 h (A), 0, 1.0 mM *rac*-ALA for 0, 12, 24, 48 h (B) or 0, 10, 50, 100 μM hydrogen peroxide for 48 h (C). The methods are described in detail in Materials and Methods. Results in A–C are means ± SD of 3 independent experiments. Values marked with a dagger or asterisk(s) are significantly different from the control of 3Y1 cells or control of each cell line at $p<0.05$, respectively. Open column shows 3Y1 cells and shaded column shows HR-3Y1-2 cells and "None" means that cells were not treated with *rac*-ALA.

cells.[4–7] These results provide new insight into the mechanism by which antioxidants could decrease excess intracellular ROS then inhibit abnormal proliferation. Our data clearly showed that HR-3Y1-2 cells have higher levels of intracellular ROS than 3Y1 cells. In addition, the growth rate, ability to adhere to fibronectin, and β1-integrin expression of HR-3Y1-2 cells were higher than those of 3Y1 cells. ROS behaves as a second messenger to activate the ERK pathway, which is a representative mitogenic signaling pathway.[18] Therefore, the observation that HR-3Y1-2 cells could proliferate faster than 3Y1 cells is convincing. Therefore, 3Y1 and HR-3Y1-2 cells are considered appropriate cell lines for evaluation of the effects of antioxidants on v-H-*ras* mediated transformation.

Several studies have shown that ALA elicits apoptotic cell death or growth arrest in several cancer cell lines.[19–22] However, the detailed mechanism remains to be elucidated. On the other hand, ALA is capable of preventing apoptotic cell death under conditions of oxidative stress because of its antioxidant activity.[23,24] The results of the present study indicated that *rac*-ALA down-regulates intracellular ROS level accompanied by inhibition of cell proliferation in HR-3Y1-2 cells, whereas *rac*-ALA does not affect ROS level or cell proliferation of 3Y1 cells at 1.0 mM. In addition, our present data revealed that *R*-(+)-ALA and *rac*-ALA had comparable ability of inhibiting proliferation of HR-3Y1-2 cells, suggesting naturally occurring ALA is capable of inhibiting the proliferation of v-H-*ras* transformed cells. Since, Smith *et al.*[25] reported that protective effect against oxidative damage of ALA is different among the type of optical isomer and cell type, our subject for further study is to demonstrate the effect of ALA on various v-H-*ras* transformed cells and apply to human cancer cells with H-*ras* mutation.

As shown in Table 1, growth-inhibitory action of *rac*-ALA on HR-3Y1-2 cells is attributable to cell cycle delay at G1 phase. Such reduction of intracellular ROS may be explained on the basis of several previous reports. First, ALA and its reduced form, dihydrolipoic acid (DHLA), are capable of scavenging a variety of ROS and reactive nitrogen species.[26] Moreover, as the reduction of ALA to DHLA requires NADPH consumption,[27] NADPH starvation may indirectly prevent NADPH oxidase-derived ROS production. Secondly, LA appears to be a potent inducer of Nrf 2-mediated antioxidant gene expression, which leads to increase or maintenance of intracellular GSH.[28,29] Actually, Moini *et al.*[30] revealed that *R*-(+)-LA significantly increased cellular reduced glutathione level and at the same time points the

doi: 10.3164/jcbn.11-67

intracellular level of oxidants was decreased.

Excess intracellular ROS controls cancer metastasis and invasion by disruption of normal expression of adhesive molecules. For example, upregulation of intracellular ROS by *Helicobacter pylori* infection leads to an increase in β1-integrin expression.[31] Our data also indicated that whole cell and cell surface β1-integrin expression is upregulated in HR-3Y1-2 cells to a greater extent than 3Y1 cells. β1-Integrin can form heterodimers with αv, α5 and α8 chain, and these complexes have been implicated in interaction with fibronectin. Therefore, upregulation of β1-integrin expression in HR-3Y1-2 cells is considered to result in high adhesive ability to fibronectin. Especially, some tumor cells express high levels of α4β1 and α5β1 integrin in association with malignant phenotypes, such as metastasis and anchorage-independent proliferation. Consistent with these reports, our data indicated that HR-3Y1-2 cells had higher ability to adhere to fibronectin than 3Y1 cells. Moreover, as the blocking experiment indicated that adhesion of 3Y1 and HR-3Y1-2 cells was substantially prevented by anti-β1-integrin antibody, β1-integrin plays a pivotal role in adhesion with fibronectin in both cell lines. Intriguingly, *rac*-ALA treatment prevented adhesion of HR-3Y1-2 cells to fibronectin at 0.1, 0.3 and 1.0 mM (Fig. 3) with downregulation of cell surface β1-expression (Fig. 5). On the other hand, although 1.0 mM *rac*-ALA surely downregulated whole cell β1-integrin expression in HR-3Y1-2, low concentrations of *rac*-ALA (0.1, 0.3 mM) could not. These data suggest that *rac*-ALA reduced adhesive ability of HR-3Y1-2 cells to fibronectin through downregulation of cell surface β1-integrin expression by regulating its cellular localization. It is increasingly evident that cell surface β1-integrin level is regulated by its dynamic internalization and recycling process.[32,33] As downregulation of cell surface β1-integrin by *rac*-ALA was apparent after 12 h of treatment as shown in Fig. 5, which was after ROS inhibition, downregulation of cell surface β1-integrin expression in HR-3Y1-2 cells is dependent on ROS-related pathways. Moreover, exogenous hydrogen peroxide is capable of upregulating cell surface β1-integrin expression in 3Y1 cells (Fig. 5C). On the contrary, exogenous hydrogen peroxide downregulates β1-integrin expression in promyelocytic leukemia.[34] These information indicate that intracellular ROS manipulates β1-integrin expression.

We can't avoid discussing about another plausible mechanism of ALA. ALA may directly interact with cysteine residue of the proteins involved in various cellular signaling pathways. For instance, ALA downregulates phosphatases such as PP2A through the redox modulation of cysteine residues because ALA occurs thiol-disulfide exchange between ALA and protein sulfhydryls.[29] As PP2A has positive role in H-*ras* dependent activation of Raf-1 which consequently activates ERK pathway,[35] ALA promises downregulation of ERK pathway through inactivation of PP2A. On the other hand, as cysteine-rich-domain in Raf-1 protein is also involved its activation and ERK2 also has cysteine residue in its active site, the interaction between ALA and cysteine residue(s) in these or other signaling proteins is mostly unknown. Further studies are needed to focus on the molecular mechanism of ALA including redox modulation of signaling molecules.

Taken together, ALA is a promising agent to decrease intracellular ROS and ameliorate the malignant characteristics of v-Ha-*ras*-transformed cells.

Acknowledgments

This work is supported by Grant-in Aid Scientific Research (C) No. 20580137.

Abbreviations

ALA	α-lipoic acid
DCF	2',7'-dichlorofluorescin
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
ROS	reactive oxygen species

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doi: 10.3164/jcbn.11-67