Our previous study showed α-lipoic acid (LA) downregulated cell surface β1-integrin expression of v-H-ras-transformed derivative of rat fibroblast with amelioration of their malignant phenotype. Here, we evaluated the ameliorating effect of LA on the malignant characters in H-ras-transformed bladder cancer cells. H-ras mutated bladder cancer line, T24 cells were incubated with LA to evaluate the inhibitory effect on proliferation, migration, invasion and β1-integrin expression. Fluorescence staining of F-actin and western blotting analyses of the related signaling pathways were also performed. LA inhibited the proliferation of T24 cells. Cell adhesion to collagen IV and fibronectin was strikingly inhibited by LA treatment accompanied by downregulation of cell surface but not whole cell β1-integrin expression. LA clearly inhibited cell migration and invasion of T24 cells, which were mimicked by extracellular signal-regulated kinase (ERK) and Akt pathway inhibition. Actually, LA significantly downregulated the phosphorylated ERK and Akt levels. Moreover, LA downregulated phosphorylated focal adhesion kinase level with disappearance of stress fiber formation. Finally, although LA induced the internalization of cell surface β1-integrin, disruption of the raft did not affect the action of LA. Taken together, LA is a promising agent to improve malignant character of bladder cancer cells through regulation of cellular β1-integrin localization.

Key Words: bladder cancer cells, α-lipoic acid, invasion, migration, β1-integrin

R as 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. 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, abnormal motility and differentiation. For instance, the HR-3Y1-2 cell line, derived from 3Y1 rat fibroblasts transformed with the v-H-ras oncogene, show anchorage-independent proliferation and changes in cellular morphology, and HR-3Y1-2 cells are capable of proliferating faster than the parental cell line 3Y1. Therefore, a component that can suppress of activated Ras can be promising agent for alleviation of malignant characters of cancer cells. In our previous study in which we explored the components that was capable of suppressing the proliferation of HR-3Y1-2 cells, α-lipoic acid (LA) was shown to selectively inhibit the proliferation of HR-3Y1-2 cells. Furthermore, LA potently suppressed the migration and invasion of HR-3Y1-2 cells accompanied with downregulation of β1-integrin expression. Therefore, LA is a promising agent for alleviation of malignant characters in ras mutated cancer cells.

LA is a naturally occurring antioxidative compound and is essential in humans, functioning as a coenzyme in various biological processes. Accumulating data have been revealed that LA has cytotoxic effect and migration inhibitory effect on cancer cells. However, their detail molecular mechanisms are not fully understood. Frequency of mutation of ras family such as H-ras, K-ras and N-ras in human tumors is highly organ specific and H-ras mutation is common in bladder cancer. Here, we evaluated the effect of LA on the proliferation, migration and invasion of bladder cancer cells. Moreover, involvement of β1-integrin in the action of LA was also evaluated.

Materials and Methods

Chemicals. LA was purchased from Sigma (St. Louis, MO). PD98059 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and LY294002 was purchased from Cell Signaling Technology (Beverly, MA). Methyl-β-cyclodextrin was purchased from Tokyo Chemical Industries (Tokyo, Japan).

Cell culture. T24 human bladder cancer cell line was purchased from the Japanese Collection of Research Bioresources (JCRB0711, Osaka, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 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 adhesion to fibronectin. Cells at growth phase were treated with FBS-free DMEM for 60 min at 37°C. The cells pretreated with or without LA were then recovered and seeded at 2.0 × 10^4 cells/well in fibronectin-coated or collagen IV-coated 24-well plates (BD Biosciences, San Jose, CA). After 60 min, floating cells were removed from the well and adherent cells were counted.

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 Na3P4O10 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). Anti-extracellular signal-regulated kinase (ERK), anti-Akt, and anti-phosphorylated focal adhesion kinase (FAK) antibodies were purchased from Cell Signaling Technology and anti-FAK antibody was purchased form Assay Bio Technology (Sunnyvale, CA). The membranes were washed with TTBS after each antibody binding reaction. Detection of each protein was performed using an ECL Prime kit (Amersham-Pharmacia).

**Cell surface β1-integrin expression.** At the end of the culture period, cells were washed with cold phosphate buffered saline (PBS), then incubated with FITC-conjugated anti-β1-integrin (Santa Cruz Biotechnology) for 30 min at 4°C. Cells were washed and resuspended into 2% FBS, 2 mM EDTA/PBS and subjected flow cytometric analysis (EPICS XL, Beckman Coulter, Fullerton, CA). Cells were stained with 10 μg/ml propidium iodide to eliminate dead cell from analysis.

**Cell migration.** Cells were cultured to confluence and dish bottom was scratched with plastic 200 μl tip to remove the cells. Then, cells were cultured with serum-free media containing LA or inhibitors for 3–12 h. After taking photographs of scratched area, area of migrated cells was calculated to evaluate the migration. The extent of migration was measured by calculating the area occupied by the cells in a scratched area, the data showing 100% when the scratched area was completely filled with migrated cells.

**Cell invasion.** Cells were pretreated with LA or inhibitors for indicated time and concentration, and additionally incubated with FBS free medium for 2 h. Then, cells suspended into FBS-free media were inoculated into matrigel coated cell insert chamber 24-well transwell chamber (8 μm pore size). Lower chamber of transwell was filled with the DMEM with 5% FBS. At the end of culture period, transwells were removed from 24-well plate, then lower surface was treated with 0.25% trypsin-EDTA and migrated cells were counted.

**Fluorescence staining.** Cells were cultured and treated with LA in glass bottom dish. At the end of culture period, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Then, cell membrane was permeablized using 0.5% Triton-X100/PBS for 5 min. F-actin was stained using commercial kit (Acti-stain 488, Cytoskelton, Denver, CO) according to the appended protocol. For immunostaining, after cell permeabilization, blocking was performed using 10% bovine serum albumin/PBS for 20 min. Thereafter, cells were stained with FITC-conjugated anti-β1-integrin (Santa Cruz Biotechnology), and finally, nuclei were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan).

**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 \( p<0.05 \), 0.01** were considered significant.

**Results**

First, we evaluated the effects of LA on the growth of T24 cells. The results indicated that 0.3 and 1.0 mM LA significantly prevented the growth of T24 cells at 24 and 48 h. (Fig. 1A and B).

As LA inhibited the proliferation of T24 cells as previously shown in v-H-ras transformed rat fibroblast, we next evaluated the effects of LA on β1-integrin expression and β1-integrin related cell adhesion. As shown in Fig. 2A and B, western blotting data showed LA did not affect whole cell integrin level at each time point (1.0 mM). On the other hand, flowcytometric analysis showed that LA dose-dependently downregulated cell surface integrin-β1 expression, whereas no obvious time-dependency was detected during 12–48 h (Fig. 2C and D). To know whether downregulation of cell surface integrin-β1 evokes a substantial decrease of cell adhesion to extracellular matrixes (ECMs), we examined the effect of LA on cell adhesion to collagen IV and fibronectin both of which are known to bind with integrin composed of β1 subunit. Adhesion to these ECMs are significantly inhibited by 48 h pretreatment with 0.3 and 1.0 mM or by 24 and 48 h pretreatment with 1.0 mM LA (Fig. 2E and F).

Integrin mediated cell adhesion activates FAK and following polymerization of G-actin. F-actin, a polymerized G-actin, filament bundle is component of stress fiber that provides a tension and contraction to the cells and regulates cell motility. Next, we tried to know the effect of LA on FAK activation and stress fiber formation. As shown in Fig. 3A and B, LA downregulated phosphorylated FAK level. When cells were treated with 1.0 mM LA, phosphorylated FAK level was significantly decreased at 12, 24 and 48 h. In Fig. 3C, F-actin was stained with phalloidin and shown in green color. Here, structured stress fiber was observed in the control cells, whereas cellular edge was clearly stained and obvious stress fiber was not observed in LA treated cells.

As stress fiber formation is an important driving force for cell motility, LA is expected to inhibit cell migration and invasion of T24 cells. As shown in Fig. 4A and B, wounding assay revealed T24 cells without any treatment actively migrated during 12 h assay period. In this wounding assay, cells were co-cultured with shown concentrations of LA during cell migration. LA dose-dependently inhibited cell migration, and representative photographs (Fig. 4A) and Fig. 4B showed 1.0 mM LA treatment
almost completely inhibited the migration of T24 cells. Moreover, invasion assay revealed that 0.3 and 1.0 mM LA pretreatment significantly inhibited T24 cell invasion ability at 24 and 48 h (Fig. 4C).

It is well-known that FAK activation leads to activation of its downstream signaling cascade ERK and Akt pathways that play pivotal role in regulating cell motility. We next tried to know the effect of LA on the phosphorylation of ERK and Akt. As shown in Fig. 5E, 40 μM PD98059 and LY294002 strikingly inhibited cell invasion of T24 cells which was comparable to that of LA. Moreover, these inhibitors significantly inhibited cell migration of T24 cells. Although, at 3 h treatment, inhibition ability was comparable among PD98059, LY294002 and LA, LA exerted the strongest ability at 6 and 12 h (Fig. 5F). Furthermore, these inhibitors

Fig. 2. α-lipoic acid reduces cell surface β1-integrin level and suppresses adhesion to collagen IV and fibronectin. (A, B) Effect of α-lipoic acid (LA) (1.0 mM) on the whole cell β1-integrin expression was evaluated and representative band pattern was shown in A and expression intensity was quantified in B (β1-integrin/β-actin). (C, D) Effect of LA (1.0 mM) on the cell surface β1-integrin expression was evaluated and representative histograms were shown in C and expression intensity was quantified in D. (E, F) Effect of cell adhesion to collagen IV (col IV) or fibronectin (Fn) on T24 cells. Cells were pretreated with 1.0 mM LA for 12, 24, 48 h (E) or with 0, 0.1, 0.3, 1.0 mM LA for 48 h then, seeded onto collagen IV or fibronectin coated plates. Results are means ± SD of 3 samples. Values marked with asterisk(s) are significantly different from the control value at *p<0.05 and **p<0.01, respectively.
were capable of downregulating cell surface β1-integrin (Fig. 5G).

As shown in Fig. 2, although LA downregulated cell surface β1-integrin level, whole cell β1-integrin level was not changed. Here, we hypothesized that LA regulated β1-integrin localization and promoted its internalization. Therefore, β1-integrin localization was examined in membrane permeabilized cells. Interestingly, whereas β1-integrin was uniformly distributed in cytosol fraction, deeply stained particles by β1-integrin antibody was observed in LA treated T24 cells, showing concentration into this particle and internalization of β1-integrin under LA treatment (Fig. 6A). To know whether this phenomenon of β1-integrin is raft dependent or not, cells were treated with methyl β-cyclo dextrin (MβCD) which removes cholesterol from plasma membrane resulting in degradation of raft structure. As shown in Fig. 6B, MβCD did not affect on β1-integrin level under LA treatment.

Discussion

Oncogenic mutations in H-ras are commonly found in bladder cancer and could be a molecular target of bladder cancer therapy.13–17 Actually, T24 cells used in this experiment possesses mutation in H-ras gene.18 This mutation evokes activation of Ras dependent signaling such as disregulated proliferation, abnormal cell adhesion and motility that are associated with cancer cell malignancy. Expression of these malignant phenotypes is associated with abnormal regulation of cellular integrin expression and activity. Our previous study revealed that cell surface β1-integrin was upregulated accompanied with elevation of cell adhesion to fibronectin, cell migration and invasion in H-ras transformed 3Y1 fibroblasts.17 In addition, several studies indicated that bladder cancer cells highly expressed β1-integrin and blocking antibody for β1-integrin prevented cell adhesion and cell migration in T24 cells.18–21 These data suggest that upregulated β1-integrin expression plays an important role in the H-ras mediated expression of malignant character of cancer cells. Present data revealed that LA suppressed cell surface β1-integrin expression in T24 cells without any change in whole cell expression. Integrin is a heterodimer molecule that consist of several α- and β-chains and binds to various ECM partners depend on a combination of α- and β-chain.22 β1-integrin forms heterodimeric complex with various α-chain to bind variety of ECMs such as collagen IV and fibronectin. Data showed that cell adhesion to fibronectin and collagen IV was suppressed under LA treatment suggesting downregulation of cell surface β1-integrin expression substantially attenuate cell adhesion to these ECMs.

Cell invasion requires cell migration and adhesion to ECM through integrins. During cell invasion, cancer cell form small projections called invadopodia that is rich in actin and plays a

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**Fig. 3.** Downregulation of phosphorylated focal adhesion kinase and stress fiber formation. (A, B) Effect of α-lipoic acid (LA) on phosphorylated focal adhesion kinase (FAK) level was evaluated. In left panel, LA was 1.0 mM and in right panel cells were treated for 48 h. Representative band pattern was shown in A and expression intensity was quantified in B (p-FAK/FAK). (C) Cells were treated with 0 or 1.0 mM LA for 48 h and actin fiber was stained with Acti-stain 488 (green) and nucleus were stained with Hoechst 33342 (blue). Results are means ± SD of 3 samples. Values marked with asterisk(s) are significantly different from the control value at *p<0.05 and **p<0.01, respectively.
pivotal role in cell motility. β1-Integrin is highly localized into invadopodia. (23, 24) Actually, in bladder cancer cells, α5β1 integrin plays a pivotal role in cell invasion through generation of contractile forces that provide cells a driving force of cell movement. (25) Data in this study show that LA suppressed migration and invasion accompanied with downregulation of cell surface β1-integrin. Therefore, it is reasonable to support that inhibition of cell adhesion, migration and invasion is attributable to downregulation of cell surface β1-integrin expression. Suppression of matrix metalloproteinase (MMP) family may be involved in invasion suppression of T24 cell, because it has been reported that LA suppressed invasion and expression of MMP-2, -9 in human breast cancer cells, MDA-MB-231 cells that possess K-ras mutation. (11)

Here, substantial effect of downregulation of cell surface β1-integrin expression on integrin-related signal was also evaluated. FAK, a tyrosine kinase, binds to intracellular domain of integrin and binding of β1-integrin with ECMs occurs its autophosphorylation at Tyr397. (26) As shown in Fig. 3, LA decreased phosphorylated FAK level, suggesting substantial attenuation of integrin signaling. As LA and dihydro-LA downregulates cell surface expression of the α4, β1-integrin in human Jurkat T cells, and intercellular adhesion molecule 1 and vascular cell adhesion protein 1 in central nervous system endothelial cells, LA might dynamically regulate cell surface protein expressions in several types of cells. (27, 28) FAK phosphorylation by integrin engagement triggers activation of downstream signal cascades such as ERK and PI3K/Akt pathway and resulting in upregulation of MMPs expression and regulation of RhoA, Cdc42, Rac1 pathway that evokes cell migration, invasion and proliferation. (29–33) As present data show that LA decreased phosphorylated ERK1/2 and Akt levels, suggesting attenuation of ERK and PI3K/Akt pathway was caused by downregulation of cell surface integrin-β1 and FAK phosphorylation. In addition, actin stress fiber formation provide cellular tensile and contractile force that enable cell motility. Stress fiber formation is regulated by RhoA which is activated by integrin signaling pathway. (34) Therefore, downregulation of phosphorylated FAK and stress fiber formation by LA shown in Fig. 3B strongly suggests the substantial inhibition of integrin signaling. Moreover, inhibitors for ERK and Akt pathways mimicked the action of LA in that they inhibited inhibition and migration of T24 cells (Fig. 4). Therefore, it is considered that ERK and Akt pathways are responsible for migration and invasion suppression by LA.

Schwartz et al. (35) have shown that LA significantly inhibited the growth of T24 cells and Fig. 1 supports this previous data. Although proliferation inhibition of bladder cancer cells was also observed in vivo mice transplanted model, (36) there is no evidence of relationship between proliferation inhibition and downregulation of β1-integrin expression. ERK and PI3K/Akt pathway are pivotal regulator for cell proliferation in bladder cancer, besides inhibition of these pathway evokes growth inhibition of bladder cancer cells. (37) Therefore, downregulation of ERK and PI3K/Akt pathway might contribute to the growth inhibitory effect of LA on T24 cells shown in Fig. 1. Moreover since LA suppressed of platelet derived growth factor induced ras related ERK signaling in vascular smooth muscle cells and transforming growth factor-β induced ERK phosphorylation, (38, 39) LA might downregulate ras related ERK activation in T24 cells.
Akt and ERK pathways are representative signaling route from Ras and these pathways are responsible for regulating integrin-ligand binding affinity. Therefore, integrin related pathway and Ras related pathway closely overlap and interact each other. It is considered that LA at least suppressed integrin related pathway because cell surface integrin level and adhesion to fibronectin and collagen IV were inhibited in T24 cells. On the other hand, it is unclear whether suppression of cell surface $\beta_1$-integrin expression is sufficient to explain the adhesion inhibition because it is regulated by its conformational change as well as transcriptional, translational and cellular localization level. Moreover, in the case of bladder cancer cells, oligosaccharide pattern of $\alpha_3\beta_1$ integrin is critical factor for determining affinity to fibronectin and cell migration.  

Integrins abundantly exist on the lipid raft regions of plasma membrane where several signaling molecules that participate in the integrin signaling are recruited in response to integrin engagement to ECM. Such localization on plasma membrane is regulated by intracellular membrane transport related proteins such as Arf/Sar family and Rab family via regulation of endocytosis and exocytosis. Coming-in and coming-out of integrin from plasma membrane to cytosolic fraction is called integrin trafficking that is the plausible target of LA because LA downregulated cell surface $\beta_1$-integrin level without any change in whole cell integrin level. Microscopic data clearly showed the particles of integrin in LA treated cells, indicating occurrence of integrin trafficking. Endocytotic mechanism of integrin is still controversial but two major pathways are proposed. One is clathrin-mediated and other is raft-mediated pathway, and latter pathway is prevented by depletion of cholesterol from the raft using MβCD. As MβCD treatment of T24 cells did not change integrin localization in T24 cells, raft dependent integrin internalization seems not occur robustly at steady state. In addition, MβCD treatment did not interfered the action of LA. Taken together, it is considered that LA suppressed cell surface $\beta_1$-integrin via raft independent internalization and further studies are needed to clarify the involvement of other endocytosis pathway and exocytosis.

Although several reports have been revealed that LA induced apoptotic cell death and inhibited cell migration in cancer cells,
putative molecular target of LA has been unclear. This report presents novel effect of LA on the bladder cancer cells and the importance of β1-integrin in the action of LA. From clinical point of view, β1-integrin suppression is significant because engagement of integrin on ECM provokes resistance to cancer drugs. T24 cells highly express β1-integrin that is largely responsible for adhesion to fibronectin and acquire drug resistance to mitomycin-C via PI3K/Akt pathway.⁵⁶–⁵⁸ Therefore LA might be useful for sensitizing bladder cancer cells to anticancer drugs.

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Abbreviations

- DMEM: Dulbecco’s modified Eagle’s medium
- ECMs: extracellular matrixes
- FAK: focal adhesion kinase
- FBS: fetal bovine serum
- LA: α-lipoic acid
- MβCD: methyl β-cyclo dextrin
- MMP: matrix metalloproteinase
- PBS: phosphate buffered saline
- TTBS: Tris-buffered saline with 0.1% Tween-20

Conflict of Interest

No potential conflicts of interest were disclosed.

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