Arginine deiminase originating from *Lactococcus lactis* ssp. *lactis* American Type Culture Collection (ATCC) 7962 induces G$_1$-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells

Jong-Eun Kim[1], Seo Young Kim[1], Ki Won Lee[2]* and Hyong Joo Lee[1]*

[1]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea*
[2]*Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, South Korea*

There are multiple lines of evidence that lactic acid bacteria (LAB) exert cancer-preventive effects. However, the underlying mechanisms are poorly understood. In the present study we found that the cytoplasmic fraction of *Lactococcus lactis* ssp. *lactis* American Type Culture Collection (ATCC) 7962 exerted the strongest antiproliferative effects (half-maximal inhibitory concentration (IC$_{50}$) = 17 µg/ml) in SNU-1 human stomach cancer cells and arginine deiminase (ADI; *EC* 3.5.3.6) activity. We also cloned, expressed and purified ADI from *L. lactis* ssp. *lactis* ATCC 7962 (LADI). Both purified ADI from *L. lactis* (PADI; IC$_{50}$ = 2 µg/ml) and recombinant ADI originating from LADI (IC$_{50}$ = 0·6 µg/ml) inhibited the proliferation of SNU-1 cells. LADI induced G$_0$/G$_1$-phase arrest, sub-G$_1$ accumulation, DNA condensation and DNA fragmentation in SNU-1 cells. 4′,6-Diamidino-2-phenylindole (DAPI) staining and DNA fragmentation data provide evidence that LADI induces apoptosis in SNU-1 cells. LADI increased the expressions of p53 and p27^{Kip1}, and decreased the expressions of cyclin D$_1$, c-myc and Bcl-xL in SNU-1 cells. However, LADI had no effects on the expressions of p21^{Cip1} and Bcl-2. Collectively, these data indicate that ADI induces apoptosis and G$_0$/G$_1$-phase arrest of SNU-1 cells, which might contribute to the chemopreventive potential of LAB.

Lactic acid bacteria: Arginine deiminase: Cell-cycle arrest: Apoptosis

Arginine enhances tumour growth. Conversely, the restriction of arginine inhibits the growth of metastatic tumours[1,2] due to this amino acid being involved in several biosynthetic pathways that significantly influence carcinogenesis and tumour biology (for example, NO generation, creatine production and polyamine synthesis). More pathways are disturbed by removing arginine than by removing most other amino acids. Also, tumour cells die rapidly in an arginine-free medium, whereas diploid fibroblasts move into quiescence, a difference that clearly offers a selective advantage in targeting tumour cells[3]. Therefore, there have been attempts to use L-arginine-degrading enzymes in cancer treatment, with arginine-depletion enzyme being regarded as a potential anticancer agent[4].

Most investigations of arginine deiminase (ADI; *EC* 3.5.3.6), which catalyses the imine hydrolysis of arginine to produce citrulline and ammonia from *Mycoplasma arginini* (MADI), have suggested that this inhibits cell proliferation in hepatocellular, melanoma, leukaemia and prostate cancer cell lines by inducing G$_0$/G$_1$ arrest[5–8], with these cells being auxotrophic to arginine[9]. In addition, MADI has been shown to affect apoptosis, inhibit NO synthesis, exert effects against TNF-α and neutralise endotoxin[5,6,10–12]. Recent phase I and II clinical studies have investigated the

effects of the pegylated form of ADI on hepatocellular carcinoma and melanoma[13,14].

Abnormal cell proliferation is strongly associated with carcinogenesis. The regulation of apoptosis, a process of controlled cellular death, is required for the maintenance of normal homeostasis, which represents the balance between cell proliferation and cell death in multicellular organisms[15]. Therefore, controlling the cell cycle and apoptosis has been considered a promising target for cancer chemoprevention[16]. c-Myc is a proto-oncogene that acts as a transcription factor stimulating both cell-cycle progression and apoptosis, and is overexpressed in a wide range of human cancers. It plays important roles in G$_1$-phase progression by regulating the expressions of p27^{Kip1}[17] and cyclin D$_1$ protein[18]. p53 is a tumour-suppressor gene that is highly mutated in many tumours. It is a key regulator of cell-cycle arrest, senescence, differentiation and apoptosis by acting as a transcription factor or interacting with other proteins such as members of the Bcl family including Bcl-2, Bcl-xL and Bax, which play a central role in apoptosis. Although members of this family have similar structures, there are two subfamilies with opposite functions: anti-apoptotic (including Bcl-2 and Bcl-xL) and pro-apoptotic (including Bax).

Lactic acid bacteria (LAB) have been used to ferment foods such as cheese, yoghurt and kimchi for at least the past 4000 years, and have recently been shown to be effective chemopreventive food ingredients against many cancer types (reviewed by de Moreno de LeBlanc *et al.* [19] and Kim *et al.* [20]). However, the active components and underlying mechanisms have not been fully elucidated. We previously screened the antitumour capacities of various cellular components – whole cells, peptidoglycans and cytoplasmic fractions – from ten types of LAB by measuring the inhibition of proliferation in diverse human tumour cell lines which exist in the gastrointestinal tract. The cytoplasmic fraction but not the peptidoglycan fraction of *Lactococcus lactis* strongly suppressed the proliferation of SNU-1 human stomach cancer cells[21]. However, the components in the cytoplasmic fraction of *Lactococcus lactis* (CFL) responsible for the antiproliferative activity were not determined. The present study investigated whether the antiproliferative activity of CFL in SNU-1 human stomach cancer cells is linked to the induction of cell-cycle arrest and apoptosis by ADI.

Materials and methods

Chemicals

Methyl thiazol tetrazolium (MTT), propidium iodide, ethidium bromide, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-borate, EDTA and the antibody for β-actin were purchased from Sigma (St Louis, MO, USA). p53, p27[Kip1], p21[Cip1], c-myc, cyclin D$_1$, Bcl-xL, Bax and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies (anti-mouse and anti-rabbit) were purchased from Zymed (San Francisco, CA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum and streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA). M17 broth was obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals were purchased from Sigma.

Cell culture

The SNU-1 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). The cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10 % (v/v) fetal bovine serum (GIBCO BRL), penicillin (1000 U/l) and streptomycin (1 mg/l) (GIBCO BRL) in a humidified incubator (Forma Scientific, Marjetta, OH, USA) with 5 % CO$_2$ at 37°C.

Preparation of lactic acid bacteria

LAB were used for screening antiproliferative and ADI activities. Lactococci and streptococci were cultured in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 5 % (w/w) glucose, lactobacilli were cultured in deMan–Rogosa–Sharpe (MRS) broth and bifidobacteria were cultured in brain-heart-infusion broth with 0·05 % (w/w) L-cysteine. The cells were then harvested by centrifugation at 4°C, washed three times with distilled water and sonicated with a cell disruptor (Sonics & Materials, Danbury, CT, USA) for 30 min in ice. The suspension was centrifuged (Hitachi,

Tokyo, Japan) at 70 000 **g** for 30 min and the supernatant fraction was used to obtain the cytoplasmic fraction (CFL).

Purification of arginine deiminase from cytoplasmic fraction of *Lactococcus lactis*

Native ADI (purified ADI; PADI) was purified from CFL. CFL was subjected to anion exchange chromatography on a Q-Sepharose Fast Flow column (5 ml; GE Healthcare, Piscataway, NJ, USA) that had previously been equilibrated with 50 mM-Tris buffer (pH 7·0). The loaded column was washed with 30 ml of the same buffer and then eluted with a linear gradient (0–1·0 M) of NaCl in 50 ml of the Tris buffer using fast protein liquid chromatography. The ADI-rich fractions were pooled, concentrated by a centrifugal concentrator (Vivaspin 15; Vivascience, Epsom, Surrey, UK) and subjected to gel permeation chromatography on a Sephacryl-S200 column (Amersham; 1·6 × 26 cm). The column was washed with 50 mM-Tris buffer (pH 7·0) containing 0·15 M-NaCl and then eluted with the same buffer using fast protein liquid chromatography. The active fractions were analysed by SDS-PAGE and Coomassie brilliant blue staining (Fig. 1).

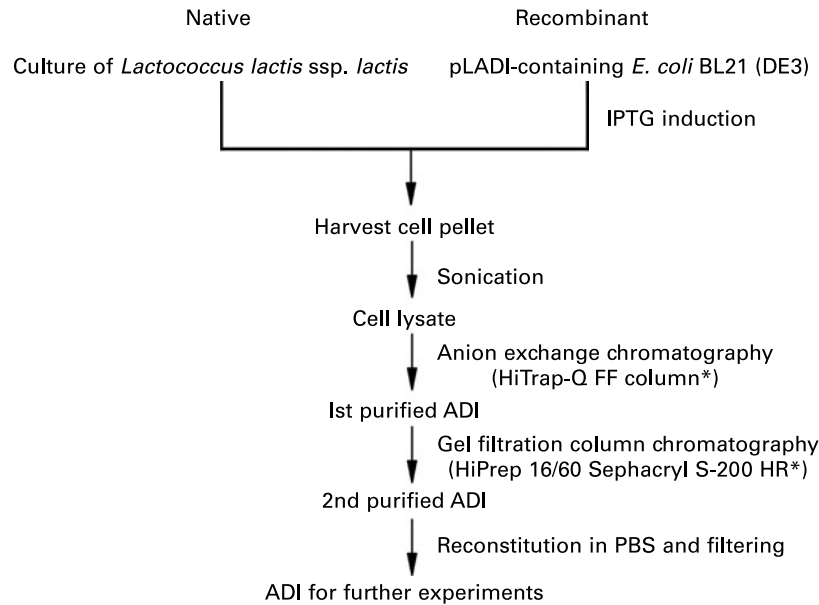### Measurement of arginine deiminase enzymic activity

ADI activity was quantified based on the amount of L-citrulline produced from L-arginine as described previously[22]. Briefly, CFL was incubated with 10 mM-arginine and 50 mM-Tris (pH 7·0) in a final volume of 1·0 ml for 3 h at 37°C. The reaction was terminated by the addition of 0·5 ml of a 1:3 mixture (v/v) of H$_2$SO$_4$ and H$_3$PO$_4$. The amount of L-citrulline formed during the incubation was determined by measuring the coloured reaction product formed from L-citrulline and diacetyl monooxime.

Expression and purification of recombinant arginine deiminase

Recombinant ADI (ADI from *L. lactis*; LADI) was expressed and purified to homogeneity, and its enzyme activity was determined[23]. Briefly, the *arcA* gene of *L. lactis* ssp. *lactis* American Type Culture Collection (ATCC) 7962 (National Center for Biotechnology Information (NCBI) accession no. DQ364637) that coded LADI was cloned. We constructed pLADI, in which the *arcA* gene was located downstream of the T7 promoter. LADI was expressed by isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) in *Escherichia coli* BL21 (DE3) harbouring pLADI. Other purification procedures were the same as that for PADI (Fig. 1).

Measuring antiproliferative activity

The effects on cell proliferation were measured by the MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded on ninety-six-well microplates and then treated with samples at different concentrations for 48 h, after which 20 μl MTT stock solution (5 mg/ml, Sigma) were added to each well and the plates were further incubated for 4 h at 37°C. The supernatant fraction was removed and 100 μl dimethyl sulfoxide were added to each well to solubilise the water-insoluble

Native   Recombinant

Culture of *Lactococcus lactis* ssp. *lactis*   pLADI-containing *E. coli* BL21 (DE3)

IPTG induction

Harvest cell pellet

Sonication

Cell lysate

Anion exchange chromatography
(HiTrap-Q FF column*)

Ist purified ADI

Gel filtration column chromatography
(HiPrep 16/60 Sephacryl S-200 HR*)

2nd purified ADI

Reconstitution in PBS and filtering

ADI for further experiments

Fig. 1. Flow chart of the procedure for purifying arginine deiminase (ADI). LADI, arginine deiminase from *Lactococcus lactis*, IPTG, isopropyl β-D-1-thiogalacto-pyranoside; *E. coli*, *Escherichia coli*. * GE Healthcare (Piscataway, NJ, USA).

purple formazan crystals. The absorbance at 570 nm was measured with a microplate reader (Multiscan MCC 340; Titertek, Huntsville, AL, USA). All the measurements were performed in triplicate. Results are expressed as the percentage proliferation with respect to untreated cells.

Cell-cycle analysis

Cells (1.0×10^5) were cultured in a 10 cm dish for 24 h and then treated with LADI at 0, 0·6 or 1·2 μg/ml for 48 h. The DNA content of nuclei was then determined by staining nuclear DNA with propidium iodide (50 μg/ml; Sigma) and measuring with a flow cytometer (Becton Dickinson, San Jose, CA, USA). The proportion of nuclei in each phase of the cell cycle was determined using FlowJo DNA analysis software (Tree Star, Ashland, KT, USA).

DNA fragmentation assay

Cells (1.0×10^5) were cultured in a 10 cm dish for 24 h, treated with LADI at 1·2 μg/ml for 48 h, and then lysed in 5 mM-Tris-HCl buffer (pH 7·4) containing 0·5 % Triton X-100 and 20 mM-EDTA for 30 min at 4°C. After centrifugation at 19 000 *g* for 30 min, supernatant fractions were extracted with phenol–chloroform and precipitated in ethanol. Samples were subjected to electrophoresis on a 1·5 % agarose gel containing ethidium bromide (Sigma). The gel was run in TBE (Tris-borate and EDTA; Sigma) buffer at 75 V (1·5 h, room temperature) and visualised on a UV light box.

Nuclear staining with 4′,6-diamidino-2-phenylindole

Cells (1.0×10^5) were cultured in a 10 cm dish for 24 h, treated with LADI at 1·2 μg/ml for 48 h and then fixed by 4 % paraformaldehyde (Sigma). After being transferred to slides, cells were dried at room temperature and then stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Sigma) for 15 min. The adhering cells were washed with PBS, air dried and mounted with 90 % glycerol. The cell nuclear morphology was observed under a fluorescence microscope (Olympus Ix70; Olympus, Tokyo, Japan).

Western blot analysis

Cells (1.0×10^5) were cultured in a 10 cm dish for 24 h and then treated with LADI at 0, 0·6 and 1·2 μg/ml for 48 h. Cell pellets obtained by centrifugation at 1000 *g* for 5 min were washed with and then re-suspended in PBS. Cell lysis was performed at 4°C for 30 min in cell lysis buffer (20 mM-Tris-HCl (pH 7·5), 150 mM-NaCl, 1 mM-Na_2EDTA, 1 mM-ethylene glycol tetraacetic acid, 1 % Triton, 2·5 mM-sodium pyrophosphate, 1 mM-β-glycerophosphate, 1 mM-Na_3VO_4, leupeptin (1 μg/ml) and 1 mM-phenylmethanesulfonylfluoride). The cell lysates were centrifuged at 23 000 *g* for 15 min and the resulting supernatant fraction was stored at −70°C before Western blot analysis. The protein concentration in each sample was measured by subjecting lysate protein (30 μg) to 10 % SDS-PAGE, with the protein electrophoretically transferred to a nitrocellulose membrane (Whatman, Clifton, NJ, USA). Protein bands were visualised by a chemiluminescence detection kit (GE Healthcare, Piscataway, NJ, USA) after hybridisation with the horseradish peroxidase-conjugated secondary antibody. Quantification of the bands was performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Where appropriate, data are expressed as mean values and standard deviations. Student's *t* test was used for single comparisons. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

Table 1. Half-maximal inhibitory concentration (IC_{50}) values of cytoplasmic fractions from lactic acid bacteria against SNU-1 cells (μg/ml)

Strain and abbreviation	IC_{50} values
Lactobacillus casei ssp. *casei* KCTC 3109 (Lcas)	>200
Lactobacillus plantarum KCTC 3099 (Lpla)	107
Streptococcius thermophillus KCTC 2185 (Sthe)	>200
Lactobacillus bulgarucus KCTC 3188 (Lbul)	>200
Bifidobacterium adolescentis ATCC 15 730 (Bado)	98
Lactobacillus lactis ssp. *lactis* ATCC 7962 (Llac)	17

KCTC, Korean Collection for Type Cultures; ATCC, American Type Culture Collection.

Results

Antiproliferative activity of cytoplasmic fraction of Lactococcus lactis in SNU-1 cells and its relationship with arginine deiminase activity

We first measured the antiproliferative activities of cytoplasmic fractions of six types of LAB. CFL exerted the strongest antiproliferative effects. The half-maximal inhibitory concentration (IC_{50}) value of CFL was 17 μg/ml, and those of other LAB were more than 200 μg/ml, except for values of 107 and 98 μg/ml for *L. plantarum* and *Bifidobacterium adolescentis*, respectively (Table 1). We measured ADI activity to isolate the potential compounds responsible for these effects. CFL exerted the strongest ADI activity in the six types of LAB (Fig. 2(a)). To elucidate the active compound underlying this effect, we used an anion exchange and gel filtration column to purify PADI (Fig. 2(b)). The inhibitory effects of purified PADI on the proliferation of SNU-1 cells were determined by the MTT assay. SNU-1 cells were treated with the final ADI fractions at 31·3, 62·5, 125, 250 and 500 μg/ml. PADI significantly decreased the proliferation of SNU-1 cells in a dose-dependent manner, with an IC_{50} value of 2 μg/ml (Fig. 2(c)). These results indicated that the antiproliferative effects of CFL were due to ADI activity.

Arginine deiminase from Lactococcus lactis induces antiproliferative effects in SNU-1 cells via arginine depletion

To verify the mechanisms underlying the antiproliferative effects of ADI in SNU-1 cells, we expressed and purified
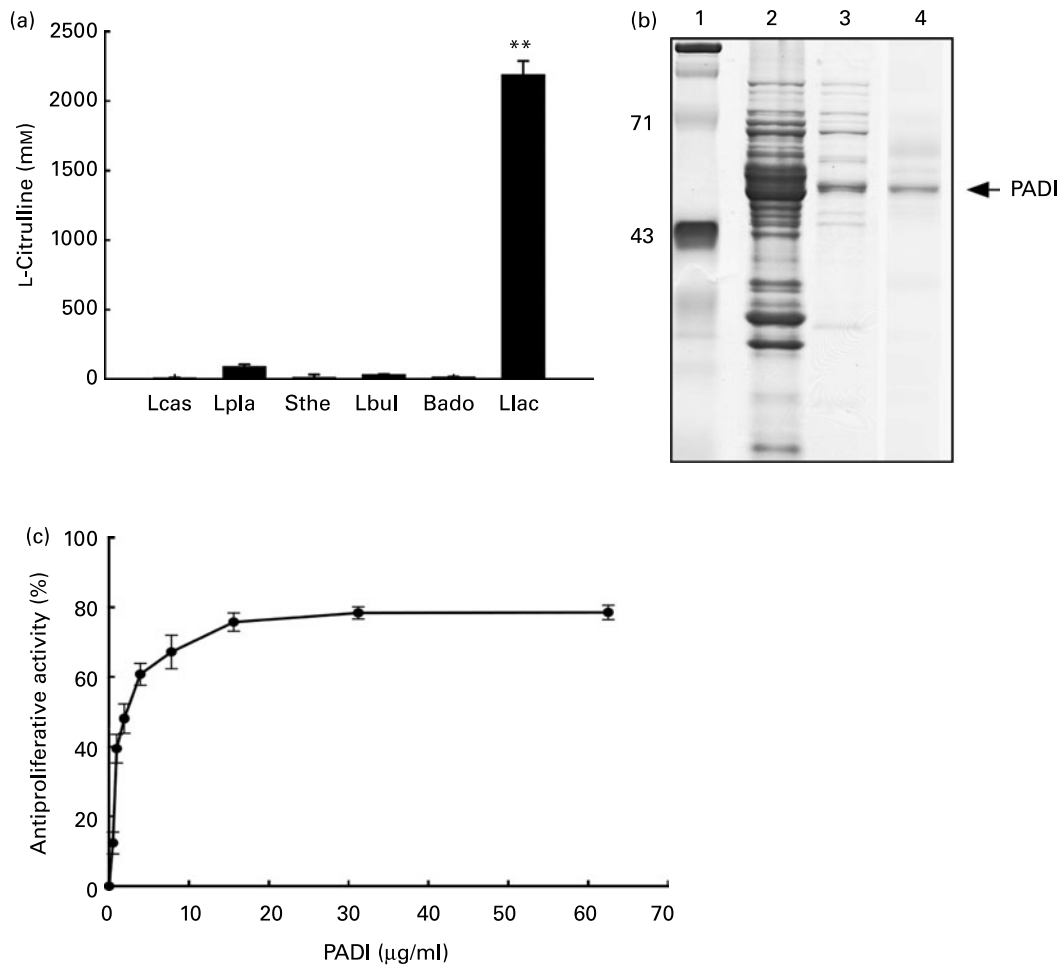
Fig. 2. Antiproliferative activity of the cytoplasmic fraction of *Lactococcus lactis* correlates with arginine deiminase (ADI) activity. (a) ADI enzymic activities of cytoplasmic fractions of several lactic acid bacteria (Lcas, *Lactobacillus casei*; Lpla, *Lactobacillus plantarum*; Sthe, *Streptococcius thermophillus*; Lbul, *Lactobacillus bulgarucus*; Bado, *Bifidobacterium adolescentis*; Llac, *Lactococcus lactis*; see Table 1) were determined by the amount of L-citrulline produced from L-arginine as described in the Materials and methods section. Data are means from triplicate tests, with standard deviations represented by vertical bars. ** $P<0.01$. (b) SDS-PAGE analysis of purified ADI (PADI). Lanes: 1, molecular-mass marker protein; 2, total cell extract; 3, eluted fraction from Q-sepharose; 4, eluted PADI from sephacryl S-200. (c) Antiproliferative activity of PADI in SNU-1 cells as measured using the methyl thiazol tetrazolium assay. Data are means from triplicate tests, with standard deviations represented by vertical bars.

LADI in an *E. coli* system[23]. The MTT assay was used to analyse the antiproliferative effects of LADI in SNU-1 cells (Fig. 3(a)). LADI dose-dependently inhibited the proliferation of SNU-1 cells with an IC_{50} of about $0.6\,\mu g/ml$. Subsequent experiments were performed using concentrations of 0.6 or $1.2\,\mu g/ml$. We added extra arginine to the culture medium to confirm whether the antiproliferative effect of LADI was mediated by depleting arginine. We found that the inhibition of proliferation of SNU-1 cells by LADI was dose-dependently recovered by arginine treatment (Fig. 3(b)).

Arginine deiminase from Lactococcus lactis *induces G_1-phase arrest and apoptosis in SNU-1 cells*

We also examined whether the antiproliferative effects of LADI involved cell-cycle arrest and apoptosis. SNU-1 cells were treated with LADI at 0, 0.6 or $1.2\,\mu g/ml$, and the distribution of cell-cycle phases was determined using flow cytometry with propidium iodide staining. The apoptotic sub-G_1-phase fraction increased from $2.54\,\%$ to $11.58\,\%$ in SNU-1 cells by LADI treatment. LADI induced cell-cycle arrest of SNU-1 cells at the G_0/G_1 phase and a progressive decline of G_2/M-phase cells (Fig. 4). These results indicate that LADI induced apoptotic cell death concurrently with cell-cycle arrest in SNU-1 cells. We also assessed apoptosis in SNU-1 cells by examining DNA fragmentation and nuclear condensation using agarose electrophoresis and the

DNA-specific fluorescent dye 4′,6-diamidino-2-phenylindole. SNU-1 cells were treated with LADI at 0 or $1.2\,\mu g/ml$ for 48 h. We found that LADI increased DNA fragmentation (Fig. 5(a)) and nuclear condensation (Fig. 5(b)) in SNU-1 cells. These results provide evidence that LADI induces cell-cycle arrest and apoptosis in SNU-1 cells.

Effects of arginine deiminase from Lactococcus lactis *on distribution of cell-cycle phases and expression of apoptosis-regulating proteins*

Different regulators working in multiple pathways tightly regulate the cell cycle, including p53, cyclin and CDKi (cyclin-dependent kinase inhibitor) proteins[24]. The Bcl-2 family of anti- and pro-apoptotic regulators is important to life-or-death decisions[25]. We used immunoblotting to detect proteins underlying the LADI-induced cell-cycle arrest and apoptosis in SNU-1 cells. LADI significantly increased the expressions of p53 and p27^{Kip1} and significantly decreased the expressions of cyclin D_1 and c-myc in SNU-1 cells. However, it had no effect on the expression of p21^{Cip1} (Fig. 6(a)). The expression of Bcl-xL but not of Bcl-2 and Bax proteins was inhibited by LADI (Fig. 6(b)).

Discussion

There is considerable evidence of the anticancer effects of LAB[20], but the underlying active compounds and mechanisms still need to be elucidated. We screened the antiproliferative effects of various species of LAB and their fractions in several cancer cell lines[21]. We found that CFL has the lowest IC_{50} in SNU-1 cells. To explain this, we searched for active compounds that exhibit differential expression in these species. We hypothesised that the active compound was the enzyme expressed only by *L. lacits*, because it is a soluble component and is effective at a relatively low concentration.

ADI is an enzyme that has been previously described as exerting antiproliferative effects in micro-organisms. ADI underlies the death of certain cell lines infected with *Mycoplasma* species[26], and its anticancer effects have also been examined[27]. It was reported that *L. lactis* ssp. *lactis* expresses ADI, and that there are important differences between the characteristics of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* [28]. We previously found that whilst there was only 35 % amino acid sequence homology between LADI and MADI, all of the important sequences involving enzyme activity were conserved[23]. LADI also exhibits anti-inflammatory effects, inhibiting lipopolysaccharide-induced cyclo-oxygenase-2 and inducible NO synthase expression in RAW 264.7 murine macrophages[29]. We therefore assessed ADI activity in the cytoplasmic fractions of several LAB and found a negative correlation between IC_{50} values and ADI activity. Due to quantity limitations, we cloned, expressed and purified LADI in *E. coli* in order to study the underlying mechanisms[23]. Both LADI and PADI inhibited cell proliferation, but the IC_{50} was lower for LADI, which could have been due to purity differences.

Cancer and normal cells exhibit differences in their metabolism of basic nutrients such as amino acids. Depleting nutrients that are important to cell growth kills cancer cells
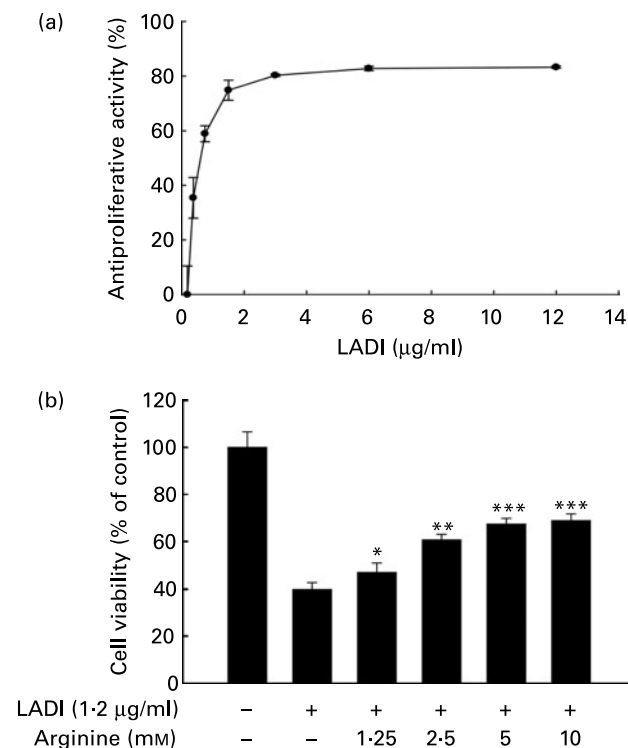
Fig. 3. Antiproliferative activity of arginine deiminase from *Lactococcus lactis* (LADI) in SNU-1 cells by arginine depletion. (a) Antiproliferative activity of LADI. Cells were treated with LADI (0, 0.375, 0.75, 1.5, 3, 6 or $12\,\mu g/ml$) for 48 h and were measured using the methyl thiazol tetrazolium assay. (b) Arginine recovered the antiproliferative activity of LADI. Data are means from triplicate tests, with standard deviations represented by vertical bars. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.
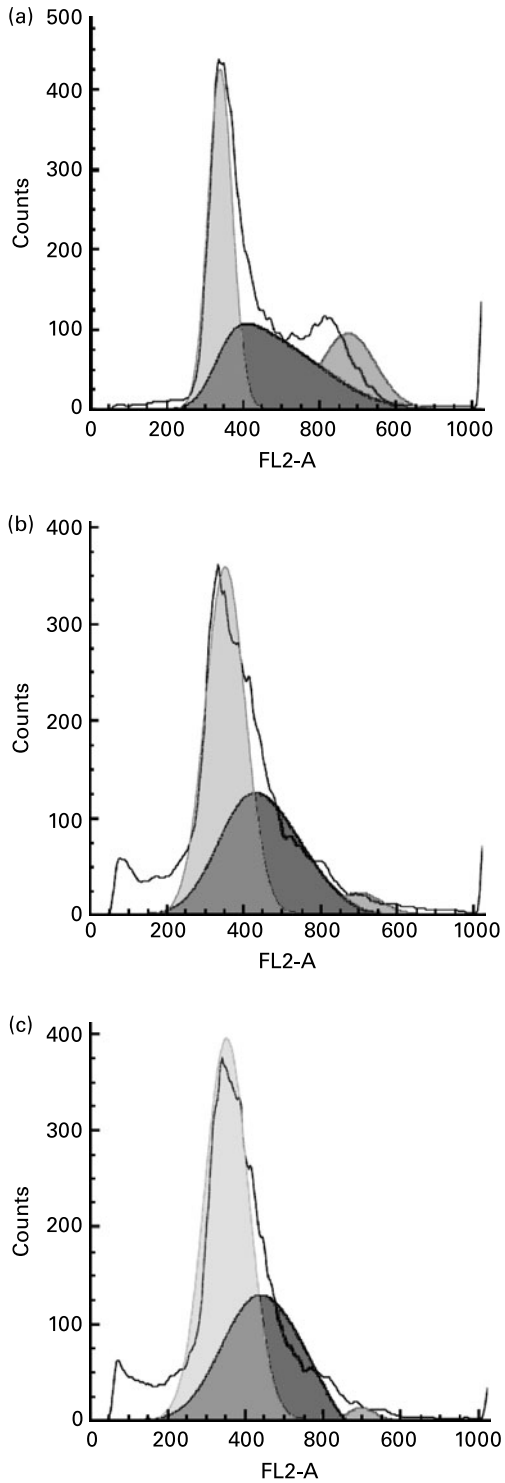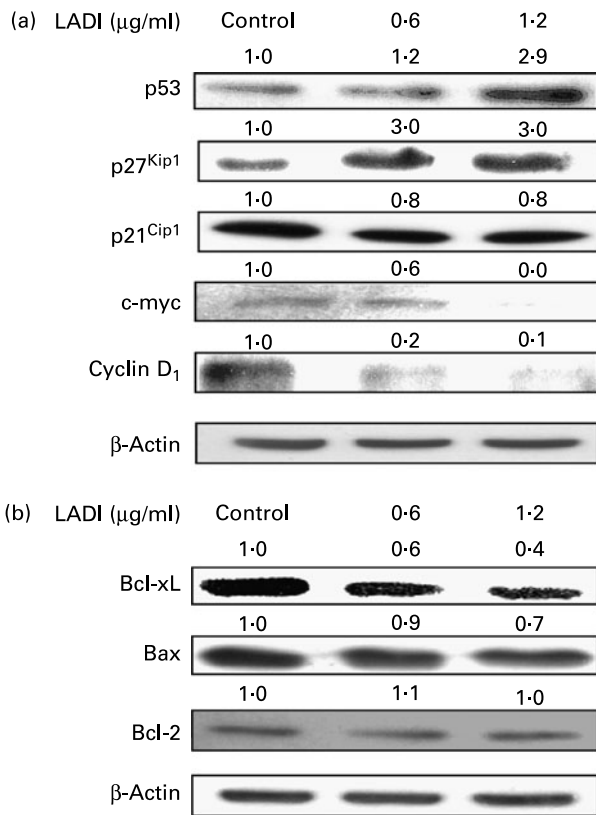
Fig. 4. Effects of arginine deiminase from *Lactococcus lactis* (LADI) on the cell cycle ($\square$, sub-G_1; $\square$, G_0/G_1; $\blacksquare$, S; $\blacksquare$, G_2/M) in SNU-1 cells after treatment with LADI. Cells were treated with LADI (0 (a) (control), 0·6 (b) or 1·2 (c) μg/ml) for 48 h, stained with propidium iodide (PI) and analysed by fluorescence-activated cell sorting. FL2-A, PI fluorescence on channel 2. All experiments were performed in duplicate and gave similar results. In the control treatment, the percentage of cells at each stage of the cell cycle was: sub-G_1, 2·54; G_0/G_1, 51·34; S, 46·63; G_2/M, 25·97. In the 0·6 μg LADI/ml treatment, the percentage of cells at each stage of the cell cycle was: sub-G_1, 10·72; G_0/G_1, 61·17; S, 42·72; G_2/M, 4·48. In the 1·2 μg LADI/ml treatment, the percentage of cells at each stage of the cell cycle was: sub-G_1, 11·58; G_0/G_1, 73·17; S, 40·92; G_2/M, 1·50.
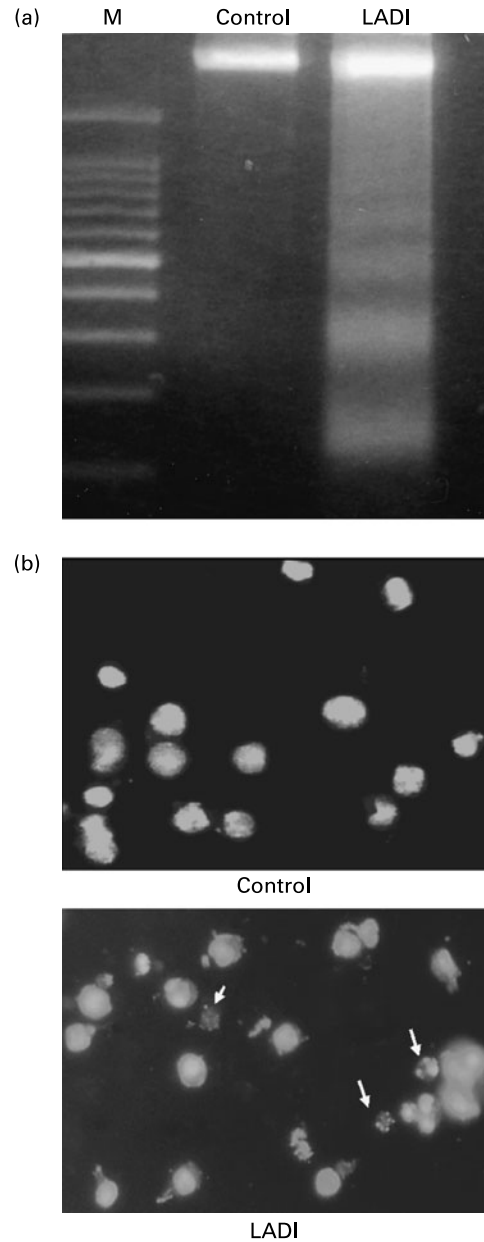
Fig. 5. Arginine deiminase from *Lactococcus lactis* (LADI) induced DNA fragmentation and chromatin condensation in SNU-1 cells. (a) LADI induced internucleosomal DNA fragmentation. SNU-1 cells were incubated with LADI at 0 or 1·2 μg/ml for 48 h. Genomic DNA was then prepared and analysed on a 1·5% agarose gel. Lane 1 shows DNA size markers (M; 100 bp DNA ladder; Invitrogen, MI, USA). (b) LADI induced chromatin condensation and segregation. After treatment with LADI (1·2 μg/ml) for 48 h, cells were washed and fixed in 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole to assess chromatin condensation.

but not normal cells, and hence this is a potential cancer therapeutic strategy[30]. Although arginine is not an essential amino acid, some tumours are auxotrophic to arginine, including melanoma and hepatocellular carcinoma. Arginine depletion arrests the cell cycle at the G_0/G_1 phase in these cell types[3]. In the present study we found that treatment with LADI arrested SNU-1 cells in the G_0/G_1 phase, and they died via apoptosis. These results indicated that SNU-1 cells are also auxotrophic to arginine because it inhibits cell growth.

Fig. 6. Effects of arginine deiminase from *Lactococcus lactis* (LADI) on G₁-phase regulation and Bcl-2-family proteins in SNU-1 cells. After treatment with LADI (0 (control), 0·6 or 1·2 μg/ml) for 48 h, cells were harvested and the levels of p53, p27^{Kip1}, p21^{Cip1}, c-myc, cyclin D₁, Bcl-xL, Bax, Bcl-2 and β-actin proteins were determined by Western blot analysis as described in the Materials and methods using specific antibodies. The scores above the bands are percentage of control, normalised by their β-actin. All experiments were performed in duplicate and gave similar results.

c-Myc is involved in the antiproliferative effects of arginine depletion, and has previously been shown to play a key role in cell proliferation and in the inhibition of cancer cell growth by amino acid depletion[31]. MADI-induced arginine depletion resulted in G₁-phase cell-cycle arrest and apoptosis, and inhibited c-myc expression in human T-cell acute lymphoblastic leukaemia CCRF-CEM cells[6]. We found that LADI also inhibited c-myc in SNU-1 cells. c-Myc controls many proteins involved in the G₀/G₁-to-S-phase transition, such as cyclin D₁, p27^{Kip1} and p21^{Cip1}. It also regulates p53, Bcl-2, Bcl-xL and Bax, which are related to apoptosis[18,32–34]. LADI increased the expressions of p53 and p27^{Kip1} and decreased the expressions of cyclin D₁ and Bcl-xL in SNU-1 cells. However, LADI had no effects on the expressions of p21^{Cip1} and Bcl-2. Previous investigations of the mechanism underlying arginine depletion showed that it inhibited cyclin D₁ expression in human diploid fibroblasts[35] and induced p27^{Kip1} and p21^{Cip1} in HepG2 human hepatoma cells[36]. Also, MADI reportedly inhibits Bcl-xL in human lymphoma cells[37]. However, how arginine depletion inhibits c-myc expression remains to be elucidated.

In conclusion, the antiproliferative effects of CFL are due to ADI. LADI induces G₁-phase cell-cycle arrest and apoptosis via arginine depletion by inhibiting c-myc, cyclin D₁ and Bcl-xL, and inducing p53 and p27^{Kip1}. The present paper is the first to reveal the putative active component underlying the anticancer effects of *L. lactis* ssp. *lactis* and their underlying mechanisms.

References

1. Gonzalez GG & Byus CV (1991) Effect of dietary arginine restriction upon ornithine and polyamine metabolism during two-stage epidermal carcinogenesis in the mouse. *Cancer Res* **51**, 2932–2939.
2. Wu G & Morris SM Jr (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336**, 1–17.
3. Wheatley DN, Scott L, Lamb J, et al. (2000) Single amino acid (arginine) restriction: growth and death of cultured HeLa and human diploid fibroblasts. *Cell Physiol Biochem* **10**, 37–55.
4. Cheng PNM, Lam TL, Lam WM, et al. (2007) Pegylated recombinant human arginase (rhArg-peg5,000 mw) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res* **67**, 309–317.
5. Gong H, Zolzer F, von Recklinghausen G, et al. (2000) Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis. *Leukemia* **14**, 826–829.
6. Noh EJ, Kang SW, Shin YJ, et al. (2004) Arginine deiminase enhances dexamethasone-induced cytotoxicity in human T-lymphoblastic leukemia CCRF-CEM cells. *Int J Cancer* **112**, 502–508.
7. Ensor CM, Holtsberg FW, Bomalaski JS, et al. (2002) Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas *in vitro* and *in vivo*. *Cancer Res* **62**, 5443–5450.
8. Kang SW, Kang H, Park IS, et al. (2000) Cytoprotective effect of arginine deiminase on taxol-induced apoptosis in DU145 human prostate cancer cells. *Mol Cells* **10**, 331–337.
9. Dillon BJ, Prieto VG, Curley SA, et al. (2004) Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation. *Cancer* **100**, 826–833.
10. Beloussow K, Wang L, Wu J, et al. (2002) Recombinant arginine deiminase as a potential anti-angiogenic agent. *Cancer Lett* **183**, 155–162.
11. Park IS, Kang SW, Shin YJ, et al. (2003) Arginine deiminase: a potential inhibitor of angiogenesis and tumour growth. *Br J Cancer* **89**, 907–914.
12. Thomas JB, Holtsberg FW, Ensor CM, et al. (2002) Enzymic degradation of plasma arginine using arginine deiminase inhibits nitric oxide production and protects mice from the lethal effects of tumour necrosis factor α and endotoxin. *Biochem J* **363**, 581–587.
13. Ascierto PA, Scala S, Castello G, et al. (2005) Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. *J Clin Oncol* **23**, 7660–7668.

14. Izzo F, Marra P, Beneduce G, *et al.* (2004) Pegylated arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: results from phase I/II studies. *J Clin Oncol* **22**, 1815–1822.

15. Kim JE, Kwon JY, Lee DE, *et al.* (2009) MKK4 is a novel target for the inhibition of tumor necrosis factor-α-induced vascular endothelial growth factor expression by myricetin. *Biochem Pharmacol* **77**, 412–421.

16. Danial NN & Korsmeyer SJ (2004) Cell death: critical control points. *Cell* **116**, 205–219.

17. Obaya AJ, Kotenko I, Cole MD, *et al.* (2002) The proto-oncogene c-myc acts through the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 to facilitate the activation of Cdk4/6 and early G_1 phase progression. *J Biol Chem* **277**, 31263–31269.

18. Perez-Roger I, Kim SH, Griffiths B, *et al.* (1999) Cyclins D_1 and D_2 mediate Myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). *EMBO J* **18**, 5310–5320.

19. de Moreno de LeBlanc A, Matar C & Perdigon G (2007) The application of probiotics in cancer. *Br J Nutr* **98**, Suppl. 1, S105–S110.

20. Kim JE, Kim JY, Lee KW, *et al.* (2007) Cancer chemopreventive effects of lactic acid bacteria. *J Microbiol Biotechnol* **17**, 1227–1235.

21. Kim JY, Woo HJ, Kim YS, *et al.* (2002) Screening for antiproliferative effects of cellular components from lactic acid bacteria against human cancer cell lines. *Biotechnol Lett* **24**, 1431–1436.

22. Boyde TRC & Rahmatullah M (1980) Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal Biochem* **107**, 424–431.

23. Kim JE, Jeong DW & Lee HJ (2007) Expression, purification, and characterization of arginine deiminase from *Lactococcus lactis* ssp. *lactis* ATCC 7962 in *Escherichia coli* BL21. *Protein Expr Purif* **53**, 9–15.

24. Vermeulen K, Berneman ZN & Van Bockstaele DR (2003) Cell cycle and apoptosis. *Cell Prolif* **36**, 165–175.

25. Cory S & Adams JM (2002) The BCL2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647–656.

26. Sasaki T, Shintani M & Kihara K (1984) Inhibition of growth of mammalian cell cultures by extracts of arginine-utilizing mycoplasmas. *In Vitro* **20**, 369–375.

27. Feun L & Savaraj N (2006) Pegylated arginine deiminase: a novel anticancer enzyme agent. *Expert Opin Investig Drugs* **15**, 815–822.

28. Niven CF, Smiley KL & Sherman JM (1942) The hydrolysis of arginine by streptococci. *J Bacteriol* **43**, 651–660.

29. Kim JE, Hur HJ, Lee KW, *et al.* (2007) Anti-inflammatory effects of recombinant arginine deiminase originating from *Lactococcus lactis* ssp. *lactis* ATCC 7962. *J Microbiol Biotechnol* **17**, 1491–1497.

30. Thompson CB, Bauer DE, Lum JJ, *et al.* (2005) How do cancer cells acquire the fuel needed to support cell growth? *Cold Spring Harb Symp Quant Biol* **70**, 357–362.

31. Yuneva M, Zamboni N, Oefner P, *et al.* (2007) Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol* **178**, 93–105.

32. Adhikary S & Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* **6**, 635–645.

33. Daksis JI, Lu RY, Facchini LM, *et al.* (1994) Myc induces cyclin D_1 expression in the absence of *de novo* protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. *Oncogene* **9**, 3635–3645.

34. Dang CV, O'Donnell KA, Zeller KI, *et al.* (2006) The c-Myc target gene network. *Semin Cancer Biol* **16**, 253–264.

35. Lamb J & Wheatley DN (2000) Single amino acid (arginine) deprivation induces G_1 arrest associated with inhibition of Cdk4 expression in cultured human diploid fibroblasts. *Exp Cell Res* **255**, 238–249.

36. Leung-Pineda V, Pan Y, Chen H, *et al.* (2004) Induction of p21 and p27 expression by amino acid deprivation of HepG2 human hepatoma cells involves mRNA stabilization. *Biochem J* **379**, 79–88.

37. Seo JH, Sung HJ, Choi CW, *et al.* (2007) Extrinsic nitric oxide donor partially reverses arginine deiminase induced cell growth inhibition through NFκB and Bcl-xL. *Invest New Drugs* **26**, 277–282.