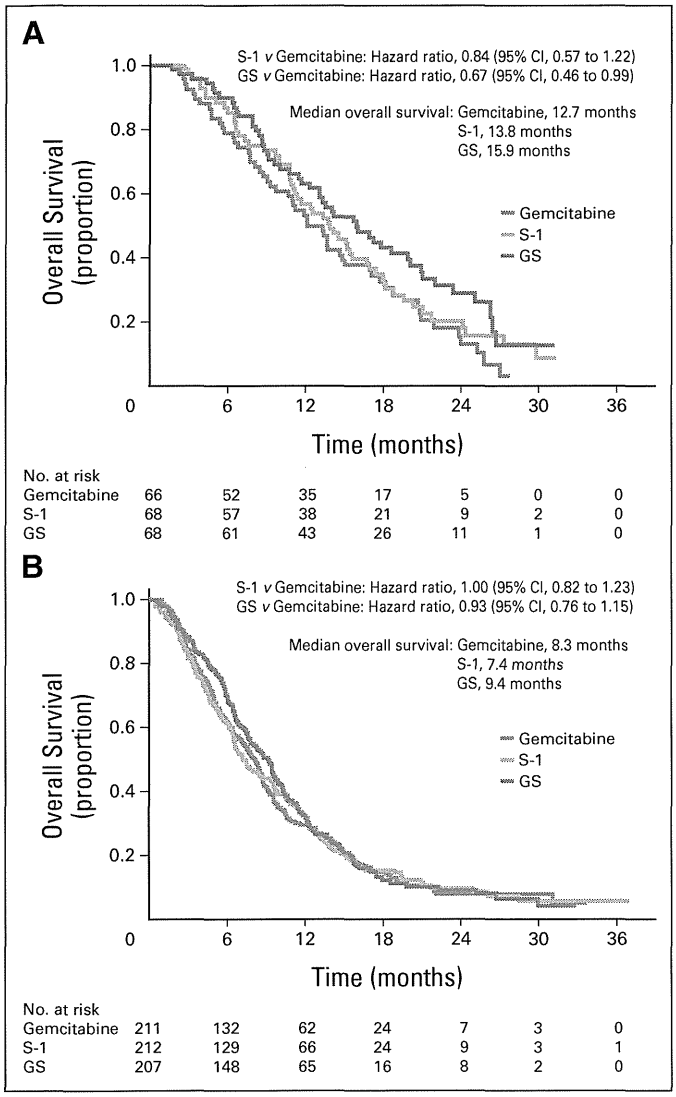

Fig 4. Kaplan-Meier estimates of overall survival in (A) locally advanced disease and (B) metastatic disease. GS, gemcitabine plus S-1.

therapy was not analyzed in this study, a phase II study of second-line S-1 in patients with gemcitabine-refractory PC showed a 15% response rate and 58% disease control rate.[25] Compared with the GS group, which had no promising second-line therapy, the use of S-1 as second-line therapy in the gemcitabine group might have contributed to prolonged survival.

The lack of a significant difference in OS between gemcitabine and GS suggests that gemcitabine and S-1 could be used sequentially rather than concurrently. However, the GS group showed a high response rate and favorable PFS, with a better HR of 0.66 compared with other gemcitabine-based combination regimens in other phase III studies (HR = 0.75 to 1.07).[3,18,20,22,24] Furthermore, the GS group showed a favorable HR for OS in patients with locally advanced disease or patients with a performance status of 1 in the subgroup analyses. Therefore, it is speculated that there may be room to select GS therapy, depending on the profile of the patients and further investigations.

Regarding oral fluoropyrimidines other than S-1, capecitabine has been studied in patients with PC, mainly in the West. In two phase III studies, a combination of gemcitabine plus capecitabine did not significantly prolong survival as compared with gemcitabine alone.[19,20] The results of a meta-analysis of these phase III studies, however, demonstrated that survival was significantly prolonged by combined treatment, with an HR of 0.86,[20] which is similar to the HR for GS in the present study (0.88).

One limitation of our study is that it is uncertain whether our results can be simply extrapolated to Western patients because pharmacokinetics and pharmacodynamics of S-1 between Westerners and East Asians may be different.[26,27] Although S-1 is available for PC only in Japan at the moment, if S-1 is used in Western patients, its effectiveness should be monitored and the dose should be carefully adjusted accordingly. Another potential limitation is that the protocol-specified noninferiority margin of 1.33 may be large. However, the result of point estimate of the HR of S-1 was 0.96 and actual upper limit of the 97.5% CI was 1.18, which was sufficiently lower than the prespecified margin of 1.33. Furthermore, Bayesian posterior probability with log HR within a stricter threshold (log 1.15) was 98%.

Given that most gemcitabine-based combination regimens have not been shown to be significantly superior to gemcitabine alone and that FOLFIRINOX has demonstrated overwhelming superiority to gemcitabine in a phase III study, reporting an HR of 0.57,[4] the development of gemcitabine-free combination regimens for first-line treatment seems to be warranted. However, because FOLFIRINOX requires the placement of a central venous access port for continuous intravenous infusion of fluorouracil, it can be expected that S-1, an oral fluoropyrimidine, will replace the continuous infusion of fluorouracil in the future.

In conclusion, this study has verified the noninferiority of S-1 to gemcitabine, thereby suggesting that S-1 can be used as first-line therapy for locally advanced and metastatic PC. Because S-1 was confirmed to be a key treatment for PC, S-1–based regimens are expected to be developed in the future to improve the management of this formidable disease.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

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Details of Adequate Organ Functions in Enrollment Criteria and Main Exclusion Criteria

Adequate organ functions were defined as follows: leukocyte count $\geq$ 3,500/μL, neutrophil count $\geq$ 2,000/μL, platelet count $\geq$ 100,000/μL, hemoglobin level $\geq$ 9.0 g/dL, serum creatinine level $\leq$ 1.2 mg/dL, creatinine clearance $\geq$ 50 mL/min, serum AST and ALT levels $\leq$ 150 U/L, and serum total bilirubin level $\leq$ 2.0 mg/dL or $\leq$ 3.0 mg/dL if biliary drainage was performed.

Main exclusion criteria were as follows: pulmonary fibrosis or interstitial pneumonia; watery diarrhea; active infection; marked pleural effusion or ascites; and serious complications such as heart failure, peptic ulcer bleeding, or poorly controlled diabetes. Pancreatic cancers other than adenocarcinoma or adenosquamous carcinoma (eg, anaplastic carcinoma) were excluded from the study.

Dosage Adjustment Guideline for Toxicities

All treatment cycles were repeated until disease progression, unacceptable toxicity, or patient refusal. If patients had a leukocyte count of less than 2,000/μL, a neutrophil count of less than 1,000/μL, a platelet count of less than 70×10^3/μL, or grade 3 or worse rash, the administration of anticancer agents was postponed. S-1 was temporarily halted both in S-1 and in GS groups if patients had a creatinine level of 1.5 mg/dL or higher or grade 2 or worse diarrhea or stomatitis. Treatment was discontinued if these events did not resolve within 4 weeks after treatment suspension. In patients who experienced febrile neutropenia, grade 4 leukopenia, neutropenia, or thrombocytopenia or grade 3 or worse rash, the dose of gemcitabine was reduced by 200 mg/m^2. In patients with febrile neutropenia; grade 4

leukopenia, neutropenia, or thrombocytopenia; a creatinine level of 1.5 mg/dL or higher; or grade 3 or worse diarrhea, stomatitis, or rash, the dose of S-1 was reduced by 20 mg/d.

Sample Size Determination: Statistical Methods

In the initial plan, the total target number of patients was set at 600, given a statistical power of 80%, an enrollment period of 3 years, and a follow-up period of 2 years. However, because patient enrollment was faster than expected, the target number of patients was revised to 750 to provide the study with a statistical power of 90%. Consequently, the final analysis was performed after the occurrence of 680 events had been confirmed. An interim analysis was not performed. Although the actual median OS in the gemcitabine group was better than initially expected, because an adequate number of patients had been enrolled, a power of $\geq$ 90% was maintained on recalculation of the power on the basis of the actual results.

Quality of Life

To assess the quality of life, the health status of patients on the EQ-5D questionnaire was converted into a single simple utility index ranging from 0 for death to 1 for complete health. Quality-adjusted life-years (QALYs) for individual patients were estimated as the product of the utility index during follow-up and survival time and were compared between the groups, using the generalized Wilcoxon test.

As a result, median QALYs were 0.401 in the gemcitabine group, 0.420 in the S-1 group, and 0.525 in the GS group. The QALY value in the S-1 group was similar to that in the gemcitabine group, and there was no statistically significant difference between the two groups ($P = .56$). The QALY value in the GS group was significantly better than that in the gemcitabine group ($P < .001$). The details of quality-of-life assessments will be reported elsewhere.

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ORIGINAL ARTICLE

α-Lipoic Acid Prevents the Induction of iNOS Gene Expression Through Destabilization of Its mRNA in Proinflammatory Cytokine-Stimulated Hepatocytes

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Abstract
Background/Aims α-Lipoic acid (α-LA) has been reported to reduce ischemia–reperfusion injury (IRI). Proinflammatory cytokines stimulate the induction of inducible nitric oxide synthase (iNOS) gene expression, leading to excess production of NO and resulting in liver injury including IRI. We hypothesized that inhibition of iNOS induction underlies the protective effects of α-LA on the liver. The objective was to investigate whether α-LA directly influences iNOS induction in cultured hepatocytes, which is used as a simple in vitro injury model, and the mechanism involved.
Methods Primary cultured rat hepatocytes were treated with interleukin (IL)-1β in the presence or absence of α-LA. The induction of iNOS and NO production and its signal were analyzed.
Results α-LA inhibited the expression of iNOS mRNA and protein dose- and time-dependently, resulting in decreases in NO production. α-LA had no effects on the degradation of IκB proteins and activation of NF-κB. In contrast, α-LA inhibited the upregulation of type I IL-1 receptor stimulated by IL-1β, although α-LA had no effect on Akt activation. Transfection experiments with iNOS promoter-luciferase constructs revealed that α-LA had no effect on the transactivation of the iNOS promoter, but decreased the stabilization of iNOS mRNA. Further, α-LA inhibited the expression of an iNOS gene antisense-transcript, which is involved in iNOS mRNA stability.
Conclusions Results indicate that α-LA inhibits the induction of iNOS gene expression at a posttranscriptional step via iNOS mRNA stabilization, rather than promoter activation. It may provide useful therapeutic effects through the suppression of iNOS induction involved in liver injury.

Keywords α-Lipoic acid · Liver injury · Inducible nitric oxide synthase · Primary cultured hepatocytes · Nuclear factor-κB · Type I interleukin-1 receptor

Introduction

α-Lipoic acid (α-LA), which is a naturally occurring compound present in the majority of pro-eukaryotic and eukaryotic cells, is believed to be a key regulator of energy metabolism in mitochondria [1]. α-LA has been used as a therapeutic agent in the treatment of diabetic neuropathy [2] and as a nutritional supplement in many countries, which is known to be without serious side-effects. Besides its well-described antioxidant effects, α-LA exhibits distinct regulatory action on signal-transduction processes playing a central role in tissue damage and protection [3–6].

Ischemia–reperfusion injury (IRI) of the liver is still a major cause of morbidity and mortality in patients undergoing liver surgery and transplantation. In animal models, there are a variety of pharmacological agents protecting the liver from IRI [7]. Among them, preconditioning of α-LA reduced IRI of the rat liver [8]. Further, α-LA reduced IRI of the liver in humans who were undergoing liver surgery [9]. In various liver injuries including IRI, lipopolysaccharide and

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proinflammatory cytokines including interleukin (IL)-1β and tumor necrosis factor (TNF)-α stimulate the induction of inducible nitric oxide synthase (iNOS) gene expression, which is followed by excess levels of nitric oxide (NO) production. Such NO production by iNOS has been implicated as one of the factors in liver injury, although NO has been reported to exert either detrimental or beneficial effects depending the insults and tissues involved.

In animal models of liver injury caused by various insults, such as IR, partial hepatectomy and endotoxin shock, the induction of iNOS and NO production is upregulated concomitantly with the production of cytokines including TNF-α, IL-6, interferon-γ and cytokine-induced neutrophil chemoattractant-1 in the liver, as we reported previously [10–14]. In these studies, drugs showing liver-protective effects inhibited the induction of iNOS and NO production as well as the decreased production of various inflammatory mediators. Furthermore, in vitro experiments with primary cultured rat hepatocytes revealed that these drugs also inhibited the induction of iNOS and NO production [12, 15, 16]. Thus, the prevention of NO production is considered to be one of the indicators of liver protection. In the present study, we examined IL-1β-stimulated cultured hepatocytes as a simple in vitro injury (or inflammation) model for in vivo animal models. We investigated to examine whether α-LA directly inhibits the induction of iNOS and NO production in this in vitro system, and if so, the mechanism involved in.

Materials and Methods

Materials

Recombinant human IL-1β (2×10^7 units/mg protein) was provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). α-Lipoic acid (α-LA; 1,2-dithiolane-3-pentanoic acid, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate-buffered saline (10 mM) and added to the culture medium. [γ-^{32}P]Adenosine-5′-triphosphate (ATP; −222 TBq/mmol) was obtained from DuPont-New England Nuclear Japan (Tokyo, Japan). Rats were kept at 22°C under a 12-h/12-h light/dark cycle, and received food and water ad libitum. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Care Committee of Kansai Medical University.

Primary Cultures of Hepatocytes

Hepatocytes were isolated from male Wistar strain rats (200–220 g; Charles River, Tokyo, Japan) by collagenase (Wako Pure Chemicals, Osaka, Japan) perfusion [17, 18]. Isolated hepatocytes were suspended in culture medium at 6×10^5 cells/ml, seeded into 35-mm plastic dishes (2 ml/dish; Falcon Plastic, Oxnard, CA, USA) and cultured at 37°C in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air. The culture medium was Williams' medium E (WE) supplemented with 10% newborn calf serum, HEPES (5 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (10 nM) and insulin (10 nM). After 5 h, the medium was replaced with fresh serum-free WE, and the cells were cultured two days before use in experiments. The numbers of cells attached to the dishes were calculated by counting the nuclei [19] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean $\pm$ SE, $n = 7$ experiments).

Treatment of Cells with α-LA

On day 2, the cells were washed with fresh serum- and hormone-free WE, and incubated with IL-1β (1 nM) in the same medium in the presence or absence of α-LA. The doses of α-LA used are indicated in the appropriate figures and their legends.

Determinations of NO Production and Lactate Dehydrogenase (LDH)

Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [20]. Culture medium was also used for measurements of LDH activity to reflect cell viability using a commercial kit (Wako Pure Chemicals).

Western-Blot Analysis

Total cell lysates were obtained from cultured cells as described previously [15] with minor modifications as follows. Cells (1×10^6 cells/35-mm dish) were lysed in 100–200 µl of solubilizing buffer (10 mM Tris–HCl, pH 7.4, containing 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)), passed through a 26-gauge needle, allowed to stand on ice for 30 min and then centrifuged (16,000 $\times$ g for 15 min). The supernatant (total cell lysate) was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final: 125 mM Tris–HCl, pH 6.8, containing 5% glycerol, 2% SDS and 1% 2-mercaptoethanol), subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity BioReagents, Golden, CO, USA), human

IκBα, human IκBβ, mouse type I IL-1 receptor (IL-1RI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat β-tubulin (internal control; Clone TUB2.1; Sigma Chemical Co., St. Louis, MO, USA), followed by visualization with an ECL blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

In the case of Akt, total cell lysates prepared from 100-mm dishes (5×10^6 cells/dish) were precleared with Protein A (Sigma Chemical Co.), and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling, Beverly, MA, USA) and Protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation overnight at 4°C, the immunocomplexes were centrifuged (16,000 × *g* for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and analyzed by Western blotting using rabbit polyclonal antibodies against human Akt and phospho-(Ser473) Akt (Cell Signaling) as primary antibodies. In the case of p65, nuclear extracts were immunoprecipitated with an anti-p65 antibody (H286; Santa Cruz Biotechnology). The bands were analyzed by Western blotting using an antibody against human NF-κB p65 (BD Transduction Laboratories, Lexington, KY, USA).

Northern-Blot Analysis and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured hepatocytes using a guanidinium–phenol–chloroform method [21] with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) or a phenol-free, filter-based total RNA isolation kit (RNAqueous Kit; Ambion, Austin, TX, USA) according to the manufacturer's instructions, and then treated with a TURBO DNA-free Kit (Ambion) if necessary. Ten micrograms of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes (Nytran, Schleider & Schuell, Dassel, Germany), and immobilized by baking (80°C, 2 h) for hybridization with DNA probes. cDNA probe of iNOS (rat vascular smooth muscle cells; RT-PCR product (830 bp) amplified with YNO12 and YNO56 primers [22]) was provided by Dr. Y. Nunokawa (Suntory Institute for Biochemical Research, Osaka, Japan). cDNAs encoding rat IL-1RI [23] and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [24] were prepared by RT-PCR [25]. The cDNAs were radiolabeled with [α-32P]dCTP by the random priming method.

For strand-specific RT-PCR analysis, cDNAs were synthesized from total RNA with strand-specific primers, and step-down PCR was performed using PC708 (Astec, Fukuoka, Japan), as previously described [25, 26] with minor modifications. For the antisense-transcript of iNOS, the sense primer 5′-CCTTTGCCTCATACTTCCTCAGA-3′ was used for RT and the primer set 5′-ACCAGGAGGCGC CATCCCGCTGC-3′ and 5′-ATCTTCATCAAGGAATTA TACACGG-3′ (211-bp product) was used for PCR. The PCR protocol was: ten cycles of (94°C, 1 min; 65°C, 1 min 30 s; 72°C, 20 s); 15 cycles of (94°C, 1 min; 60°C, 1 min 30 s; 72°C, 20 s); and 5 cycles of (94°C, 1 min; 55°C, 1 min 30 s; 72°C, 20 s). The amplified products were analyzed by 3% agarose gel electrophoresis with ethidium bromide, and the levels of antisense-transcript were semi-quantified using a UV transilluminator. The antisense-transcript levels were measured in triplicate by real-time PCR using an iCycler System (Bio-Rad Laboratories). SYBR Green I (Roche Diagnostics) was included in the reaction mixture, and the following touchdown protocol was applied: 1 cycle of 94°C for 1 min; and 50 cycles of 94°C for 30 s, (72–0.3 × *n*)°C for 1 min where *n* is number of cycles, and 72°C for 30 s. The cDNA for the rat antisense-transcript was deposited in DDBJ/EMBL/GenBank under Accession No. AB250952.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared according to Schreiber et al. [27] with minor modifications [28]. Briefly, the dishes were placed on ice, washed with Tris–HCl-buffered saline, harvested with the same buffer using a rubber policeman and centrifuged (1,840 × *g* for 1 min). The precipitate (2×10^6 cells from two 35-mm dishes) was suspended in 400 μl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasylol, 0.5 mM PMSF and 1 mM dithiothreitol) and incubated on ice for 15 min. After addition of Nonidet P-40 (final: 0.625%), the cells were lysed by vortexing (2–3 times for 1 min each) and centrifuged (15,000 × *g* for 1 min). The nuclear pellet was resuspended with extraction buffer (10 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasylol, 0.5 mM PMSF and 1 mM dithiothreitol), followed by continuous mixing for 20 min and centrifugation (15,000 × *g* for 5 min). Aliquots of the supernatant (nuclear extract) were frozen in liquid nitrogen and stored at −80°C until use.

Binding reactions (total: 15 μl) were performed by incubating nuclear extract aliquots (4 μg of protein) in reaction buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 10% glycerol and 1 mg of poly(dI-dC)) with the probe (approximately 40,000 cpm) for 20 min at room temperature. In the case of supershift assays, the nuclear extracts were incubated in the presence of anti-p50 and anti-p65 antibodies (nuclear factor (NF)-κBp50 (NLS) and NF-κBp65 (H286); Santa Cruz Biotech) or cold probes as a competitor (250-fold excess) for 30 min at 4°C, followed by incubation with the labeled probe. The products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in

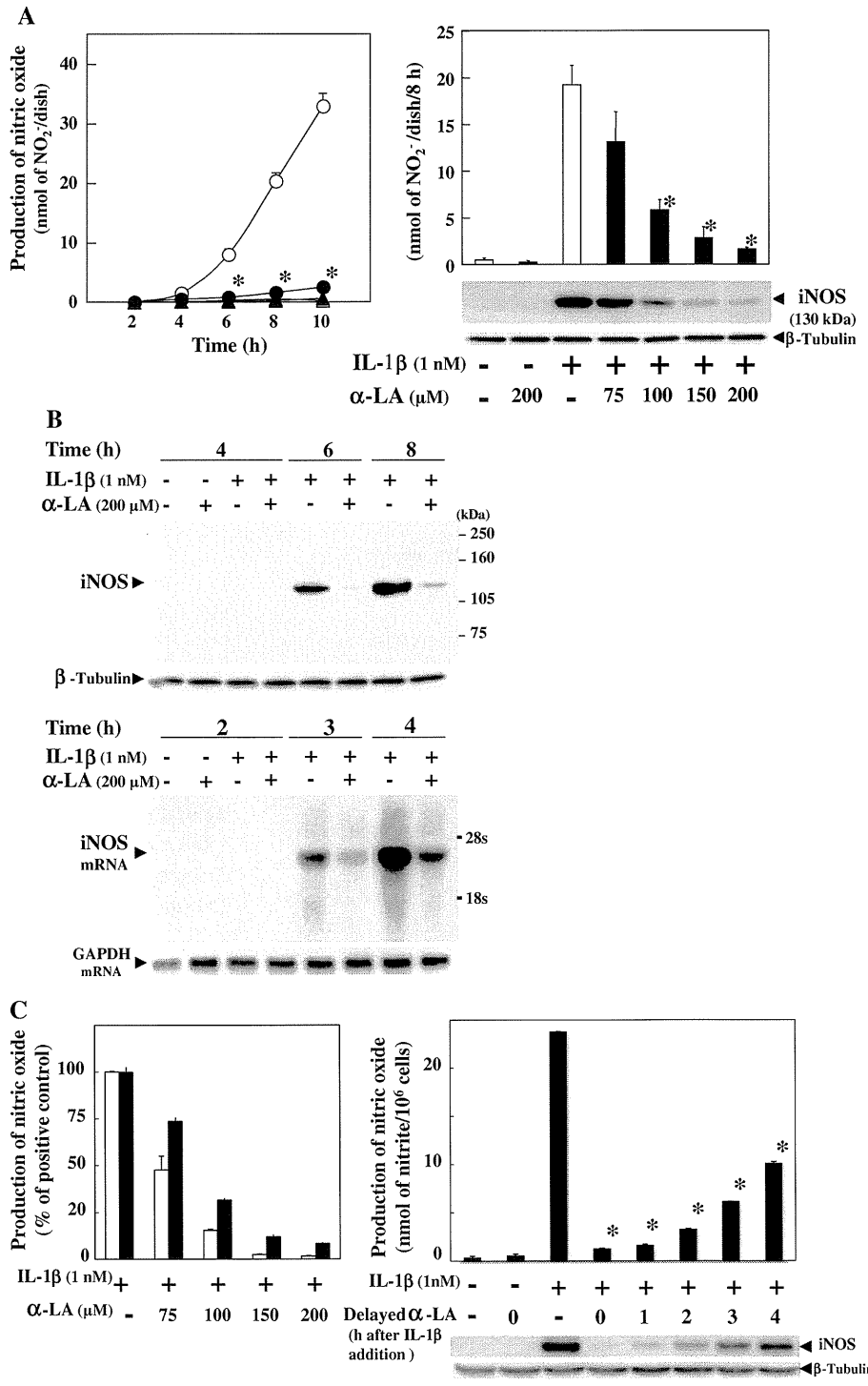

Fig. 1 Effects of α-lipoic acid on the induction of NO production and iNOS in hepatocytes. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of α-LA (75–200 μM). **a/b** Simultaneous addition; effects of α-LA (200 μM) for the indicated times on NO production (**a**, *left*) (IL-1β, *open circles*; IL-1β ± α-LA, *closed circles*; α-LA, *closed triangles*; controls (without IL-1β and α-LA), *open triangles*), iNOS protein and iNOS mRNA (**b**). Effect of various doses of α-LA (75–200 μM) over 8 h on NO production and iNOS protein (**a**, *right*). **c** Pretreatment and delayed treatment; cells were treated with simultaneous addition of IL-1β and α-LA (75–200 μM) (*left*, *closed*) or pretreated with α-LA for 1 h before IL-1β addition (*left*, *open*), and treated with α-LA (200 μM) at 0–4 h after IL-1β addition (*right*). The effects of α-LA on NO production and iNOS protein were analyzed at 8 h after IL-1β addition. The levels of nitrite were measured in culture medium (data are means ± SD with $n = 3$ dishes/point; $^*p < 0.05$ vs. IL-1β alone). In the Western-blotting panel, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. Total RNA (10 μg) was analyzed by Northern blotting, and the filters were probed with labeled inserts for iNOS or GAPDH cDNA

high ionic strength buffer (50 mM Tris–HCl, 380 mM glycine, 2 mM EDTA, pH 8.5) and the dried gels were analyzed by autoradiography. An NF-κB consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC-3′) from the mouse immunoglobulin κ light chain was purchased (Promega, Madison, WI, USA) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford [29] with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.

Construction of Luciferase Reporter Plasmids and Expression Plasmids

The 1.2-kb 5′-flanking region including the TATA box of the rat iNOS gene was inserted into the pGL3-Basic vector

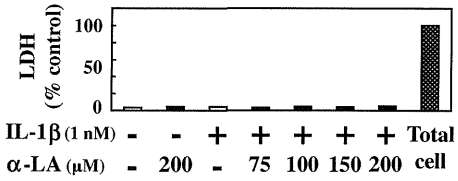

Fig. 2 Effects of α-lipoic acid on cellular cytotoxicity. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of α-LA (75–200 μM) for 8 h. LDH were measured in culture medium (data are means ± SD with $n = 3$ dishes/point)

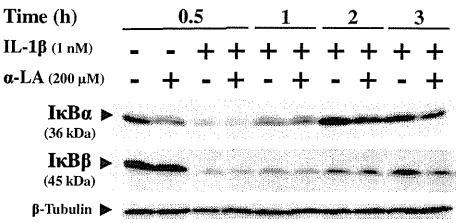

Fig. 3 Effects of α-lipoic acid on the degradation of IκB proteins. Cells were treated with IL-1β (1 nM) in the presence or absence of α-LA (200 μM) for the indicated times. Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IκBα, anti-IκBβ, or anti-β-tubulin antibody

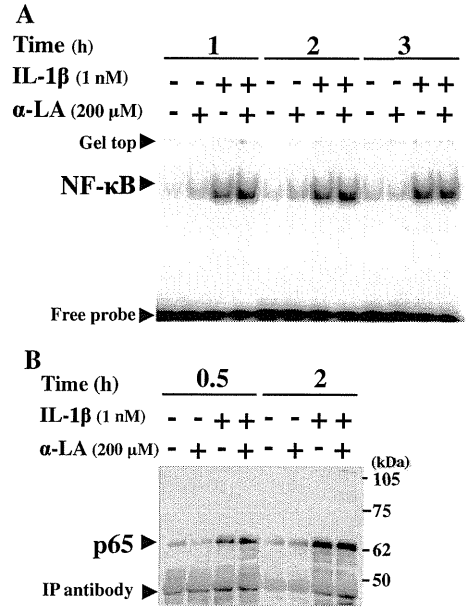

Fig. 4 Effects of α-lipoic acid on the activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of α-LA (200 μM) for the indicated times. **a** Activation of NF-κB. Nuclear extracts (4 μg of protein) were analyzed by EMSAs. **b** Nuclear translocation of NF-κB subunit p65. Nuclear extracts were immunoprecipitated, and the immunoprecipitates were analyzed by Western blotting with an anti-p65 antibody

(Promega) to create pRiNOS-Luc-SVpA [30]. A rat cDNA for the 3′-untranslated region (UTR) of the iNOS mRNA was amplified with the primers 5′-tgctctaGACAGTGAG GGGTTTGGAGAGA-3′ and 5′-gcggatcctttaTTCTTGATC AAACACTCATTTT-3′, and the resultant cDNA was digested with BamH I and Xba I. This cDNA for the iNOS 3′-UTR (submitted to DDBJ/EMBL/GenBank under Accession No. AB250951) was used to replace the SV40 polyadenylation signal (SVpA) of pRiNOS-Luc to create pRiNOS-Luc-3′UTR.

Transfection and Luciferase Assay

Transfection of cultured hepatocytes was performed as described previously [30, 31]. Briefly, hepatocytes were cultured at 4×10^5 cells/dish (35 × 10 mm) in WE supplemented with serum, dexamethasone and insulin for 7 h, before being subjected to magnet-assisted transfection (MATra). Reporter plasmids pRiNOS-Luc-SVpA or pRiNOS-Luc-3′UTR (1 μg) and the CMV promoter-driven β-galactosidase plasmid pCMV-LacZ (1 ng) as an internal control, and plasmid expressing NF-kB (0.125 μg of each pCMV-p65 and pCMV-p105) were mixed with MATra-A reagent (1 μl; IBA GmbH, Göttingen, Germany). After incubation for 15 min on a magnetic plate at room temperature, the medium was replaced with fresh WE containing serum. The cells were cultured overnight, and then treated with or without IL-1β in the presence or absence of α-LA. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

Statistical Analysis

The results shown in the figures are representative of 3–4 independent experiments yielding similar findings. Differences were analyzed by the Bonferroni-Dunn test, and values of $p < 0.05$ were considered to indicate statistical significance.

Results

α-Lipoic Acid Inhibits iNOS Induction in IL-1β-Stimulated Hepatocytes

The proinflammatory cytokine IL-1β stimulated the induction of iNOS gene expression in primary cultures of rat hepatocytes as reported previously [32, 33]. Simultaneous addition of α-LA (75–200 μM) with IL-1β decreased the production of NO time- and dose-dependently (Fig. 1a). α-LA had a maximal effect (more than 80% inhibition) at 200 μM, but showed no cellular cytotoxicity as evaluated by release of LDH into the culture medium

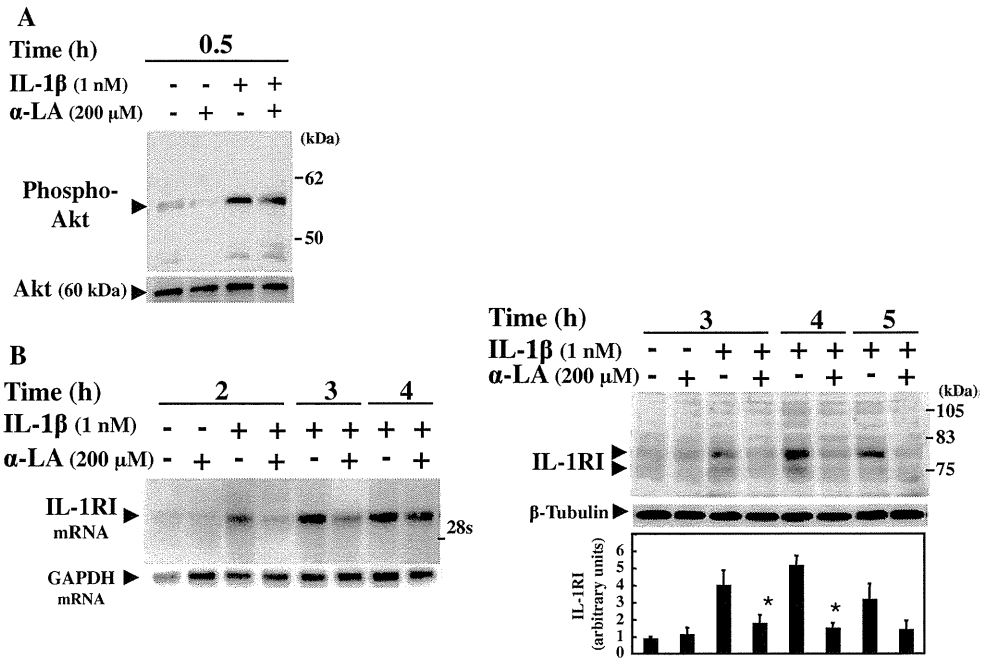

Fig. 5 Effects of α-lipoic acid on the upregulation of IL-1RI. Cells were treated with IL-1β (1) in the presence or absence of α-LA (200 μM) for the indicated times. **a** Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting with an anti-phospho-Akt or anti-Akt antibody. **b** Total RNA (10 μg) was analyzed by Northern blotting, and the filters were probed with labeled inserts for IL-1RI or GAPDH cDNA. **c** Cell lysates (50 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti-β-tubulin antibody. The bands corresponding to IL-1RI were quantified by densitometry (lower, means ± SD for $n = 3$ experiments; $^*p \leq 0.05$ vs. IL-1β alone)

(Fig. 2) and Trypan blue exclusion in hepatocytes (data not shown). Western- and Northern-blot analyses revealed that α-LA decreased the levels of iNOS protein (Fig. 1a, b, upper) and iNOS mRNA (Fig. 1b, lower), suggesting that it inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

Pretreatment of α-LA, which was added to the medium 1 h before IL-1β addition, increased its inhibitory effects as compared with simultaneous addition (Fig. 1c, left). Further, we examined whether delayed treatment of α-LA influences NO production. α-LA was added to the medium 1–4 h after the addition of IL-1β. Although the magnitude of inhibition decreased time-dependently, delayed treatment of α-LA up to 4 h after IL-1β addition still markedly inhibited NO production (Fig. 1c, right).

α-Lipoic Acid Has No Effect on NF-κB Activation But Reduces the Type I IL-1 Receptor (IL-1RI) Upregulation

We examined the mechanism involved in the inhibition of iNOS induction. IL-1β enhances the degradation of IκBα and IκBβ proteins, followed by translocation of the transcription factor NF-κB from the cytoplasm to the nucleus, and its DNA binding (NF-κB activation). α-LA had no effects on IκB degradation and NF-κB activation (Figs. 3, 4a). In support of these observations, Western blotting of nuclear extracts revealed that α-LA did not influence the levels of NF-κB subunit p65 in the nucleus (Fig. 4b).

IL-1β also stimulates upregulation of IL-1RI through the activation of phosphatidylinositol-3 kinase (PI3K)/Akt [34], which is essential for the induction of iNOS gene expression in concert with NF-κB activation signaling in hepatocytes. α-LA had no effect on the phosphorylation (activation) of Akt (Fig. 5a), a downstream kinase of PI3K. However, α-LA reduced the levels of IL-1RI mRNA and its protein in time-dependent manners (Fig. 5b). Taken together, these results suggest that α-LA can influence the signal of IL-1RI upregulation but not of NF-κB activation.

α-Lipoic Acid Affects iNOS mRNA Stability but not iNOS Promoter Activation

Next, we carried out transfection experiments with iNOS promoter-firefly luciferase (Luc) constructs, namely pRiNOS-Luc-SVpA and pRiNOS-Luc-3′UTR, which detect iNOS mRNA synthesis and its stability [12, 16, 31], respectively (Fig. 6a). IL-1β increased both of the luciferase activities, while α-LA significantly decreased the

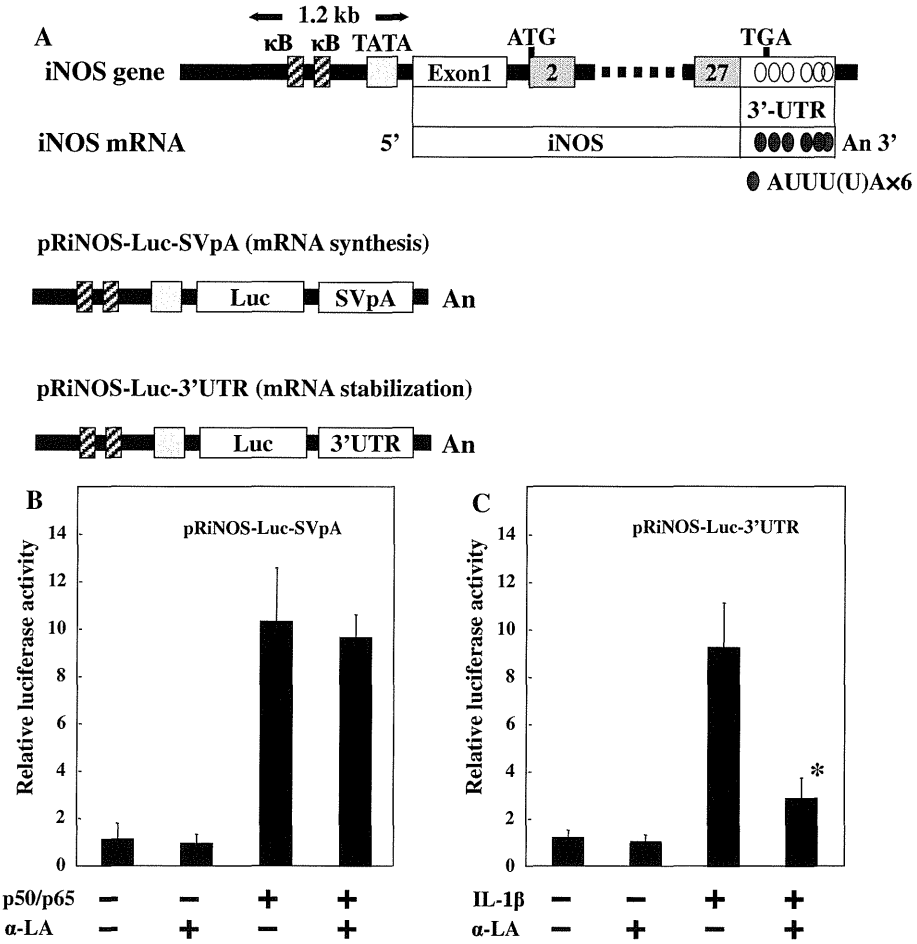

Fig. 6 Effects of α-lipoic acid on the transactivation of the iNOS promoter. **a** Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), luciferase gene and SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3′-UTR (pRiNOS-Luc-3′UTR). 'An' indicates the presence of a poly(A) tail. The iNOS 3′-UTR contains AU-rich elements (AUUU(U)A × 6), which contribute to mRNA stabilization. **b** Cotransfected cells with pRiNOS-Luc-SVpA and p50/p65 were treated in the presence or absence of α-LA (200 μM) without IL-1β for 8 h. **c** Transfected cells (pRiNOS-Luc-3′UTR) without p50/p65 were treated with IL-1β (1 nM) in the presence or absence of α-LA (200 μM) for 5 h. The luciferase activities were normalized by the β-galactosidase activity. The fold activation was calculated by dividing the luciferase activity by that of the control (without IL-1β and α-LA). Data are means ± SD ($n = 4$ dishes). *$p < 0.05$ versus IL-1β alone

iNOS mRNA stability (Fig. 6c) but not iNOS mRNA synthesis (Fig. 6b). Recently, we reported that the natural antisense-transcript of the iNOS gene upregulates iNOS mRNA stability in IL-1β-stimulated hepatocytes [35]. RT-PCR and quantitative real-time PCR experiments revealed that IL-1β increased the expression of the iNOS gene antisense-transcript time-dependently with increased levels of iNOS mRNA, and that α-LA decreased the levels of the antisense-transcript (Fig. 7a, b).

Discussion

It is known that the induction of iNOS gene expression is regulated by transactivation of the iNOS promoter and posttranscriptional modifications [36]. In experiments with iNOS promoter constructs, α-LA was found to have no effect on iNOS promoter transactivation (Fig. 6b) but to inhibit iNOS induction at the step of its mRNA stabilization (Fig. 6c) in proinflammatory cytokine-stimulated hepatocytes. In support of the former, α-LA did not inhibit the degradation of IκBα and IκBβ (Fig. 3), nuclear translocation of NF-κB subunit p65 (Fig. 4b) and DNA binding of NF-κB (Fig. 4a), indicating that α-LA cannot influence the iNOS mRNA synthesis through NF-κB activation.

Regarding the iNOS mRNA stabilization, the 3′-UTR of the iNOS mRNA in rats has six AREs (AUUU(U)A), which are associated with ARE-binding proteins such as HuR and heterogeneous nuclear ribonucleoproteins L/I

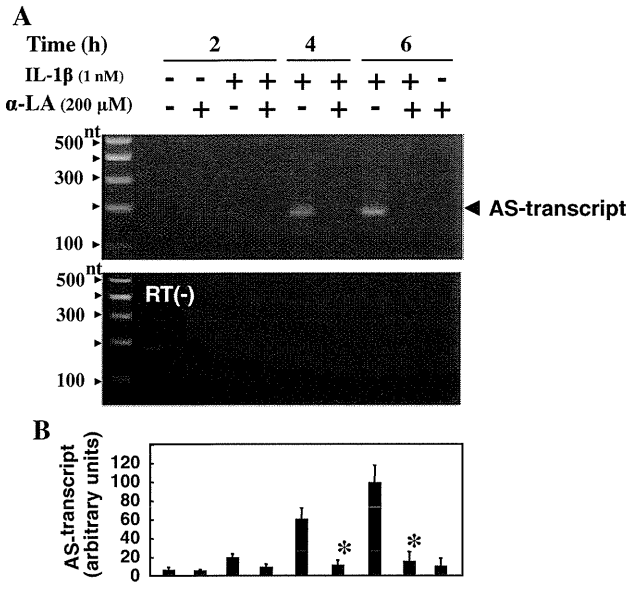

Fig. 7 Effects of α-lipoic acid on the expression of the iNOS gene antisense-transcript in hepatocytes. Cells were treated with IL-1β (1 nM) in the presence or absence of α-LA (200 μM) for the indicated times. **a** Total RNA was analyzed by strand-specific RT-PCR to detect the iNOS gene antisense-transcript (AS-T). **b** Quantitative RT-PCR was carried out for the iNOS gene AS-T, and the copy number of the iNOS gene AS-T was normalized by that of a negative PCR control using total RNA without RT [RT(−)]. Data are means ± SD ($n = 3$ experiments). *$p < 0.05$ versus IL-1β alone

(PTB), thus contributing to the stabilization of the mRNA [37]. Recently, we found that the antisense strand corresponding to the 3′-UTR of iNOS mRNA is transcribed from the iNOS gene, and that the iNOS mRNA antisense-transcript plays a key role in stabilizing the iNOS mRNA by interacting with the 3′-UTR and ARE-binding proteins [35]. In our in vitro model, α-LA destabilized the iNOS mRNA through the inhibition of iNOS gene antisense-transcript expression (Fig. 7). Drugs such as edaravone (free radical scavenger) [16], FR183998 (Na^+/H^+ exchanger inhibitor) [12] and insulin-like growth factor I [13] were found to inhibit iNOS induction partly by suppressing iNOS antisense-transcript production in damaged liver and primary cultured hepatocytes.

In concert with NF-κB activation, the upregulation of IL-1RI is required for the induction of the iNOS gene expression, as we reported previously [34]. In the present study, we found that α-LA decreased the expressions of IL-1RI mRNA and its protein (Fig. 5b), although α-LA had no effect on Akt activation (Fig. 5a), presumably leading to the inhibition of iNOS antisense-transcript expression (Fig. 7) and resulting in decreased activities of iNOS mRNA stabilization. Thus the destabilization of iNOS mRNA largely contributed to the decreased levels of iNOS mRNA and protein. However, it cannot negate the possibility that α-LA may affect iNOS mRNA stability through other signal pathway rather than IL-1RI upregulation.

These results suggest that α-LA can inhibit the induction of iNOS expression in liver injury, which is involved in the liver-protective effects of α-LA. Muller et al. [I-8] reported that α-LA attenuated IRI I in both perfused liver and IR model of rats. In contrast to our findings in hepatocytes, they found that α-LA preconditioning increased the phosphorylation of Akt and reduced NF-κB activation, resulting in the liver-protective effects in the liver. α-LA-treated livers showed no increased phosphorylation of endothelial NOS, which has been shown to play a protective role in hepatic IRI [38]. They did not mention the effect of α-LA on iNOS induction at all. Whole liver is composed of parenchymal cells, hepatocytes, and non-parenchymal cells such as Kupffer, Ito and sinusoidal endothelial cells. It seems likely that α-LA also may have the liver-protective effects through various non-parenchymal cells as well as hepatocytes in a different mechanism.

Delayed treatment with α-LA was found to cause a significant reduction in NO production and iNOS induction (Fig. 3c). These observations may be of clinical importance, since the initiation of therapeutic α-LA treatment is usually delayed from the onset of diseases. Our simple in vitro experiment with cultured hepatocytes may be adequate for screening of liver-protective drugs, because it is rapid and inexpensive compared with in vivo animal models of liver injury. However, there are a variety of factors involved in liver injury including proinflammatory cytokines and chemokines such as TNF-α, IL-6, interferon-γ, and IL-8, in addition to iNOS induction and NO production. The liver-protective effects of drugs deduced from this model need to be examined and supported in in vivo animal models.

In conclusion, α-LA inhibited iNOS gene expression at posttranscriptional step in cultured rat hepatocytes in an in vitro liver injury model. α-LA may have therapeutic potential for various liver injuries including IRI.

Conflict of interest There is no conflict of interest.

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Neo-adjuvant Chemoradiation Therapy Using S-1 Followed by Surgical Resection in Patients with Pancreatic Cancer

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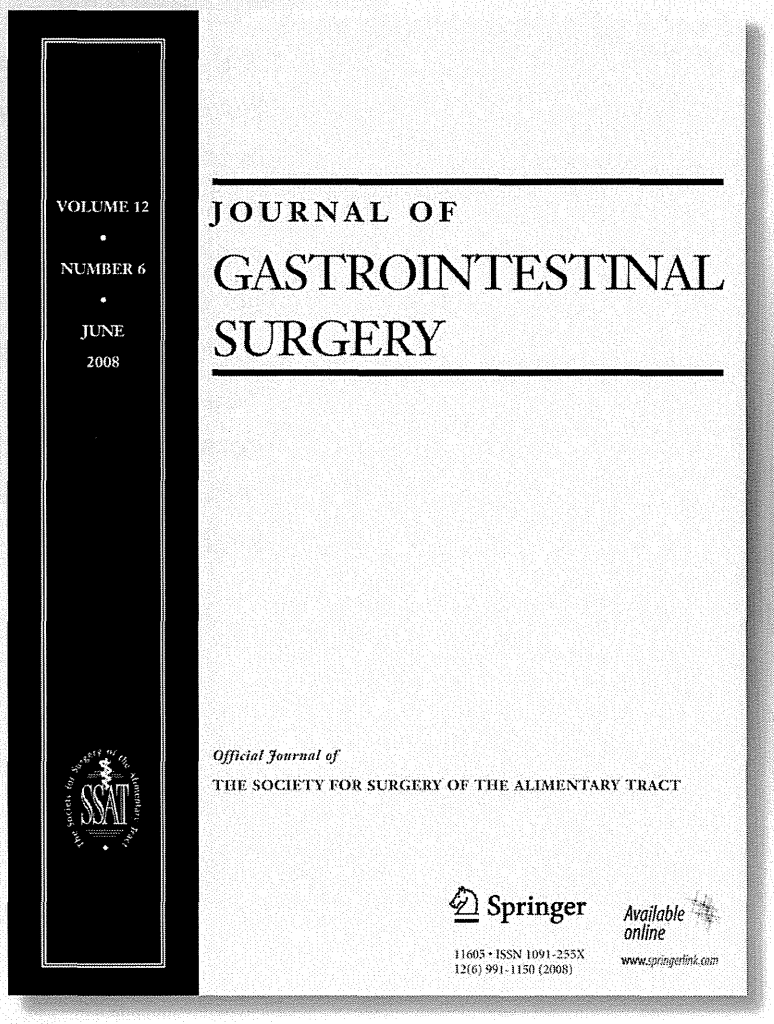

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ORIGINAL ARTICLE

Neo-adjuvant Chemoradiation Therapy Using S-1 Followed by Surgical Resection in Patients with Pancreatic Cancer

Sohei Satoi · Hideyoshi Toyokawa · Hiroaki Yanagimoto · Tomohisa Yamamoto · Minoru Kamata · Chisato Ohe · Noriko Sakaida · Yoshiko Uemura · Hiroaki Kitade · Noboru Tanigawa · Kentaro Inoue · Yoichi Matsui · A-Hon Kwon

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Abstract
Objective The aim of this study was to compare short-term surgical results in pancreatic cancer patients who underwent surgical resection after neo-adjuvant chemoradiation therapy (NACRT) using S-1.
Methods The study population comprised 77 patients with pancreatic cancer between 2006 and 2010. Out of 34 patients who underwent staging laparoscopy between 2008 and 2010, 31 patients without occult distant organ metastasis underwent chemoradiation and of whom 30 underwent pancreatectomy (NACRT group). Of the other 43 patients, 36 underwent surgical resection in 2006–2008, followed by adjuvant therapy (adjuvant group). The primary endpoint was frequency of pathological curative resection (R0).
Results The new regimen of NACRT was feasible and safe. Twenty-eight of 30 (93%) patients in the NACRT group had R0 resection, which was significantly higher than in the adjuvant group (21 of 36 patients, 58%, p=0.005). The number and extent of metastatic lymph nodes in the NACRT group (1 (0–25), N0/1; 18 of 38) was significantly lower than in the adjuvant group (2 (0–19), N0/1; 23 of 30), p=0.0363). The frequency of intractable ascites in the NACRT group (eight of 30) was significantly higher than in the adjuvant group (two of 36, p=0.035).
Conclusion Neo-adjuvant chemoradiation therapy using S-1 followed by pancreatectomy can improve the rate of pathologically curative resection and reduces the number and extent of lymph node metastasis.

Keywords Chemoradiation · S-1 · Adjuvant chemotherapy · Residual tumor grading · Mortality and morbidity

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Introduction

Pancreatic cancer is a lethal disease with a poor prognosis, even in patients who have undergone curative resection. The results of surgical therapy alone for ductal pancreatic adenocarcinoma are disappointing, and the 5-year actual survival rate ranges from 3% to 17%, even after surgical resection.[1–5] Bradley proposed that further improvements in the numbers of long-term survivors from this dread disease, or increases in the number of actual cures, are unlikely to result from modifications of current surgical techniques.[6] To achieve a 5-year survival rate exceeding 50% in patients with pancreatic cancer, Traverso[7] advocated appropriate patient selection for curative resection by accurate staging, balanced resection, centralized treatment in high-volume centers, and the use of an effective adjuvant or neo-adjuvant therapy.

Neo-adjuvant chemoradiation therapy (NACRT) has several possibilities, such as improved patient selection after the re-staging evaluation, increased resectability rate with clear margins (R0 resection), and a decreased rate of metastatic lymph nodes (LN) and local relapse.[8] We previously reported that NACRT increased the resectability rate with clear margins and decreased the rate of metastatic spread to the lymph nodes, resulting in a significant improvement of the 5-year actual survival rate in curative cases with pancreatic cancer and who had not received adjuvant therapy.[9–11] However, there were three limitations and/or issues associated with our previous study that need to be addressed. Firstly, approximately 20% of patients who underwent NACRT did not undergo subsequent surgical resection because of tumor progression or newly developed distant organ metastases. Secondly, no partial or complete responses were observed after pre-operative chemoradiation using low-dose 5-fluorouracil and cisplatin or gemcitabine (400 mg/m^2, three times in 4 weeks). Finally, although the actual disease-free survival rate at 1 year was approximately 50%, this was similar to that of the surgery-alone group. To address these issues, we have introduced a new strategy of treating patients with pancreatic cancer.

The objective of this study was to investigate the short-term results in patients with pancreatic cancer after surgical resection following NACRT using S-1[12], an orally administered drug consisted of a combination of tegafur, 5-chloro-2,4-dihydroxypyridine, and oteracil potassium.

Patients and Methods

Between January 2006 and September 2010, 103 consecutive patients with a clinical diagnosis of pancreatic ductal adenocarcinoma met our resectability criteria[9,10] and were regarded as potentially or borderline resectable or unresectable pancreatic cancer patients, as defined by the National Comprehensive Cancer Network (NCCN) guideline.[13] This diagnosis was made using cine-imaging multi-detector row CT (MDCT) at Kansai Medical University Hospital. The patients who were expected to achieve pathologically curative resection by Appleby operation[14] or distal pancreatectomy with the celiac axis (CA) resection[15] were also eligible for this study. Cases involving an endocrine tumor of the pancreas, intraductal papillary mucinous cancer, acinar cell cancer, anaplastic cancer, duodenal cancer, distal common bile duct cancer, or ampullary cancer were excluded. The period during which the patient was treated determined which group they were classified under, namely adjuvant (2006–2008) or NACRT (2008–2010) groups (Fig. 1).

Adjuvant Group Between January 2006 and September 2008, among 48 consecutive patients, 43 with T3/T4 pancreatic cancer (International Union Against Cancer (UICC) classification, sixth edition[16]) who met our resectability criteria[9,10] were classified as the adjuvant group, as shown in Fig. 1. Peri-operatively, all 43 patients had pathological evidence of pancreatic ductal adenocarcinoma. It was planned that all patients in the adjuvant group who underwent pancreatectomy would then receive adjuvant chemotherapy comprising weekly gemcitabine (1,000 mg/m^2) with three times in 4 weeks and a total of 18 times of gemcitabine administration.

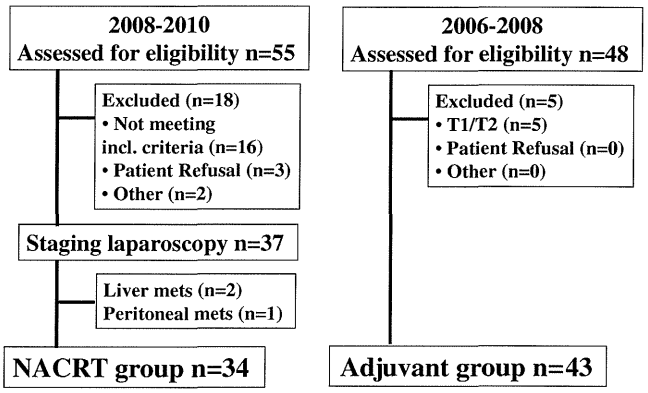

Fig. 1 Study profile

NACRT Group In October 2008, we introduced a new strategy for treating T3/T4 pancreatic cancer patients (UICC classification, sixth edition[16]) who met our resectability criteria,[9,10] and all patients since that date have been treated with this method, as described below. The main criteria for inclusion in this NACRT group were (1) T3/T4 pancreatic cancer (UICC classification, sixth edition[16]) and coincided with our resectability,[9,10] (2) confirmation of pathological evidence of pancreatic cancer, (3) no distant organ metastasis under the staging laparoscopy, and (4) introduction of adjuvant chemotherapy. There were 55 consecutive patients with clinically diagnosed pancreatic cancer between October 2008 and September 2010. Eighteen patients were excluded due to no pathological evidence (n=4), pre-operative diagnosis of lower bile duct cancer (n=4), localized tumor within pancreatic parenchyma (n=3), patients' refusal (n=3), poor performance status (n=2), and other reasons (n=2). The remaining 37 patients underwent staging laparoscopy, following which three additional patients with occult liver (n=2) and peritoneal (n=1) metastases were also excluded. Thus, eventually, 34 patients underwent the planned NACRT (described below) and were classified as the NACRT group (Fig. 1). The tumor extension in these patients was re-evaluated by cine-imaging MDCT 3 weeks after NACRT. It was planned that all patients in whom the MDCT did not show progressive disease or the development of newly distant organ metastasis would undergo

pancreatectomy at approximately 1 week after this re-evaluation. All patients who underwent pancreatectomy following NACRT were to undergo adjuvant chemotherapy with the same regimen as patients in the adjuvant group.

Regimen of NACRT Following the result of the phase I trial of S-1 with concurrent radiotherapy by Ikeda et al.,[12] radiotherapy was administered by 10 or 15 MV photons using three-dimensional treatment planning. A total dose of 50.4 Gy was delivered in 28 fractions over 5.5 weeks. The clinical target volume (CTV) included only the gross primary tumor and nodal involvement enlarged over 10 mm, as detected by computed tomography. Elective nodal irradiation was not used. The planning target volume was defined as CTV plus a 10-mm margin in the lateral direction and 10–20-mm margin in the craniocaudal direction to account for respiratory organ motion and daily setup error. The four-field technique was used. S-1 was administered orally, twice daily (80 mg/m^2/day) on the day of irradiation (Monday to Friday) during radiotherapy.

Extent of Lymph Node and Nerve Plexus Dissection LN dissection around the CA, superior mesenteric artery (SMA), middle colic artery (MCA), superior mesenteric vein (SMV), para-aortic region, and of the hepatoduodenal ligament and right-sided dissection of the nerve plexus around the CA and the SMA was carried out in patients who underwent pancreaticoduodenectomy. In patients who underwent distal pancreatectomy, LN dissection around the CA, SMA, MCA, SMV, and para-aortic region was performed in all patients, while left-sided dissection of the nerve plexus around the CA and the SMA was limited to patients with pancreatic body cancer. In a few cases, distal pancreatectomy with celiac axis resection was performed.

Post-operative morbidity and mortality, defined as in-hospital death due to any cause, were also recorded. Informed consent was obtained from all patients according to institutional regulations, and this study was approved by the local ethics committee. Patient data were obtained from the prospective database of pancreatobiliary disease at Kansai Medical University Hospital.

Endpoints and Statistical Analysis The primary endpoint was the frequency of pathological curative resection (R0) defined by residual tumor grading. The specimen was serially cut with the thickness of 5 mm. All of these were histologically examined according to "General Rules for the study of pancreatic Cancer[17]." Within the general rule, when all of surgical margin factors, such as the pancreatic and bile duct transection margins and dissected peripancreatic tissue margin, were negative, we determined no residual tumor (R0). If at least one of them was positive, pathological residual tumor (R1) was determined. Furthermore, the stumps of the nerve plexus and the retroperitoneal tissue were pathologically examined independently of the resected specimen, in order to evaluate, in detail, the extent of resection (namely R0 or R1) in the NACRT group. Secondary endpoints were feasibility of NACRT and its associated adverse effects, response rate defined by Response Evaluation Criteria in Solid Tumor,[18] pathological tumor grading defined by Evans classification,[19] and safety of pancreatectomy following NACRT. The study design to predict the number of patients necessary for statistical validity (two-sided) was based on the premise of improving the rate of pathologically curative resection from 70% to 90%, with the α set at 0.05 and the β set at 0.2, yielding a power of 80%. It was calculated that 30 patients were required in this study group. The countable data were expressed as the median and range. The countable data using Mann–Whitney U test or the category data using Fisher's exact test or chi-square test were compared between the NACRT and adjuvant groups. Results were considered significant at $p<0.05$.

Results

Clinical Courses of Patients in NACRT and Adjuvant Groups As shown in Fig. 2, among 34 patients in the NACRT group, one patient withdrew her consent to continue NACRT treatment due to grade 1 nausea and fatigue; she underwent pancreatectomy 19 days after discontinuation of NACRT. MDCT for re-evaluation showed the presence of multiple liver metastases in two patients, and one patient refused subsequent pancreatectomy due to poor performance status. Among 31 patients who underwent open laparotomy,

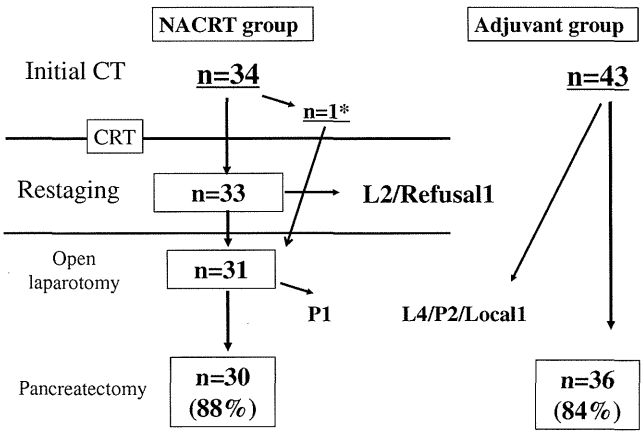

Fig. 2 Clinical course of NACRT and adjuvant groups. *Asterisk* the patient who had refused to continue this regimen underwent pancreatectomy without re-evaluation. *L* liver metastasis, *P* peritoneal metastasis, *Local* locally advanced tumor

one patient incidentally had occult peritoneal metastases. Consequently, 30 out of the 34 patients underwent pancreatectomy in NACRT group.

In the adjuvant group, seven patients did not undergo pancreatectomy because of liver metastasis (n=4), peritoneal metastasis (n=2), and progressive local disease (n=1) which became apparent during open laparotomy. Thus, eventually, 36 of 43 patients underwent surgical resection in the adjuvant group. There was no difference in the surgical resectability rate between the two groups.

Radiological Response and Adverse Effects of Chemoradiotherapy A total of 33 out of 34 patients completed the regimen of NACRT and were evaluated for efficacy in terms of radiological response at 3 weeks after the end of CRT. The patient who had refused to continue this regimen underwent pancreatectomy without re-evaluation. Complete response was not observed in any patient. Partial response and stable disease were achieved in six and 11 patients, respectively. The overall response rate and disease control rate were 18% and 88.0%, respectively. Twenty-five of 34 patients in NACRT group had pancreatic cancer with radiographic findings of portal or superior mesenteric vein (PV/SMV) invasion at pre-NACRT period. Six of nine patients with PV/SMV involvement demonstrating tumor abutment had tumor shrinkage with no radiographic evidence of PV/SMV abutment after NACRT. Three of 16 patients with PV/SMV involvement with impingement and narrowing of the lumen had tumor shrinkage with no radiographic evidence of PV/SMV abutment after NACRT. A total of 33 patients were evaluated for toxicity of NACRT as shown in Table 1. Adverse events were reported in 18 (55%) patients. No on-treatment deaths or grade 4 toxicity occurred. The most severe hematologic toxicity was leukocytosis (grade 3), reported in only one patient (3.0%). Grade 3 anorexia and fatigue were each seen in one patient (3.0%). Problems with biliary stenting were seen in seven patients (21%), who underwent procedures to replace the stenting. All toxicities were tolerable and reversible after temporarily withholding therapy.

Comparisons of Surgical and Pathological Results Between NACRT and Adjuvant Groups There were no significant differences in the clinical backgrounds between NACRT and adjuvant groups, apart from the resectability status defined by NCCN[12] as shown in Table 2. A significantly higher frequency of borderline resectable and unresectable pancreatic cancer was seen in the NACRT group relative to the adjuvant group (p=0.022). No significant differences were seen in operative factors between the two treatment groups, as shown in Table 3. Resection of other organs, including vascular resection, was carried out in 17 of 36 patients in the adjuvant group and 19 of 30 patients in NACRT group.

Regarding the rate of pathologically curative resection (R0), which was the primary endpoint in this study, 28 of 30 (93%) patients in NACRT group had R0 resection, which was significantly higher than the rate in the adjuvant group, where 21 of 36 (58%) patients had R0 resection (p=0.005). The reason of R1 resections of two patients in the NACRT group was the SMA margin.[20] The SMA margins were positive in 12 of 13 patients with R1 resections and in all patients with R2 resections (n=2). The neck margins were positive in the residual one of 13 patients with R1 resections and in one of two patients with R2 resections.

The number of metastatic lymph nodes in the NACRT group was significantly lower than the adjuvant group (p= 0.0363). When comparing the extent of metastatic lymph nodes, the frequency of N0/1 in the NACRT group was higher than in the adjuvant group (p=0.041). The lymph node ratio in the NACRT group was significantly lower than that in the adjuvant group (p=0.032). There was a tendency for a lower rate of negative lymph nodes in the NACRT group relative to the adjuvant group, but the difference did not reach statistical significance. In the NACRT group, there were three patients with T1/2 defined by pathological findings, with evidence of down-staging. Pathological effect, as defined by Evans classification[19], was grade IIA (n=21), IIB (n=7), and III (n=2).

Comparisons of Post-operative Mortality and Morbidity Between NACRT and Adjuvant Groups With one exception, there were no significant differences in mortality and morbidity between the two groups (Table 4). The exception was rate of intractable ascites, defined as drug resistance or ascites needed paracentesis, which was significantly higher in the NACRT group (eight of 30 patients, 27%) compared with the adjuvant group (two of 36 patients, 6%) (p=0.035). Diarrhea needing oral administration of loperamide hydrochloride and tincture of opium was reported in five of 36 (14%) patients in the adjuvant group and in nine of 30 (30%) patients in the NACR group, but the difference was not significant (p=0.138). There were three in-hospital deaths in the NACRT group. They had borderline resectable pancreatic cancer that needed vascular resection and/or other organ resection such as colon, adrenal gland, or stomach. They had adverse events of grade 2 anorexia and/or fatigue during NACRT. Postoperatively, three patients had anastomotic failure of the colon followed by liver failure, massive ascites followed by aspiration pneumonia, or fungemia followed by multiple organ dysfunction syndrome.

Discussion

In the majority of patients with pancreatic cancer, the tumor is classified as unresectable at diagnosis, and only