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ORIGINAL PAPER

Nucleolin as cell surface receptor for tumor necrosis factor-α inducing protein: a carcinogenic factor of *Helicobacter pylori*

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Abstract
Purpose Tumor necrosis factor-α inducing protein (Tipα) is a unique carcinogenic factor released from *Helicobacter pylori* (*H. pylori*). Tipα specifically binds to cells and is incorporated into cytosol and nucleus, where it strongly induces expression of *TNF-α* and *chemokine* genes mediated through NF-κB activation, resulting in tumor development. To elucidate mechanism of action of Tipα, we studied a binding protein of Tipα in gastric epithelial cells.
Methods Tipα binding protein was found in cell lysates of mouse gastric cancer cell line MGT-40 by FLAG-pull down assay and identified to be cell surface nucleolin by flow cytometry using anti-nucleolin antibody. Incorporation of Tipα into the cells was determined by Western blotting and expression of *TNF-α* gene was quantified by RT-PCR.
Results Nucleolin was co-precipitated with Tipα-FLAG, but not with del-Tipα-FLAG (an inactive mutant). After treatment with Tipα-FLAG, incorporated Tipα was co-immunoprecipitated with endogenous nucleolin using anti-nucleolin antibody. The direct binding of Tipα to recombinant His-tagged nucleolin fragment (284–710) was also confirmed. Although nucleolin is an abundant non-ribosomal protein of the nucleolus, we found that nucleolin is present on the cell surface of MGT-40 cells. Pretreatment with anti-nucleolin antibody enhanced Tipα-incorporation into the cells through nucleolin internalization. In addition, pretreatment with tunicamycin, an inhibitor of N-glycosylation, decreased the amounts of cell surface nucleolin and inhibited both internalization of Tipα and expression of *TNF-α* gene.
Conclusions All the results indicate that nucleolin acts as a receptor for Tipα and shuttles Tipα from cell surface to cytosol and nuclei. These findings provide a new mechanistic insight into gastric cancer development with Tipα.

Keywords Gastric cancer · TNF-α · NF-κB · *Helicobacter pylori* · Tumor promotion

Abbreviations

H. pylori	*Helicobacter pylori*
Tipα	TNF-α inducing protein
CagA	Cytotoxin associated antigen
LC–MS	Liquid chromatography–mass spectrometry
RBD	RNA binding domain
NF-κB	Nuclear factor-kappa B
NEMO	NF-κB essential modulator

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that colonizes in the mucosa of human stomach, resulting in induction of chronic gastritis, peptic ulcer, and stomach cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 1994, Peek and Blaser 2002). Key criteria of these clinical outcomes are the severity and persistence of inflammation caused by *H. pylori*-infection, associated with strong induction of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukine-1 (IL-1) and chemokines (El-Omar et al. 2000; Peek 2008; Snaith and El-Omar 2008). It is well accepted that inflammatory cytokines contribute to maintain cancer microenvironment (Balkwill 2009; El-Omar et al. 2003), and among the inflammatory cytokines, TNF-α plays a master role as an endogenous tumor promoter in carcinogenesis (Balkwill 2009; Moore et al. 1999; Suganuma et al. 1999). Moreover, TNF-α released from the cells acts as an instigator of a cytokine network sequence, from TNF-α to IL-1 and IL-6 and back to TNF-α, maintaining inflammation in the process of tumor promotion (Suganuma et al. 2002).

To extend the concept, a new gene, *TNF-α inducing protein* (*Tipα*) gene, was cloned from the genome of *H. pylori* strain 26695. Tipα directly induces *TNF-α* gene expression in gastric epithelial cells (Suganuma et al. 2005, 2006, 2008). The unique features of Tipα protein are as follows: (1) *H. pylori* lacking *Tipα* gene reduced the colonization levels of *H. pylori* in the stomach of mice (Godlewska et al. 2008); (2) Vaccination with Tipα significantly reduced colonization of *H. pylori* in mice associated with high levels of Tipα-specific antibody (Inoue et al. 2009); (3) Tipα protein is secreted from *H. pylori* but not mediated through Type IV secretion system (Suganuma et al. 2005); and (4) clinical isolates of *H. pylori* obtained from gastric cancer patients secreted Tipα protein in larger amounts than did *H. pylori* from patients with simple gastritis (Suganuma et al. 2008), strongly suggesting that Tipα plays an important role in *H. pylori*-induced inflammation and cancer development in human stomach (Balkwill 2009). All these features are different from those of other virulence factors, such as the cag pathogenicity island (cagPAI), CagA (cytotoxin associated antigen) and VacA (vacuolating cytotoxin A).

Members of the *Tipα* gene family include *Tipα* itself, *H. pylori-membrane protein 1* (*HP-MP1*), and *jph0543*, and these do not have any obvious homologues in other species (Suganuma et al. 2005; Yoshida et al. 1999). Tipα protein consists of 172 amino acids with a molecular weight of 19 kDa, and it forms a homodimer via two disulfide bonds with two cysteine residues in the N-terminal region. We previously reported that homodimer formation of Tipα is essential for induction of *TNF-α* gene expression in gastric epithelial cells (Suganuma et al. 2008) and also for transformation of Bhas 42 (v-H-*ras* transfected BALB/3T3) cells (Suganuma et al. 2005). To extend our experiments, we made two inactive Tipα mutants: a deletion mutant of Tipα (del-Tipα) that deleted six amino acids including two cysteine residues from native Tipα, and C5A/C7A double mutant (C5A/C7A-Tipα), two cysteine residues of Tipα are replaced by two alanines. The two mutated Tipα proteins induced *TNF-α* gene expression less strongly than native Tipα did (Suganuma et al. 2008). The crystal structures of del-Tipα and truncated forms of Tipα were recently reported by three independent groups, which revealed that they take dimerized forms, although they do not have the full length of protein (Jang et al. 2009; Tosi et al. 2009; Tsuge et al. 2009). If so, it is understandable that del-Tipα has weak activity.

We also found that fluorescence-labeled Tipα specifically binds to the surface of MGT-40 cells and enters into the cytosol and nuclei, whereas del-Tipα and C5A/C7A-Tipα bind weakly to the cells (Suganuma et al. 2008). In the light of this evidence, we think that homodimers of Tipα can easily bind to a specific receptor molecule on the cell surface of gastric epithelial cells. We identified nucleolin as a specific receptor of Tipα on the cell surface using pull-down assay with anti-FLAG antibody against FLAG-tagged Tipα protein. Nucleolin is a well-known major non-ribosomal protein consisting of 710 amino acids in nucleolus, and it has three different structural domains: an N-terminal domain containing highly acidic residues, a central domain containing four RNA recognition motifs, and a C-terminal domain containing Arg-Gly-Gly (RGG) repeats (Ginisty et al. 1999). Nucleolin is known to have multi-functions, including chromatin remodeling, DNA recombination, DNA replication, RNA transcription by RNA polymerase I and II, rRNA processing, mRNA stabilization, cytokinesis and apoptosis (Ginisty et al. 1999; Storck et al. 2007). Furthermore, recent evidence indicates that nucleolin is present on the surface of a wide range of cancer cells and some other types of the cells, and acts as receptors for several molecules (Hirano et al. 2005; Hoja-Lukowicz et al. 2009; Hovanessian et al. 2000; Legrand et al. 2004; Reyes-Reyes and Akiyama 2008). To investigate the specific interaction of nucleolin with Tipα, we conducted experiments to characterize localization of nucleolin, and studied the internalization of Tipα and subsequent induction of *TNF-α* gene expression. This paper reports for the first time that nucleolin is clearly involved in the carcinogenic process of *H. pylori* as a major cellular receptor of Tipα protein.

Materials and methods

Cell culture and reagents

Mouse gastric cancer cell line MGT-40 was maintained in DMEM with 10% fetal bovine serum (JRH Bioscience) and MITO+ serum extender (Becton–Dickinson Labware), as described previously (Ichinose et al. 1998). Human gastric cancer cell line MKN-1 and human monocytic leukemia cell line THP-1 were grown in RPMI 1640 medium with 10% fetal bovine serum. Anti-Tipα antibody was raised in rabbits by immunizing a synthetic peptide of 19 amino acids (from 11 to 29) of Tipα, and anti-nucleolin antibody (anti-NUC295) was raised in rabbits by immunizing a syntheic peptide of eight amino acids (from 295 to 302) of nucleolin, as described previously (Hirano et al. 2005; Suganuma et al. 2005). Other anti-nucleolin antibodies were purchased from Santa Cruz Biotethnology and Bethyl Laboratories, Inc. Anti-HSP90, anti-epidermal growth factor (EGF) receptor, anti-TNF receptor 2 and anti-lamin B antibodies were purchased from Santa Cruz Biotethnology. Anti-FLAG antibody was obtained from Sigma.

Preparation of three different *Tipα* genes tagged with FLAG

Three genes encoding Tipα-FLAG, del-Tipα-FLAG and C5A/C7A-Tipα-FLAG were obtained by PCR of pET28(a)$^+$-Tipα (Suganuma et al. 2005) containing oligonucleotide primers: Tipα-FLAG_F (5′-AGAGCATATGCT GCAGGCTTGCACTTGCCC) and Tipα-FLAG_R (5′-GG ATCCTACTTATCGTCGTCATCCTTGGTAGTCCATG GCTATAGG), del-Tipα-FLAG_F (5′-AGAGCATATGC CAAACACTTCACAAAGGAA), del-Tipα-FLAG_R (5′-G GATCCTACTTATCGTCGTCATCCTTGGTAGTCCAT GGCTATAGG), and C5A/C7A-Tipα-FLAG_F (5′-CA GCCATATGCTGCAGGCTGCCACTGCCCCAAACA C), C5A/C7A-Tipα-FLAG_R (5′-GGATCCTACTTATCG TCGTCATCCTTGGTAGTCCATGGCTATAGG). Three amplified fragments were separately cloned into a pET28(a)$^+$ expression vector (Invitrogen).

Preparation of three FLAG-tagged Tipα proteins

Each FLAG-tagged protein was expressed in *E. coli* (DE3) transfected pET28(a)$^+$ expression vector containing each of the corresponding genes above mentioned. They were induced with isopropyl-β-D-thiogalactopyranoside, and then purified by Ni^{2+} chelating resin (Ni Sepharose 6 Fast Flow, GE Healthcare), as reported previously (Suganuma et al. 2005). Tipα-FLAG, del-Tipα-FLAG and C5A/C7A-Tipα-FLAG all carry a tag of six histidines at the N-terminal region and also a FLAG-tag at the C-terminal region (Fig. 1a). All three recombinant Tipα proteins were more than 98% pure on SDS-PAGE. To conduct Ni^{2+} affinity pull-down assay, His-tag-removed Tipα-FLAG and His-tag-removed C5A/C7A-Tipα-FLAG proteins were prepared as follows: His-tagged Tipα-FLAG and C5A/C7A-Tipα-FLAG proteins were cleaved at a thrombin cleavage cite

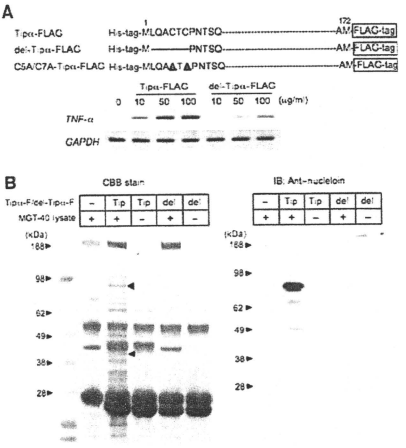

Fig. 1 Identification of nucleolin as Tipα binding protein. **a** Schematic representation of Tipα-FLAG, del-Tipα-FLAG and C5A/C7A-Tipα-FLAG proteins (*top*). Induction of *TNF-α* gene expression with Tipα-FLAG and with del-Tipα-FLAG in MGT-40 cells (*bottom*). Total RNAs were isolated from MGT-40 cells 1 h after treatment with Tipα-FLAG and with del-Tipα-FLAG, and the levels of TNF-α and GAPDH mRNAs were determined by semi-quantitative RT-PCR, as described in Materials and methods. **b** Representative results of FLAG pull-down assay. After incubation of MGT-40 cell lysates with Tipα-FLAG (Tip) and with del-Tipα-FLAG (del), Tipα-FLAG and del-Tipα-FLAG were immunoprecipitated with anti-FLAG antibody. The polypeptides that co-immunoprecipitated with Tipα-FLAG and with del-Tipα-FLAG were resolved in 4–12% NuPAGE and then stained with Quick CBB (*left panel*) and immunoblotted with anti-nucleolin antibody (IB: *right panel*). **c** Amino acid sequence of mouse nucleolin. Amino acids with *red characters* are assigned to the sequences determined by LC–MS analysis. *Underlined sequences* are recognition sites of anti-NUC295

using Thrombin cleavage capture kit (Novagen). Then the cleaved His-tag-peptide and uncleaved protein were separated using Ni^{2+} chelating resin (Tsuge et al. 2009).

Preparation of His-tagged nucleolin protein fragment

His-tagged nucleolin gene fragment (*NUC284*), containing both residues from 284 to 710 of human nucleolin and C-terminal His-tag, was expressed in *E. coli* transfected pBAD/Thio-E/NUC284 expression vector, and purified by Ni^{2+} chelating resin, as described previously (Hirano et al. 2005).

Expression of *TNF-α* gene

MGT-40 and THP-1 cells were treated with recombinant protein for 1 h, and total RNAs obtained from the cells were isolated with ISOGEN reagent (Nippon Gene). Expressions of *TNF-α* gene and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as a control were determined by both semi-quantitative RT-PCR and real-time RT-PCR, as described previously (Suganuma et al. 2005). The values are expressed as the average of three separate experiments.

FLAG pull-down assay

MGT-40 cell lysates were prepared with NP-40 lysis buffer containing 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, and the lysates (600 μg/ml) were incubated with Tipα-FLAG (200 μg/ml) and del-Tipα-FLAG (200 μg/ml) in buffer A containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin at 4°C for 2 h. After addition of 20 μl anti-FLAG M2 Gel (Sigma), the mixture was further incubated at 4°C for 2 h, and then the resin was washed with buffer A containing 1% Triton X-100. The polypeptides associated with the resin were resolved in 4–12% NuPAGE (Invitrogen), and were determined using staining with Quick CBB (Wako). The control experiments were similarly conducted without using Tipα-FLAG and del-Tipα-FLAG.

LC–MS analysis

Gel sections containing polypeptides co-precipitated with Tipα-FLAG were subjected to proteolysis with 2 μg/ml trypsin (Wako) at 25°C overnight, and the digestion was stopped by adding an elution solution (50% acetonitrile, 5% formic acid). Each sample was analyzed using Nano-ESI-Ion trap MS (HCT plus, Bruker Daltonics), according to manufacturer's instruction (Bruker application note). The data were analyzed by a protein database search on MASCOT (Matrix Science).

Ni^{2+} affinity pull-down assay

His-tagged nucleolin fragment (NUC284) was incubated with His-tag-removed Tipα-FLAG or His-tag-removed C5A/C7A-Tipα-FLAG in NP-40 lysis buffer containing 10 mM imidazole at 4°C for 2 h. Twenty microlitre of Ni^{2+} chelating resin was then added to the mixture, which was further incubated at 4°C for 2 h. After washing the resin with NP-40 lysis buffer containing 40 mM imidazole, the complex of nucleolin fragment (NUC284) with Tipα-FLAG or with C5A/C7A-Tipα-FLAG were determined by Western blotting using anti-nucleolin (H-250, Santa Cruz) and anti-Tipα antibodies, respectively.

Incorporation of Tipα into cells

MGT-40 cells were treated with Tipα, and then lysed in lysis buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Cell lysates were resolved in 12% SDS-PAGE. Incorporation of Tipα into the cells was determined by Western blotting using anti-Tipα antibody (Suganuma et al. 2008).

Analysis of subcellular fractionation

Homogenates of MGT-40 and THP-1 cells were fractionated into membrane, cytosol, and nuclei using Qproteome cell compartment kit (Qiagen), according to the manufacturer's instruction. Each fraction was subjected to Western blotting, using anti-nucleolin, anti-HSP90 (a marker for cytosol), anti-EGFR or anti-TNF receptor 2 (for membrane) and anti-lamin B antibodies (for nuclei).

Immunoprecipitation

MKN-1 and THP-1 cells were treated with Tipα-FLAG and del-Tipα-FLAG at a concentration of 100 μg/ml at 37°C for 1 h, and then lysed as described above. Cell lysates (about 400 μg) were incubated with anti-nucleolin antibody (A300-711A, Bethyl Lab, Inc.) at 4°C for 2 h. The immunocomplex was captured with protein A sepharose (GE Healthcare) at 4°C overnight, and then washed with NP-40 lysis buffer. The immunocomplex was applied to 12% SDS-PAGE. Tipα-FLAG, del-Tipα-FLAG and nucleolin were determined by Western blotting using anti-FLAG and anti-nucleolin antibodies (MS-3, Santa Cruz).

Flow cytometry

MGT-40 and THP-1 cells (1×10^6 cells/ml) in PBS were incubated with 2 μg/ml anti-NUC295 antibody and 10 μg/ml Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) on ice for 30 min. Then cells were subjected to flow cytometry (Epics XL, Beckman Coulter).

Statistical analysis

The data were compared using Student's *t* test.

Results

Identification of nucleolin as a Tipα-binding protein

To characterize the nature of the specific binding protein for Tipα, Tipα tagged with FLAG at C-terminus (Tipα-FLAG) and del-Tipα tagged with FLAG at C-terminus (del-Tipα-FLAG)—the latter with six amino acids deleted including two cysteine residues from N-terminal region of Tipα—were used for the experiments (Fig. 1a). Tipα-FLAG protein induced *TNF-α* gene expression in mouse gastric cancer cells (MGT-40), while del-Tipα-FLAG was over ten times weaker than Tipα-FLAG. Thus, Tipα-FLAG and del-Tipα-FLAG showed the same biological activity as did recombinant Tipα and del-Tipα (Fig. 1a).

The mixtures of MGT-40 cell lysates with Tipα-FLAG and with del-Tipα-FLAG were separately subjected to pull-down assay using resin conjugated with anti-FLAG antibody. Thirteen polypeptide bands on SDS-PAGE were found to be co-precipitated with Tipα-FLAG, but not with del-Tipα-FLAG (Fig. 1b). Each polypeptide band was subjected to LC–MS analysis after tryptic digestion, and it turned out that the amino acid sequences of two polypeptides, with 88 and 40 kDa, were similar to that of mouse nucleolin, as shown in Fig. 1c. The results showed that the polypeptide with 88 kDa is nucleolin and the other polypeptide with 40 kDa is a fragment of nucleolin. Three polypeptides with less than 40 kDa were derived from Tipα, and another polypeptide with less than 50 kDa was identical to ribosomal protein L4 fragment; the others could not be confirmed by LC–MS.

The polypeptide with 88 kDa was further confirmed to be nucleolin using immunoblot analysis with anti-nucleolin antibody, but the polypeptide with 40 kDa did not react with anti-nucleolin antibody (Fig. 1b), probably because the latter peptide did not contain recognition sites of the antibody. Although several polypeptides with 50–70 kDa reacted with anti-nucleolin antibody, we think that there were degradation fragments of nucleolin co-precipitated with Tipα-FLAG. The results strongly suggest that nucleolin acts as a specific binding protein of Tipα.

Interaction of incorporated Tipα with endogenous nucleolin in the cells

The binding of Tipα to nucleolin at cellular levels was examined by immunoprecipitation using anti-nucleolin antibody. Since the affinity of the anti-nucleolin antibody for human nucleolin is higher than that for mouse nucleolin, we used cell lysates of both human gastric cancer cell lines MKN-1 and human monocytic leukemia cell line THP-1 for the experiments. Significant amounts of Tipα-FLAG interacted with the lysates of MKN-1 and THP-1 cells, but the amounts of del-Tipα-FLAG interacted less with their cell lysates, which shows that both Tipα-FLAG and del-Tipα-FLAG were incorporated into the cells (Fig. 2a). Using anti-nucleolin antibody, these cell lysates were further subjected to immunoprecipitation: nucleolin was immunoprecipitated with anti-nucleolin antibody associated with Tipα-FLAG but not del-Tipα-FALG in both MKN-1 and THP-1 cells (Fig. 2a). These results suggest that Tipα directly binds to native and endogenous nucleolin in the cells, and the differences of binding ability between Tipα-FLAG and del-Tipα-FLAG to nucleolin are comparable to their inducing potencies of *TNF-α* gene expression.

Direct interaction of nucleolin with Tipα

Next, we studied whether Tipα directly binds to recombinant human nucleolin fragment NUC284, which consists of amino acids from 284 to 710 containing four RNA binding domains. His-tag-removed Tipα-FLAG was incubated in vitro with NUC284 fragment and Ni^{2+} chelating resin, and we found that Tipα-FLAG significantly co-precipitated with NUC284 fragment, although small amounts of Tipα-FLAG precipitated with Ni^{2+} chelating resin only (Fig. 2b). However, His-tag-removed C5A/C7A-Tipα-FLAG did not co-precipitate with NUC284 fragment, suggesting that the homodimer form of Tipα is necessary for direct binding to nucleolin: We think that the homodimer of Tipα directly binds to two-thirds of C-terminal nucleolin, without any scaffold proteins.

Cell surface localization of nucleolin on MGT-40 cells

We previously reported that FITC-labeled Tipα specifically binds to the cell surface of MGT-40 cells (Suganuma et al. 2008). Since nucleolin is present on both nucleolus and surface of the cells (Barel et al. 2008; Hirano et al. 2005; Hoja-Lukowicz et al. 2009: Hovanessian et al. 2000; Legrand et al. 2004; Reyes-Reyes and Akiyama 2008),

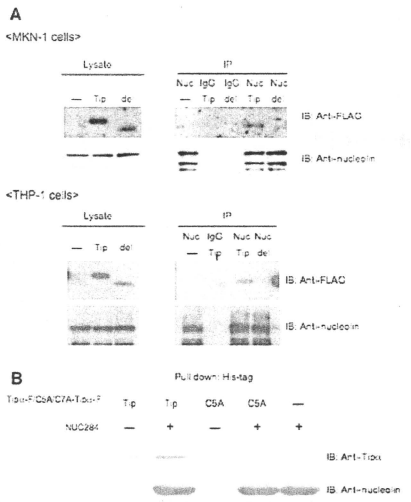

Fig. 2 Direct interaction of nucleolin with Tipα. **a** Tipα was immunoprecipitated with endogenous human nucleolin in MKN-1 and THP-1 cells. MKN-1 and THP-1 cells were treated with 100 μg/ml Tipα-FLAG (Tip) and with del-Tipα-FLAG (del) at 37°C for 1 h. Tipα-FLAG and del-Tipα-FLAG significantly incorporated into the cells (*left panels*). Each cell lysate was immunoprecipitated with anti-nucleolin antibody (NUC) and with rabbit IgG (as a control, IgG). Immunoprecipitates were resolved in 12% SDS-PAGE and immunobotted (IB) with anti-FLAG antibody and anti-nucleolin antibody (*right panels*). **b** Direct interaction of recombinant human nucleolin fragment with Tipα in vitro. His-tag removed Tipα-FLAG (Tip) and His-tag removed C5A/C7A-FLAG (C5A), which were prepared as described in Experimental procedures, were incubated with a 6-His-tag fused recombinant human nucleolin fragment containing 284–710 amino acid residues (NUC284) and then subjected to pull-down assay using Ni^{2+} chelating resins. The precipitates were resolved in 12% SDS-PAGE and analyzed by Western blotting with anti-Tipα antibody and with anti-nucleolin antibody

we first confirmed the sub-cellular localization of nucleolin in MGT-40 cells and THP-1 cells. Although most of the nucleolin was present in the nuclear fraction of MGT-40 cells, significant small amounts of nucleolin were found in membrane and cytosol fractions (Fig. 3a), while THP-1 cells showed large amounts of nucleolin in the membrane fraction (Fig. 3a). Moreover, nucleolin localized on cell surface was further determined by flow cytometry using anti-nucleolin antibody (anti-NUC295). A study of the MGT-40 cells using flow cytometry revealed that the fluorescent peak dramatically shifted to a high fluorescent peak by treatment with anti-NUC295 antibody, and that

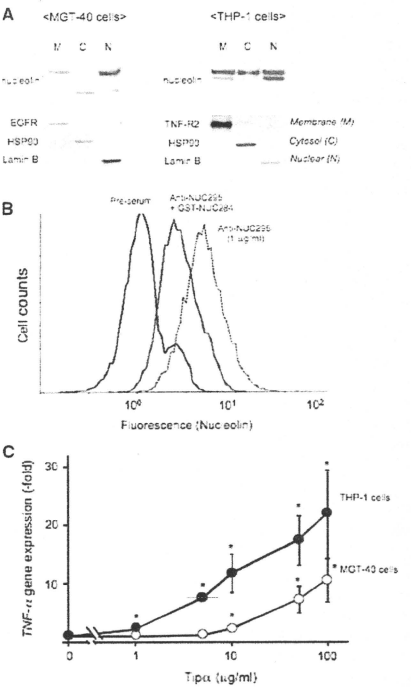

Fig. 3 Localization of nucleolin on cell surface of MGT-40 and THP-1 cells. **a** Subcellular localization of nucleolin analyzed by cell fractionation. MGT-40 and THP-1 cells were fractionated into membrane (M), cytosolic (C) and nuclear (N) fractions, and each fraction was immunoblotted with anti-nucleolin antibody. Each fraction was confirmed by Western blotting with antibodies for fractionation-marker proteins: EGFR for membrane of MGT-40 cells, TNF-R2 for membrane of THP-1 cells, HSP90 for cytosol and lamin B for nuclei. **b** Detection of nucleolin on cell surface shown by flow cytometry. MGT-40 cells were incubated with 1 μg/ml anti-NUC295 (Anti-NUC295) and with pre-immune serum (Pre-serum) as a control in the presence of 10 μg/ml Alexa Fluor 488-conjugated goat rabbit IgG on ice for 30 min. Preincubation of Anti-NUC295 with recombinant nucleolin fragment (Anti-NUC+GST-NUC284) significantly reduced fluorescence. **c** Strong induction of *TNF-α* gene expression with Tipα in THP-1 cells (*filled circle*) and MGT-40 cells (*open circle*). One hour after treatment with Tipα at various concentrations, expression of *TNF-α* and *GAPDH* genes was determined by semi-quantitative RT-PCR. Relative expression of *TNF-α* gene is shown as fold change compared with control after normalization of GAPDH mRNA levels. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical levels between non-treated and Tipα-treated cells were shown to be significant $*P < 0.01$

this fluorescent peak was significantly reduced by preincubation of anti-NUC295 with recombinant GST-nucleolin fragment (GST-NUC284) containing amino acids from 284 to 710 (Fig. 3b). This indicated that nucleolin on the cell surface had interacted with anti-NUC295. Further, we found that Tipα induced dose-dependently *TNF-α* gene expression in MGT-40 and THP-1 cells, based on the results that nucleolin localized on cell surface of both cells (Fig. 3c). The relationship between the amounts of cell surface nucleolin and the potency of Tipα on *TNF-α* gene expression will be reported elsewhere.

Effects of anti-NUC295 on *TNF-α* gene expression induced by Tipα

We studied how anti-NUC295 antibody affects the induction of *TNF-α* gene expression in MGT-40 cells treated with Tipα. First, the treatment with rabbit IgG and anti-nucleolin H-250 antibodies—the latter of which does not recognize nucleolin on cell surface—did not affect the levels of *TNF-α* gene expression induced by Tipα. However, treatment with anti-NUC295 antibody dose-dependently enhanced the *TNF-α* gene expression induced by Tipα up to twofold (Fig. 4a), and treatment with anti-NUC295 antibody dose-dependently enhanced incorporation of Tipα into the cytosol of MGT-40 cells (Fig. 4b). From our results showing that anti-NUC295 antibody internalized into MGT-40 cells as determined by flow cytometry (data not shown), we think that the complex of nucleolin, Tipα and anti-NUC295 internalized into the cells and then induced *TNF-α* gene expression.

Inhibitory effects of down-regulated cell surface nucleolin on biological activity of Tipα

Nucleolin on the cell surface is a glycoprotein containing *N*- and *O*-glycans (Carpentier et al. 2005), and the N-glycosylation of nucleolin is essential for localization on cell surface (Losfeld et al. 2009). We found that treatment of MGT-40 cells with 5 μg/ml tunicamycin, an inhibitor of the N-linked glycosylation of protein, significantly reduced the amounts of nucleolin on the cell surface as determined by flow cytometry (Fig. 5a): The levels of cell surface nucleolin were reduced by approximately 50%. Moreover, pretreatment with tunicamycin inhibited about 50% *TNF-α* gene expression induced by Tipα because tunicamycin reduced the incorporated amounts of Tipα into MGT-40 cells (Fig. 5b, c). The reduced amounts of nucleolin correlated well with reduction of *TNF-α* gene expression. Cell surface nucleolin is thus a functional receptor of Tipα associated with incorporation of Tipα into the cells and subsequent *TNF-α* gene expression.

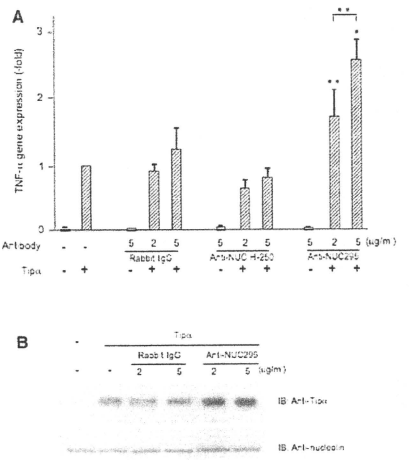

Fig. 4 Significant enhancement of Tipα-induced *TNF-α* gene expression and Tipα incorporation in cells induced by anti-NUC295. **a** MGT-40 cells were previously incubated with rabbit IgG, with anti-NUC H-250 and anti-NUC295 antibodies at 4°C for 1 h, and further treated with 50 μg/ml Tipα at 37°C for 1 h. Relative *TNF-α* gene expression is shown as fold change compared with that of cells treated with 50 μg/ml Tipα after normalization of *GAPDH* gene expression levels. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical significance of effects of anti-NUC295 in TNF-α induction by Tipα compared with non-treated were shown as $*P < 0.01$ and $**P < 0.05$, and the difference between 2 and 5 μg/ml of anti-NUC295 was significant at the level of $**P < 0.05$. **b** Incorporation of Tipα was determined by Western blotting with anti-Tipα antibody. Nucleolin levels were also determined by anti-nucleolin antibody

Discussion

Considering our 1993 discovery that TNF-α is an endogenous tumor promoter in carcinogenesis (Komori et al. 1993), we first cloned a new gene of TNF-α inducing protein from *H. pylori* genome (Suganuma et al. 2005). We also reported that the active form of Tipα is a homo-dimer that induces *TNF-α* gene expression in the cells, resulting in a cancer microenvironment (Suganuma et al. 2006, 2008). Furthermore, Tipα is now widely accepted as a carcinogenic factor of *H. pylori* (Balkwill 2009). This paper reports that Tipα directly binds to nucleolin on the cell surface, and that the complex of Tipα with nucleolin then internalizes into the cells. The results suggest that cell surface nucleolin acts as a receptor of Tipα: nucleolin is mainly localized in the nucleolus, but significant amounts are present on the cell surface, including various cancer

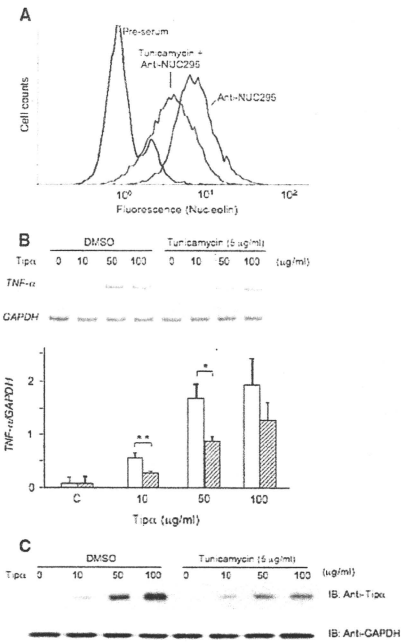

Fig. 5 Inhibition of Tipα-induced *TNF-α* gene expression and Tipα incorporation in cells induced by down-regulation of cell surface nucleolin. **a** MGT-40 cells were treated with or without 5 μg/ml tunicamycin in DMSO at 37°C for 5 h. The cell surface nucleolin on MGT-40 cells was visualized using flow cytometry with anti-NUC295, as described in Materials and methods. **b** Inhibition of *TNF-α* gene expression induced by Tipα after pretreatment with tunicamycin. After pretreatment of MGT-40 cells with or without 5 μg/ml tunicamycin in DMSO at 37°C for 5 h, the cells were treated with various concentrations of Tipα at 37°C for 1 h. The levels of *TNF-α* and *GAPDH* gene expression in MGT-40 cells were determined by semi-quantitative RT-PCR. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical levels were significant $*P < 0.01$ and $**P < 0.05$. **c** Inhibition of Tipα-incorporation into MGT-40 cells by pretreatment with tunicamycin. After treatment with tunicamycin, both MGT-40 cells treated with various concentrations of Tipα and cell lysates were resolved in 12% SDS-PAGE solution and analyzed by Western blotting with anti-Tipα antibody and anti-nucleolin antibody (IB)

cells and proliferating cells (Hirano et al. 2005; Hoja-Lukowicz et al. 2009; Hovanessian et al. 2000; Legrand et al. 2004; Reyes-Reyes and Akiyama 2008). It is well-known that cell surface nucleolin has an important role as a receptor for various extracellular ligands, including human immunodeficiency virus (HIV) particles (Nisole et al. 2002), midkine (Hovanessian 2006; Said et al. 2002), and elongation factor-TU of *Francisella tularensis* (Barel et al. 2008), lactoferrin (Legrand et al. 2004), and endostatin (Shi et al. 2007): Nucleolin acts as a shuttling molecule between cell surface, cytoplasm, and nucleus (Borer et al. 1989). Moreover, it is of interest to note that a specific DNA aptamer of nucleolin, AS1411, is the most well-investigated anti-cancer aptamer, which initially binds to cell surface nucleolin and then internalizes into the cells (Ireson and Kelland 2006). Based on evidence, we think that nucleolin shuttles Tipα, which is supported by the results that some Tipα are present in the nuclei of MGT-40 cells after treatment with Tipα protein. Although the precise function of Tipα in nucleus is not well understood, we found that Tipα directly binds to DNA oligomers in Biacore assay (Kuzuhara et al. 2007). Thus, our understanding on the Tipα function is extended by the several findings, such as nucleolin as the receptor, the translocation of Tipα into the nuclei, and the induction of *TNF-α* gene expression in the cells.

Although it is not well-known how nucleolin translocates across the membrane and how it attaches to the cell surface, glycosylation is assumed to be an essential biochemical modification for nucleolin to localize on the cell surface (Losfeld et al. 2009). In our experiments, tunicamycin significantly reduced the level of cell surface nucleolin in MGT-40 cells—although the nucleolin in nucleoli was not much reduced—and then inhibited both internalization of Tipα and *TNF-α* gene expression. But it is still not clear whether N-glycosylation of nucleolin is involved in Tipα binding because Tipα directly binds to recombinant nucleolin fragment (NUC284) without any glycosylation. Since NUC284 fragment contains four RNA binding domains (RBDs) and a RGG domain—which are well conserved in human, mouse, and rat (Ginisty et al. 1999)—we think that Tipα binds to one of these domains. To understand more precisely the nature of Tipα and nucleolin binding, we used anti-nucleolin antibody (Anti-NUC295) (Hirano et al. 2005): pretreatment with anti-NUC295 unexpectedly enhanced internalization of Tipα and induction of *TNF-α* gene expression by Tipα, indicating that the Tipα binding site of nucleolin is different from epitope of nucleolin and that anti-NUC295 enhances internalizing of nucleolin with Tipα. These findings support the previously reported results with another anti-nucleolin antibody (mAb D3), which recognizes cell surface nucleolin and induces clustering and internalization of nucleolin together with mAb D3 antibody (Hovanessian et al. 2000). Since, we obtained the results that pretreatment with methyl-β-cyclodextrin, which inhibits endocytosis by depletion of cholesterol from membrane, inhibited induction of *TNF-α* gene expression with Tipα (unpublished results), we think that anti-NUC295

enhances endocytosis of Tipα. To prove evidence that nucleolin acts as a specific receptor of Tipα, we conducted a knockdown experiment with shRNA using lentiviral vector, and the growth of THP-1 cells was inhibited, by the complete down-regulation of nucleolin (data not shown).

Several polypeptides co-precipitated with Tipα-FLAG, but did not with del-Tipα-FLAG, in pull-down assay. Although the specificity of the co-precipitation was relatively high, we found that ribosomal protein L4 is an additional binding protein. That is well-known to be a protein interacting with nucleolin. As for interaction of Tipα with nucleolin, the N-terminal portion of Tipα is thought to be an important domain: (1) disulfide bond formation in the N-terminal of Tipα is essential for the interaction, and monomer of del-Tipα does not bind to nucleolin, and (2) we successfully identified nucleolin as the Tipα-binding protein because we used FLAG-tagged at the C-terminal position of Tipα as bait, but did not use the His-tagged at the N-terminal position.

The TNF-α inducing activity of Tipα should be briefly mentioned in connection with *H. pylori*-infection in human stomach cancer development. Tipα-deficient *H. pylori* reduces colonization in mouse gastric mucosa (Godlewska et al. 2008), and vaccinations with Tipα and del-Tipα also effectively prevented colonization of *H. pylori* in the stomach of mice (Inoue et al. 2009). Therefore, we think that targeting molecules, which inhibit the interaction of Tipα and cell surface nucleolin, will be useful tools for the prevention of inflammation induced by *H. pylori* infection and of *H. pylori*-infection itself. For example, lactofferin, which binds to nucleolin (Legrand et al. 2004), is effective in suppression of *H. pylori* colonization (Okuda et al. 2005), which suggests that lactofferin inhibits the binding of Tipα and nucleolin.

How nucleolin is involved in the induction of *TNF-α* gene expression induced by Tipα is an important subject, since TNF-α is a major mediator of cancer-related inflammation in the cancer microenvironment (Balkwill 2009; Komori et al. 1993; Suganuma et al. 1999). A specific DNA aptamer of nucleolin, AS1411 (Ireson and Kelland 2006; Soundararajan et al. 2008) blocks both TNF-α induced- and constitutive-NF-κB activation in human cancer cell lines by forming a complex of nucleolin with an NF-κB essential modulator (NEMO) (Girvan et al. 2006). This indicates that nucleolin regulates NF-κB activation through interaction with NEMO, so it is possible that Tipα incorporated with nucleolin interferes in the interaction of nucleolin with NEMO and affects NF-κB signaling.

Tipα family genes and protein products show carcinogenic activity in combination with v-H-*ras* oncogene: Transfection of *HP-MP1* gene into Bhas 42 cells (v-H-ras transfected BALB/3T3) induces highly malignant transformed cells (Bhas/mp-1): these cells have strong tumorigenicity associated with a high grade of angiogenesis in nude mice (Suganuma et al. 2001). Interestingly, it has been reported that overexpression of nucleolin cooperates with oncogenic mutant Ras in a rat embryonic fibroblast transformation assay (Takagi et al. 2005). And midkine and pleiotrophin, which are ligands of nucleolin, transformed cells (Muramatsu 2002). Nucleolin is specifically expressed on the cell surface in proliferating endothelial cells (Shi et al. 2007), and it is also well-known that nucleolin protein is expressed at high levels on the cell surface of rapid proliferation cells, including cancer cells such as MCF-7 (breast cancer) (Soundararajan et al. 2008), HeLa (cervical cancer) (Li et al. 2009), colo-320 (colon adenocarcinoma) (Reyes-Reyes and Akiyama 2008) and THP-1 cells (Barel et al. 2008; Hirano et al. 2005). We also found that nucleolin is expressed on the surface of mouse and human gastric cancer cell lines (MGT-40 and MKN-1). The study on the expression levels and localization of cell surface nucleolin during development of gastric cancer by *H. pylori*-infection will surely provide a new insight into the identification of high-risk *H. pylori* carriers in more detail. Since 50% of the world population is infected with *H. pylori* (Snaith and El-Omar 2008), our results with Tipα indicate that nucleolin on the cell surface will prove useful as a high-risk biomarker for gastric cancer. This paper is the first report that nucleolin serves as a receptor of Tipα, the carcinogenic factor of *H. pylori*: further results with a complex of Tipα with nucleolin will intensify the understanding of this new carcinogenic mechanism on gastric cancer development in humans.

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Research Paper

Replacement of cisplatin with nedaplatin in a definitive 5-fluorouracil/ cisplatin-based chemoradiotherapy in Japanese patients with esophageal squamous cell carcinoma

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Abstract

Objective: The effects of replacing cisplatin (CDDP) with *cis*-diammineglycolatoplatinum (nedaplatin, NDP), a second-generation platinum complex, on the pharmacokinetics of 5-fluorouracil (5-FU) were investigated in Japanese patients with esophageal squamous cell carcinoma, who were treated with a definitive 5-FU/CDDP-based chemoradiotherapy.

Methods: Fifty-six patients were enrolled, 49 treated with CDDP and 7 treated with NDP. A course consisted of continuous infusion of 5-FU at 400 mg/m^2/day for days 1-5 and 8-12, infusion of CDDP or NDP at 40 mg/m^2/day on days 1 and 8, and radiation at 2 Gy/day on days 1 to 5, 8 to 12, and 15 to 19, with a second course repeated after a 2-week interval. Plasma concentrations of 5-FU were determined by high performance liquid chromatography at 5 PM on days 3, 10, 38 and45, and at 5 AM on days 4, 11, 39 and 46.

Results and conclusions: The circadian rhythm in plasma concentrations of 5-FU observed in the case of CDDP was altered when NDP was used instead. The clinical response can be predicted by monitoring plasma concentrations of 5-FU in the CDDP group, but not in the NDP group.

Key words: nedaplatin, chemoradiotherapy, esophageal squamous cell carcinoma, 5-fluorouracil, plasma concentration

Introduction

A clinical report published in 1999, the RTOG (Radiation Therapy Oncology Group) 85-01 trial involving 134 patients with T1-3, N0-1 and M0 esophageal cancer, is of great interest in terms of clinical outcome because it demonstrated a 5-year survival rate of 26 % [1-4]. This treatment consists of infusion of 5-fluorouracil (5-FU) and cisplatin (CDDP), and concurrent radiation, without pre- or post-surgical resection. Simultaneously in Japan, a modified version was proposed by Ohtsu and his co-workers for advanced metastatic esophageal cancer [5,6]. Two independent clinical investigations have shown curative potential using this regimen for unresectable esophageal squamous cell carcinoma (ESCC) with T4

or M1a [5,6]. A long-term evaluation of efficacy and toxicity with 139 patients resulted in a complete response (CR) rate of 56%, along with a 5-year survival rate of 29% [7-9]. Currently, a definitive 5-FU/CDDP-based chemoradiotherapy (CRT) is recognized as one of the most promising treatments for esophageal cancer [10].

A series of studies has been performed to find a marker predictive of clinical outcome after treatment with a definitive 5-FU/CDDP-based CRT [11-13]. A total of 8 measurements of the plasma concentration of 5-FU were made per patient, and it was concluded that the average value was predictive of clinical response, but not of severe acute leucopenia, stomatitis and cheilitis. Additionally, it has been suggested that clinical response and severe acute toxicities may be predicted on the basis of genetic polymorphisms.

CDDP is one of the antitumor agents most widely used against several types of solid tumors. However, its clinical use is limited by its potent nephrotoxicity, which can lead to acute renal failure. Nedaplatin (NDP), *cis*-diammineglycolatoplatinum, is a second-generation platinum complex that is approximately 10 times as soluble in water as CDDP [14-16]. As such, NDP is considered to have more pronounced activity against solid tumors, but less nephrotoxicity and gastrointestinal toxicity than CDDP [14]. In phase II clinical studies, NDP was found to be highly effective against solid tumors, including non-small cell lung cancer, small cell lung cancer, head and neck cancer and esophageal cancer [15]. The replacement of CDDP with NDP might be of value for a certain subpopulation of patients. Although little information is yet available, it was recently reported that NDP was comparable to CDDP with regards to clinical response and survival, and also to acute and late toxicity in the treatment of ESCC [16]. In this study, the effects of replacing CDDP with NDP on the pharmacokinetics of 5-FU were investigated in ESCC patients treated with a definitive 5-FU/CDDP-based CRT.

Patients and Methods

Patients

Fifty-six ESCC patients were enrolled in this study based on the following criteria: 1) ESCC treated at Kobe University Hospital from August 2002 to June 2006; 2) clinical stage T1 to T4, N0 or N1, and M0 or M1a according to the International Union Against Cancer tumor node metastasis (TNM) classification; 3) age less than 85 years; 4) an Eastern Cooperative Oncology Group performance status of 0 to 2; 5) adequate bone marrow, marrow, renal, and hepatic function; 6) no prior chemotherapy; 7) no severe medical complications; and 8) no other active malignancies (except early cancer). The tumors were histologically confirmed to be primary, and no patients with recurrence were included in this study.

Protocol

The protocol is presented in Figure 1. A course consisted of continuous infusion of 5-FU at 400 mg/m^2/day for days 1-5 and 8-12, infusion of CDDP or NDP at 40 mg/m^2/day on days 1 and 8, and radiation at 2 Gy/day on days 1 to 5, 8 to 12, and 15 to 19, with a second course repeated after a 2-week interval [5,6]. If disease progression/recurrence was observed, either salvage surgery, endoscopic treatment, or another regimen of chemotherapy was scheduled. Forty-nine of 56 patients were treated with CDDP (the CDDP group), and the remaining 7 patients were treated with NDP (the NDP group). This study was conducted with the authorization of the institutional review board and followed the medical research council guidelines of Kobe University.

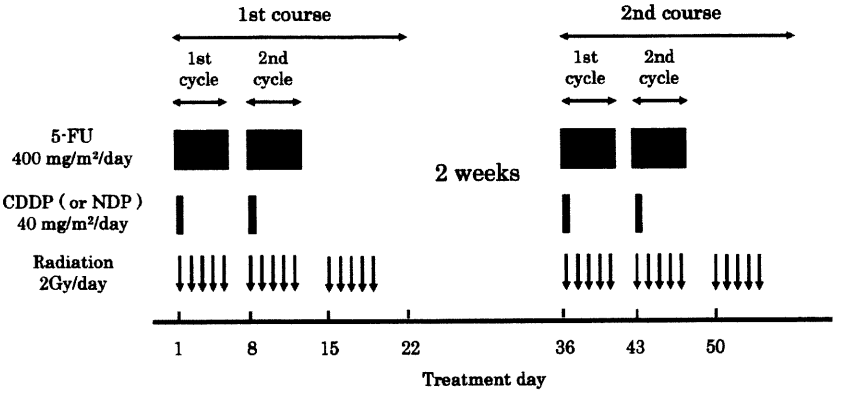

Figure 1. Protocol of a definitive 5-fluorouracil (5-FU)/ cisplatin (CDDP) or nedaplatin (NDP)-based chemoradiotherapy. One course of treatment consisted of protracted venous infusions of 5-FU (400 mg/m^2/day for days 1-5 and 8-12) and CDDP (or NDP) (40 mg/m^2/day on days 1 and 8), and radiation (2 Gy/day on days 1-5, 8-12, and 15-19), with a second course (days 36-56) was repeated after a 2-week interval.

Pharmacokinetics of 5-FU

Aliquots (5 mL) of blood were collected into etylenediaminetetraacetic acid-treated tubes at 5 PM on days 3, 10, 38 and 45, and at 5 AM on days 4, 11, 39 and 46 [11-13]. The plasma concentration of 5-FU was determined by high-performance liquid chromatography as described previously [11-13]. The apparent elimination half-life of 5-FU is approximately 10 minutes [17], and the plasma concentration will reach a steady-state within a few hours of starting continuous infusion. The systemic exposure to 5-FU during each of 4 cycles was assessed as the area under the concentration time curve for 120 hours (AUC_{120h}), calculated as 120 hours x the average of 2 measurements within a cycle.

Clinical Response

A CR was defined as the complete disappearance of all measurable and assessable disease at the first evaluation, which was performed 1 month after the completion of CRT to determine whether the disease had progressed. The clinical response was evaluated by endoscopy and chest and abdominal computed tomography (CT) scans in each course. A CR at the primary site was evaluated by endoscopic examination when all of the following criteria were satisfied on observation of the entire esophagus: 1) disappearance of the tumor lesion; 2) disappearance of ulceration (slough); and 3) absence of cancer cells in biopsy specimens. If small nodes of 1 cm or less were detected on CT scans, the recovery was defined as an "uncertain CR" after confirmation of no progression for at least 3 months. An "uncertain CR" was included as a CR when calculating the CR rate. When these criteria were not satisfied, a non-CR was assigned. The existence of erosion, a granular protruded lesion, an ulcer scar, and 1.2 w/v% iodine/glycerin-voiding lesions did not prevent an evaluation of CR. The evaluations were performed every month for the first 3 months, and when the criteria for CR were not satisfied at 3 months, the result was changed to non-CR. Follow-up evaluations were performed thereafter every 3 months for 3 years by endoscopy and CT scan. After 3 years, patients were seen every 6 months. During the follow-up period, a routine course of physical examinations and clinical laboratory tests was performed to check the patient's health.

Severe Acute Toxicities

A definitive 5-FU/CDDP-based CRT is associated with acute toxicities, predominantly leucopenia, stomatitis, and cheilitis [5-9,18]. Toxicity was evaluated using criteria defined by the Japan Clinical Oncology Group [19]. These criteria were based on the National Cancer Institute Common Toxicity Criteria. Toxicity was assessed on a 2 to 3 day basis during the CRT and subsequent hospitalization period and on every visit after the completion of CRT. Episodes of leucopenia, stomatitis, and cheilitis during the first 2 courses and subsequent 2 weeks (until day 70) were recorded as acute toxicities and those of grade 3 or more as severe acute toxicities.

Data Analysis and Statistics

All values reported are the mean±standard deviation (SD). The association of disease stage with the rates of CR and severe acute toxicities were analyzed with Fisher's exact test. Circadian variations of plasma concentrations of 5-FU were analyzed with the Wilcoxon signed-rank test. The unpaired Student's *t*-test/Welch's test or Mann-Whitney's *U* test was used for two-group comparisons of the plasma concentrations or AUC_{120h} values of 5-FU. P values of less than 0.05 (two tailed) were considered to be significant.

Results

Demographic and clinicopathologic characteristics of 56 ESCC patients are summarized in Table 1. The ratio of T1/T2/T3/T4 was 17/6/21/12, that of N0/N1 was 23/33, and that of M0/M1a was 45/11, resulting in a stage I/II/III/IVa ratio of 13/10/22/11. There was no significant difference between the 2 groups; the CDDP group (N=49) and the NDP group (N=7).

The results of clinical outcome are summarized in Table 2. The overall CR rate was 44.6%, and depended on disease stage; 84.6%, 70.0%, 27.3% and 9.1% for stage I, II, III and IVa, respectively ($P<0.05$). NDP was comparable to CDDP with respect to clinical response, but the treatment with NDP achieved a CR at stage IVa (data not shown). Episodes of severe acute leucopenia, stomatitis and cheilitis occurred in 42.9%, 12.5% and 14.3% of cases, respectively, and each rate was independent of disease stage (data not shown). Replacement of CDDP with NDP had no effect on the rates of these severe acute toxicities (data not shown).

Table 1. Demographic and Clinicopathologic Characteristics of 56 Japanese Patients with Esophageal Squamous Cell Carcinoma

Characteristics	Values
Age, yr	64.3±7.5 (48 -78)
Height, cm	163.1±6.7 (150-180)
Weight, kg	55.9±9.4 (33-79)
Sex	Male/Female = 51/5
Race	Japanese
Performance status	0/1/2/unknown = 28/22/4/2
Histological type	squamous cell carcinoma
Differentiation	well/moderate/poor/unknown = 8/31/9/8
TNM score	T1/T2/T3/T4 = 17/6/21/12
	N0/N1 = 23/33
	M0/M1a = 45/11
Stage	I/II/III/IVa = 13/10/22/11

The values are the mean±SD, with the range in parentheses. TNM score: tumor, node, metastasis. Patients with noncervical primary tumors with positive supraclavicular lymph nodes were defined as M1a.

Table 2. Clinical Outcome in 56 Japanese Patients with Esophageal Squamous Cell Carcinoma

	N	%
Clinical Response		
Complete response (CR) rate	25	44.6
Partial response (PR) rate	24	42.9
Severe Acute Toxicities		
Leucopenia	24	42.9
Stomatitis	7	12.5
Cheilitis	8	14.3

The plasma concentrations of 5-FU are shown in Figure 2. The values of AUC_{120h} are summarized in Table 3. In the 1st cycle/1st course, plasma concentrations of 5-FU were significantly lower at 5 AM (0.076±0.040 μg/mL) than at 5 PM (0.109±0.060 μg/mL) in the CDDP group ($P<0.05$, $\beta=0.907$). A similar tendency was observed in the 2nd cycle/1st course ($P=0.134$, $\beta=0.390$). In the NDP group, however, concentrations tended to be higher at 5 AM than at 5 PM in both the 1st and 2nd cycle/1st course ($P=0.249$, $\beta=0.106$, $P=0.463$, $\beta=0.138$, respectively), whereas the AUC_{120h} value of 5-FU in the CDDP group was almost the same as that in the NDP group in the 1st as well as 2nd cycle/1st course (Table 3). In the 1st course, the plasma concentrations of 5-FU at both 5 PM and 5 AM were significantly higher in the 2nd cycle than the 1st cycle in the CDDP group ($P<0.05$, $\beta=0.951$, $P<0.05$, $\beta=0.999$, respectively). Similarly in the NDP group, the concentration of 5-FU tented to increase in the 2nd cycle, but not significantly ($P=0.116$, $\beta=0.205$, $P=0.173$, $\beta=0.211$, respectively). These phenomena found in the 1st course were also found in the 2nd course, for both groups.

The correlation between the CR rate and the plasma concentration of 5-FU was evaluated, and the results obtained with the average value of 8 measurements are summarized in Table 4. In the CDDP group, the plasma concentrations of 5-FU were significantly higher in the patients with CR than those with non-CR ($P<0.05$), but the inclusion of 7 patients treated with NDP resulted in no statistically significant difference ($P=0.090$). The association with severe acute toxicities was also evaluated, and the results on leucopenia are summarized in Table 5. There was no difference in the plasma concentrations of 5-FU between the patients with and without severe acute leucopenia, in either groups. Similarly, the plasma concentrations of 5-FU in the patients with severe acute stomatitis or cheilitis were comparable to those in the patients without (data not shown).

Table 3. Area Under the Concentration-Time Curve Values (AUC_{120h}, mg*h/L) of 5-Fluorouracil (5-FU) in 56 Japanese Patients with Esophageal Squamous Cell Carcinoma

	CDDP	NDP
	N=49	N=7
1st cycle / 1st course	11.1±4.8	11.0±4.6
2nd cycle / 1st course	16.8±6.4	15.3±7.3
1st cycle / 2nd course	10.7±5.2	10.6±4.4
2nd cycle / 2nd course	16.0±5.4	15.9±6.8

CDDP: cisplatin, NDP: nedaplatin. Systemic exposure to 5-FU was assessed as the AUC_{120h}, calculated as 120 hours x the average of 2 measurements. There was no significant difference between the 2 groups at each of the 4 cycles.

Table 4. Plasma Concentrations of 5-Fluorouracil (5-FU) in the Patients with and without a Complete Response (CR).

	CR		non-CR		
	N	5-FU, μg/mL	N	5-FU, μg/mL	P
CDDP	23	0.124±0.035	26	0.105±0.044	0.045
NDP	2	0.078 , 0.117	5	0.116±0.038	-
total	25	0.122±0.035	31	0.107±0.043	0.090

CDDP: cisplatin, NDP: nedaplatin. The average of 8 measurements made per patient is listed as the data. In the CDDP group, plasma concentrations of 5-FU were significantly higher in the patients with CR than those without (non-CR), but the inclusion of 7 patients treated with NDP resulted in no significant differences.

Table 5. Plasma Concentrations of 5-Fluorouracil (5-FU) in the Patients with and without Severe Acute Leucopenia.

	Severe Acute Leucopenia		No Severe Acute Leucopenia		
	N	5-FU, μg/mL	N	5-FU, μg/mL	P
CDDP	21	0.116±0.036	28	0.113±0.033	0.785
NDP	3	0.114±0.053	4	0.109±0.021	0.869
total	24	0.115±0.037	32	0.112±0.031	0.746

CDDP: cisplatin, NDP: nedaplatin. The average of 8 measurements made per patient is listed as the data. There was no difference between the patients with and without severe acute leucopenia, in either group.

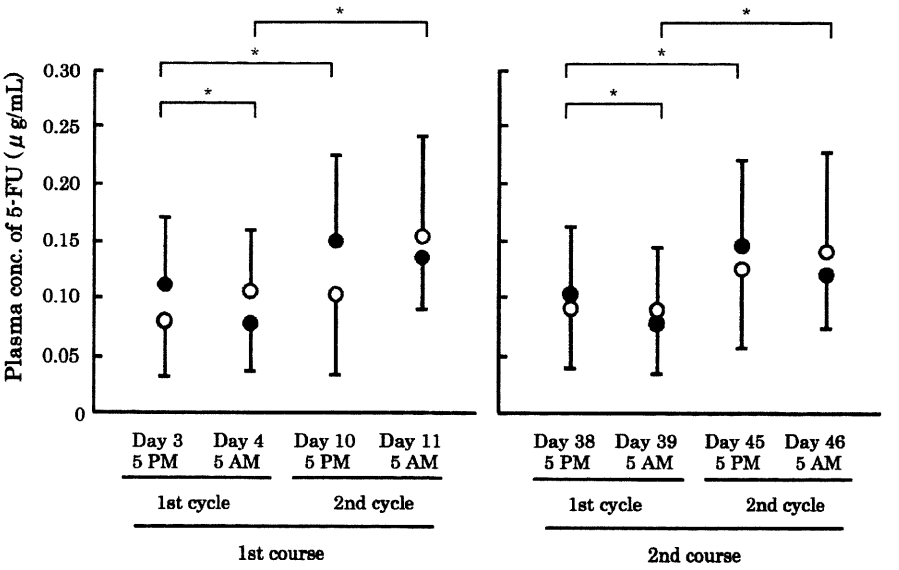

Figure 2. Plasma concentrations of 5-fluorouracil (5-FU) in 56 patients with esophageal cancer. A total of 8 measurements were made per patient: 5 PM on days 3, 10, 38 and45, and 5 AM on days 4, 11, 39 and 46. Closed circle: the cisplatin (CDDP) group (N=49), open circle: the nedaplatin (NDP) group (N=7). The bars represent the SD. * P<0.05; significant differences were observed in the CDDP group, but not in the NDP group.

Discussion

Esophageal cancer is the 8th most common cancer in the world and one of the most lethal [10]. Symptoms include dysphagia, odynophagia, and progressive weight loss. The two predominant histological subtypes are adenocarcinoma and squamous cell carcinoma, and treatment depends on the location of the primary tumor, the disease stage, patient characteristics and co-morbidities, and occasionally, histological subtype. There is no consensus on an optimal treatment strategy for esophageal cancer, and treatments include surgical procedures, radiation, chemotherapy, and combinations thereof [10]. In patients with localized squamous cell carcinoma, a definitive 5-FU/CDDP-based CRT is one of the most promising ways to achieve a complete pathologic response. The treatment might be improved further through modification of the treatment schedule, dose escalation and the replacement of 5-FU and CDDP. Capecitabine or tegafur/uracil might provide better results than 5-FU, and oxaliplatin and NDP are potential substitutes for CDDP.

In this study, we investigated the effects of replacing CDDP with NDP in 56 ESCC patients treated with a definitive 5-FU/CDDP-based CRT, and found no significant differences in clinical outcome, i.e., the CR rate and the severe acute toxicities, in the NDP group, when compared with the CDDP group. Although multi-center, cross-over style clinical investigations should be conducted on the replacement, NDP may be beneficial to ESCC patients, especially those with renal disease. Yamashita et al. [16] also reported that NDP did not differ from CDDP with regards to overall survival, progression-free survival and severe acute leucopenia in the treatment of locally advanced and metastatic esophageal cancer.

Herein, it was clarified that NDP has substantial effects on the pharmacokinetics of 5-FU. It is well-known that there is a circadian rhythm in drug metabolism, cellular proliferation and physiological function, and the suprachiasmatic nuclei, a hypothalamic pacemaker clock, is important for the rhythm [20-22]. As a result, both the toxicity and efficacy of over 30 anticancer agents vary as a function of dosing time [20-22]. More than 80 % of the administered 5-FU is eliminated by the rate-limiting enzyme, dihydropyrimidine dehydrogenase (DPD). The DPD activity is found in most tissues, but is highest in the liver. The activity of DPD of diurnally active cancer patients varies significantly during a 24-hour time period, and is greatest from midnight to early morning [21-24], being consistent with the findings of this study. However, in the NDP group, the pattern of circadian rhythm in 5-FU pharmacokinetics was certainly different from that in the CDDP group, although the AUC_{120h} values were not altered (Table 3). The interaction of DPD with CDDP might be different from that of NDP, but there is no rational explanation for these phenomena. Further clinical and non-clinical investigations should be conducted.

The plasma concentrations of 5-FU were predictive of clinical response, but not of severe acute toxicities, in the CDDP group (Tables 4, 5), however the inclusion of 7 patients treated with NDP affected predictions, presumably because clinical response cannot be predicted on the basis of plasma concentrations of 5-FU in the NDP group. A number of clinical investigations on colorectal cancer and head and neck cancer have revealed that the plasma concentrations of 5-FU were associated with treatment efficacy and toxicity, and the target level of 5-FU concentrations to ensure a certain efficacy was presented [25]. The target level might be proposed also for ESCC, but when using NDP instead of CDDP, it is necessary to look for some marker capable of indicating clinical response.

In conclusion, only a small number of patients were enrolled in this study, especially in the NDP group, and we had no conclusions on the replacement of CDDP with NDP in terms of clinical outcome after the definitive 5-FU/CDDP-based CRT. The circadian rhythm in plasma concentrations of 5-FU observed with CDDP was altered when NDP was used instead, and clinical response can be predicted on the basis of the plasma concentrations of 5-FU in the CDDP group, but not in the NDP group.

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Competing Interest

The authors declare that no conflict of interest exists.

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原　著

食道がん化学放射線療法における5-フルオロウラシル血漿中濃度と副作用との相関

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Association of the Plasma Concentration of 5-Fluorouracil with Toxicity after FP+RT Chemoradiotherapy for Esophageal Squamous Cell Carcinoma in Japanese

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ABSTRACT

The association of plasma concentration of 5-fluorouracil (5-FU) with severe acute toxicities, including leucopenia, stomatitis, and cheilitis (radiodermatitis), after treatment with one of the standard protocols of 5-FU/cisplatin (CDDP)-based chemoradiotherapy, i.e., FP+RT therapy, was evaluated in 51 Japanese patients with esophageal squamous cell carcinoma (ESCC). Here, one course of treatment consisted of protracted venous infusions of 5-FU (400 mg/m^2/24 h for day 1-5 and 8-12) and CDDP (40 mg/m^2/3 h on day 1 and 8), and radiation (2Gy/day on day 1-5, 8-12 and 15-19), and a second course was successively repeated after a 2-week interval. Eight measurements of the plasma concentration of 5-FU was done per patient, and toxicity was evaluated using the criteria defined by the National Cancer Institute Common Toxicity Criteria version 2.0. Severe acute leucopenia, stomatitis and cheilitis were found in 39.2%, 13.7% and 19.6%, respectively, of ESCC patients, and their frequencies were independent of disease stage. The 8-point average values of plasma concentration tended to be higher in the patients with severe acute cheilitis than those without such toxicities (p=0.080), and the concentrations at 17：00 on day 10 (p=0.036) and at 5：00 on day 46 (p=0.022) were predictive of severe acute cheilitis. Together with the correlation with clinical efficacy in our previous report, the dose adjustment of 5-FU based on the plasma concentration at 17：00 on day 10 is one of promising way to ensure a certain effect without severe acute cheilitis.

Keywords：esophageal squamous cell carcinoma, chemoradiotherapy, severe acute toxicities, 5-fluorouracil, plasma concentration

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要　旨

食道がんに対する標準的治療のひとつである化学放射線療法（以下，FP+RT療法）に関して，51症例を対象に，副作用の発現状況，および副作用発現と病期との関係を評価するとともに，5-フルオロウラシル（以下，5-FU）血漿中濃度との相関も解析した。FP+RT療法とは，5-FUの120時間持続点滴投与（400 mg/m^2/day，1～5日目，8～12日目），シスプラチン（以下，CDDP）の3時間点滴投与（40 mg/m^2/day，1日目，8日目），放射線照射（2 Gy/day，1～5日目，8～12日目，15～19日目）を1コースとし，2週間の休薬期間を設け，2コース目を繰り返す療法である。白血球減少，口内炎，口唇炎（放射線皮膚炎）に着目し，米国国立がん研究所の共通副作用基準NCI-CTC ver.2.0を用いて評価した。その結果，1）重症の白血球減少の発現頻度は39.2%であり報告者間で差異が認められること，2）重症の口内炎，口唇炎の発現頻度は，各々，13.7%，19.6%であること，3）重症の白血球減少，口内炎，口唇炎の発現頻度は病期に依存しないこと，4）重症の口唇炎が発現した症例では，軽症群と比較して，5-FU血漿中濃度が高い傾向にあること（p=0.080），5）治療開始10日目17時（p=0.036）あるいは46日目5時（p=0.022）に，5-FU血漿中濃度モニタリングを行うことにより，重症の口唇炎の発現を回避できること，が明らかとなった。これまでの検討と併せて総合的に判断した結果，10日目17時に採血を行い，5-FU血漿中濃度が0.19 μg/mL程度になるように以後の投与量を調整すれば，優れた治療効果を確保しつつ，重篤な口唇炎を回避できるものと結論した。

索引用語：食道がん，化学放射線療法，副作用，5-フルオロウラシル，血漿中濃度

I　はじめに

本邦における食道がん治療は，1999年，Ohtsuらにより，5-フルオロウラシル（以下，5-FU），シスプラチン（以下，CDDP）および放射線照射を併用する化学放射線療法（以下，FP+RT療法，**図1**）[1]が提唱されて以降，大きく展開したとされる。すなわち，それまでは早期発見，外科的処置が主流であった食道がん治療において，非侵襲的な治療法が選択肢として生じ，以来，FP+RT療法に関する情報収集と，どの患者にどちらの方法を選択すべきか，選択基準の設定に多大な努力が注がれてきた[2-5]。食道がんは，局所に腫瘤を形成するとともに，早期の段階でリンパ節転移をきたし，全身疾患としての性格をもつことから，FP+RT療法は，局所治療としての放射線療法と全身治療としての化学療法の併用という理解の上，理にかなった治療戦略として推進されており[6]，現在，食道がん，特に進行性の食道がんに対する標準的治療法のひとつであると認識されている。しかしながら，FP+RT療法については，がん病巣の消失，完全治癒を期待できる一方で，重篤な副作用が発現し，時として死亡に至ることから，治療効果，副作用発現における非常に大きな個人差を理由に，外科的処置を推奨する意見も少なからずあり，FP+RT療法の選択基準の設定が火急の課題となっている。

我々はこれまでに，FP+RT療法の最適化を目的として，5-FU血漿中濃度もしくは各種因子の遺伝子型に着目し，様々な検討を行ってきた[7-10]。治療効果の指標と

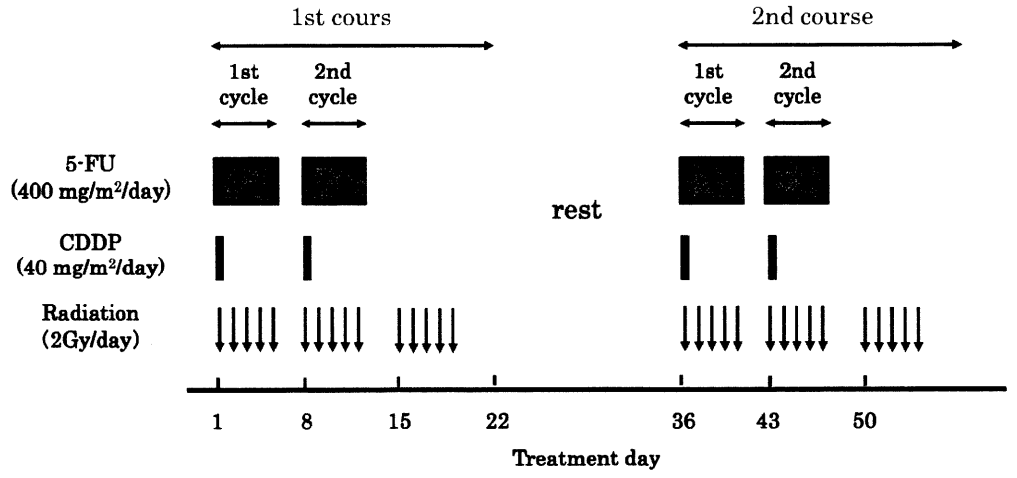

図1　FP+RT療法の標準スケジュール
5-フルオロウラシル（5-FU）の5日間持続点滴投与（400 mg/m^2/day，1～5日目，8～12日目），シスプラチン（CDDP）の点滴投与（40 mg/m^2/day，1日目，8日目），放射線照射（2 Gy/day，1～5日目，8～12日目，15～19日目）を1コースとし，2週間の休薬期間を設け，引き続き2コース目を施行する。