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
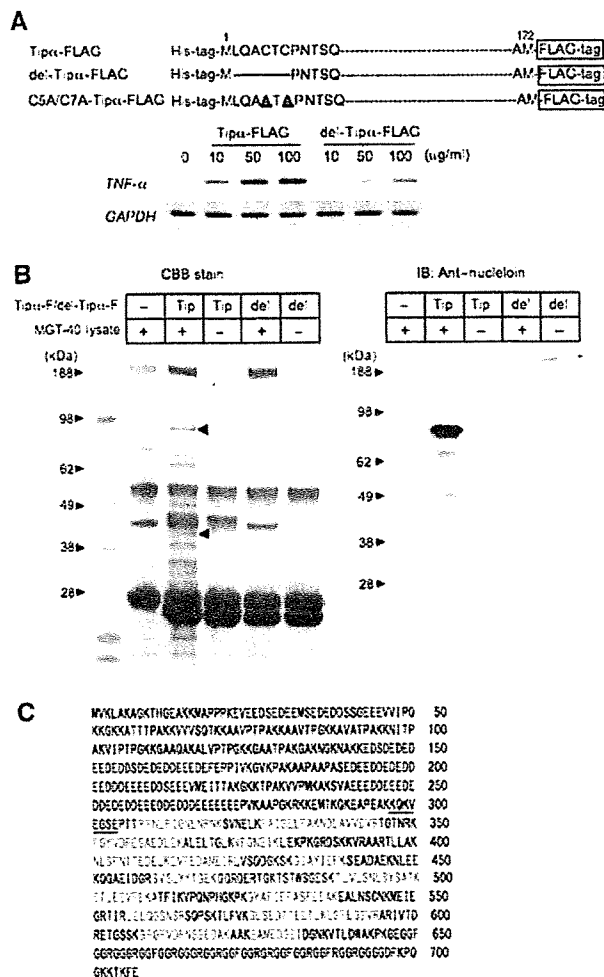

Fig. 1 Identification of nucleolin as Tipα binding protein. a Schematic representation of Tipα-FLAG, del-Tipα-FLAG and C5A/C7A-Tipα-FLAG proteins (top). Induction of TNF-α gene expression with Tipα-FLAG and with del-Tipα-FLAG in MGT-40 cells (bottom). Total RNAs were isolated from MGT-40 cells 1 h after 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),
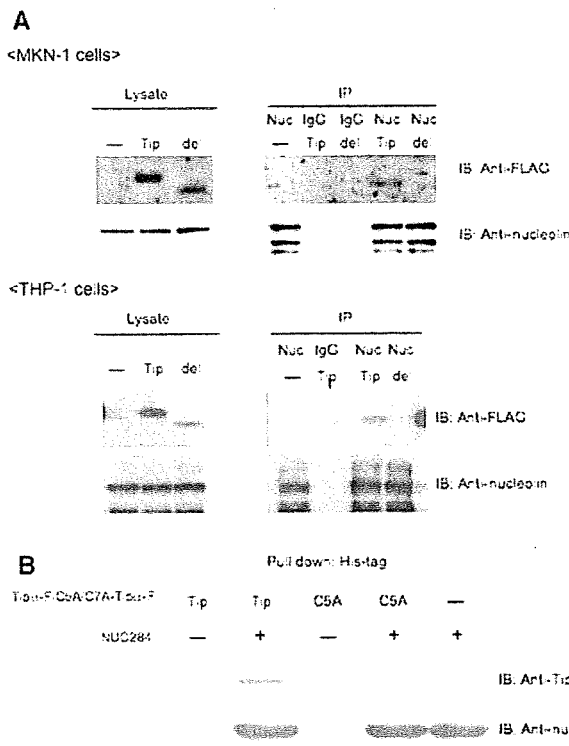
this fluorescent peak was significantly reduced by preincubation of anti-NUC295 with recombinant GST-nucleolin fragment (GST-NUC284) containing amino acids from 284 to 710 (Fig. 3b). This indicated that nucleolin on the cell surface had interacted with anti-NUC295. Further, we found that Tipα induced 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.
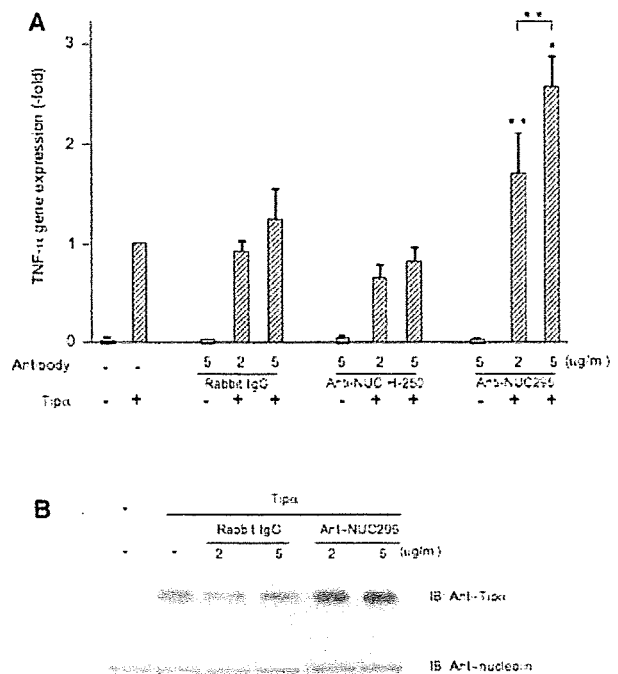
Fig. 4 Significant enhancement of Tipα-induced *TNF-α* gene expression and Tipα incorporation in cells induced by anti-NUC295. a MGT-40 cells were previously incubated with rabbit IgG, with anti-NUC H-250 and anti-NUC295 antibodies at 4°C for 1 h, and further treated with 50 μg/ml Tipα at 37°C for 1 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
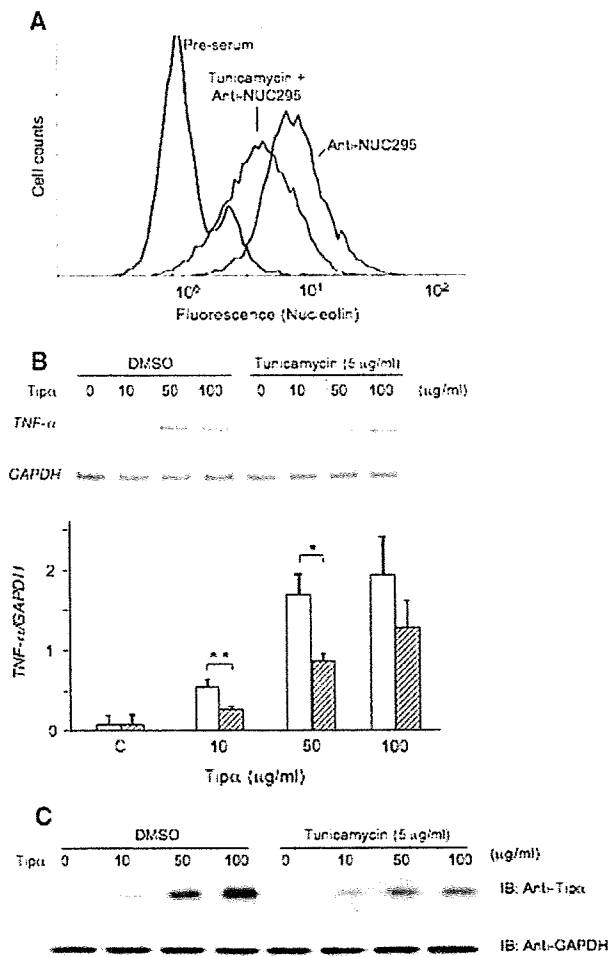
Fig. 5 Inhibition of Tipα-induced TNF-α gene expression and Tipα incorporation in cells induced by down-regulation of cell surface nucleolin. a MGT-40 cells were treated with or without 5 μg/ml tunicamycin in DMSO at 37°C for 5 h. The cell surface nucleolin on MGT-40 cells was visualized using flow cytometry 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, lactoferrin, 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 co-operates 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 and 45, 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/m²/day for days 1-5 and 8-12, infusion of CDDP or NDP at 40 mg/m²/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/m²/day for days 1-5 and 8-12) and CDDP (or NDP) (40 mg/m²/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.
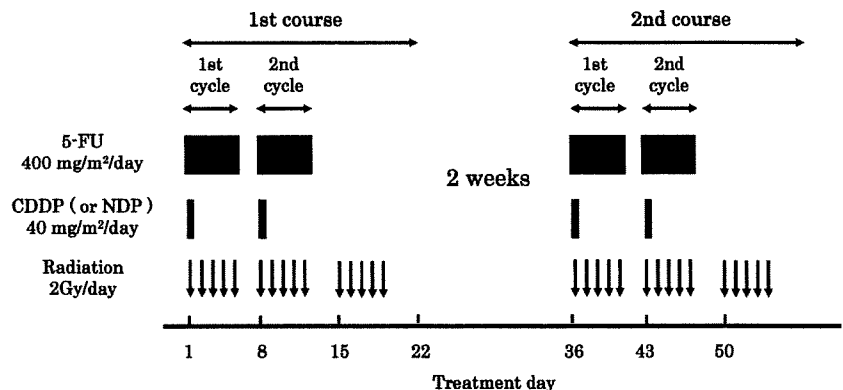
Pharmacokinetics of 5-FU

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 sig-

nificantly ($P=0.116$, $\beta=0.205$, $P=0.173$, $\beta=0.211$, 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 N=49	NDP 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.
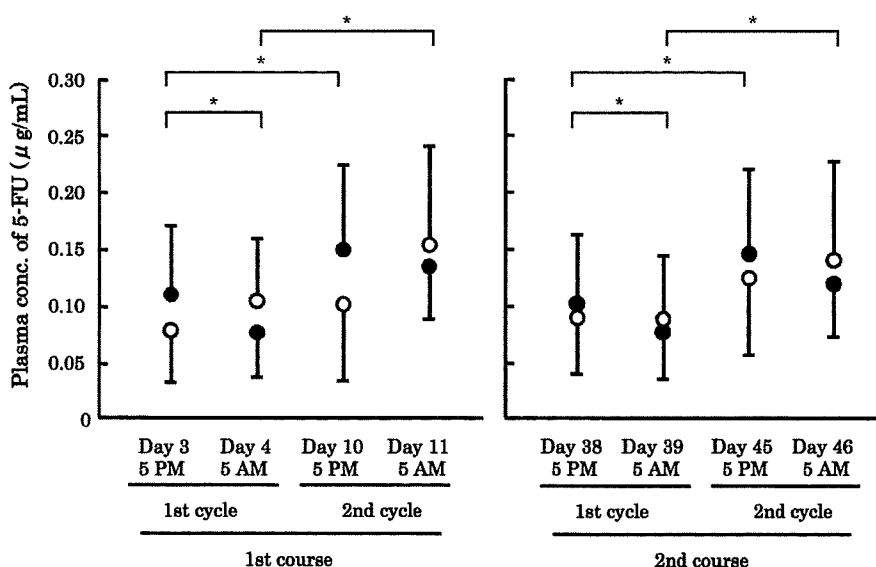
Discussion

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.

Acknowledgements

Competing Interest

The authors declare that no conflict of interest exists.

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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。

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，局所に腫瘍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
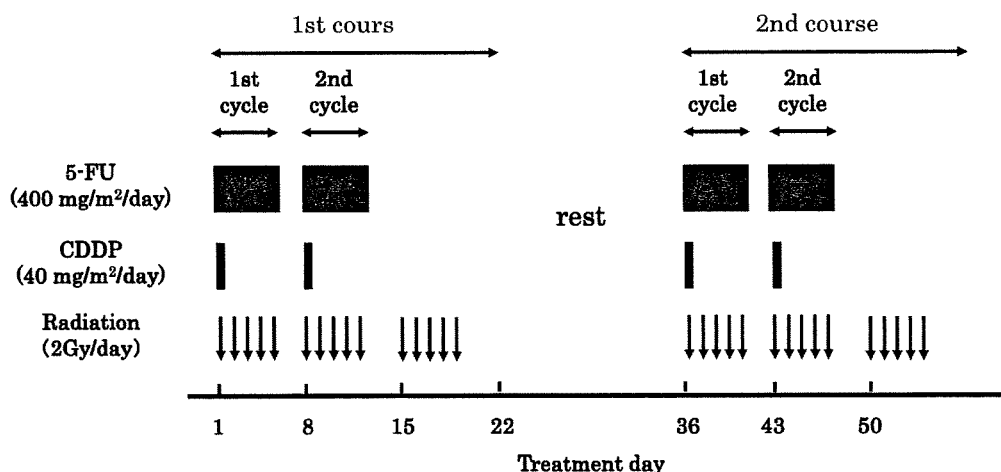

図1　FP+RT療法の標準スケジュール
5-フルオロウラシル（5-FU）の5日間持続点滴投与（400 mg/m^2/day，1～5日目，8～12日目），シスプラチン（CDDP）の点滴投与（40 mg/m^2/day，1日目，8日目），放射線照射（2 Gy/day，1～5日目，8～12日目，15～19日目）を1コースとし，2週間の休薬期間を設け，引き続き2コース目を施行する。

TDM研究

して，治療終了約4週間後に抗腫瘍効果（以下，レスポンス）を評価するとともに，2年間の予後の追跡調査を行い，その結果，1）レスポンス，予後が病期に依存すること[1]，2）レスポンスと予後が相関すること[9]，3）5-FU血漿中濃度の個体内変動，個体間変動が大きいこと[7]，4）病期と5-FU血漿中濃度が相関しないこと[10]，5）レスポンス，予後が5-FU血漿中濃度と相関すること[10]，6）レスポンス，予後と相関する遺伝子型の組み合わせがあること[8]，を示唆もしくは明らかにした。すなわち，FP+RT療法適用の可否を判定する方法として，病期の判定や遺伝子診断が有用であり，さらに，FP+RT療法を適用した場合には，5-FU血漿中濃度モニタリングにより，より高い治療効果を確保できることを明らかにした。また前報[10]において，治療効果の予測精度が高い採血ポイントの候補として，投与開始4日目5時，10日目17時をリストアップしており，いずれかの日時に5-FU血漿中濃度を評価し，その値に基づいて以後の投与量を調整するという治療戦略を提唱した。もっとも，5-FUを増量した場合に，副作用の発現頻度，重篤度がどのように変化するかについての情報がないことが課題として残された。

そこで本研究では，神戸大学医学部附属病院（以下，当院）にてFP+RT療法が施行された食道がん患者51例を対象に，副作用発現に関する詳細な情報を収集するとともに，うち30例について，副作用発現と5-FU血漿中濃度との関係も解析した。ここでは，特に，重篤な転帰をたどる骨髄抑制の指標とされる白血球減少，Quality of Life（以下，QOL）に大きな影響を与え，治療を中断せざるを得なくなる口内炎，口唇炎（放射線皮膚炎）に着目した。

II 対象と方法

1. 解析対象患者

2002年8月から2005年5月の間に治療を受け，標準スケジュールを完遂した患者51例（男性49例，女性2例）を対象とした。対象患者の平均身長は164.6±6.3 cm，平均体重は55.9±9.3 kg，平均年齢は63.6±7.3才，平均観察期間は18.1±13.9カ月であり，PSの内訳は0／1／2／不明 ＝ 23/24/2／2，病期の内訳はI/II/III/IV ＝ 11/8／17/10（術後再発は5例）であった。なお，病期分類は，UICC（The International Union Against Cancer）のTNM分類に基づいて行った。本研究は，ヘルシンキ宣言の趣旨を尊重し，研究の対象となる個人の人権の尊重，理解を求める同意を得る方法，研究対象となる個人への不利益および危険性について詳述した上で，研究内容について理解を求め，被験者となる同意を得る方法を明記した研究計画書を神戸大学医学部医学倫理委員会に提出し，承認を得ている。研究目的で利用される診療情報については，別途設定した個人情報管理者により連結可能匿名化されており，新たに定義されたコード番号で取り扱われるため，個人情報は保護されている。

2. FP+RT療法の標準スケジュール

標準スケジュールを図1に示した。FP+RT療法とは，5-FUの5日間持続点滴投与（400 mg/m²/day，1～5日目，8～12日目），CDDPの点滴投与（40 mg/m²/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治療レジメンである。なお，患者の状態に応じて，5-FU投与量を500もしくは550 mg/m²/dayに増量した（以下，Dose escalation例）。Dose escalation例は51例中6例であった。また，奏効が期待でき，重篤な副作用が認められない症例に対しては，標準スケジュール完遂後，2週間の休薬期間を設け，同様の治療を追加した（以下，追加治療例）。基本的には，標準スケジュールに従ったが，放射線照射を行わず，5-FUの持続点滴投与，CDDPの点滴投与のみとした。追加治療例は51例中19例であった。

3. 5-FU血漿中濃度データ

前報[10]において，51例中の30例を対象として，治療効果と5-FU血漿中濃度との相関解析を行っており，そのデータを用いて，副作用との関係を解析した。なお，この30例には，Dose escalation例，追加治療例は含まれなかった。採血ポイントは，患者ひとりあたり8点であり，具体的には，1コース目における3日目，10日目の17時，4日目，11日目の5時，2コース目における38日目，45日目の17時，39日目，46日目の5時であった。

4. 副作用の評価

がん化学療法施行時には必発し，重篤な転帰をたどる骨髄抑制の指標とされる白血球減少，並びに，5-FU使用時および放射線療法時に高頻度に発現し，QOLに大きな影響を与え，治療を中断せざるを得なくなる口内炎，口唇炎（放射線皮膚炎）に着目した。各々の重篤度は，米国国立がん研究所（National Cancer Institute：NCI）による共通毒性基準（Common Toxicity Criteria）NCI-CTC ver.2.0を用いて評価した。グレード3以上のものを重症，一方，グレード2以下のものを軽症とした。

5. データ処理および統計処理

重症の白血球減少，口内炎，口唇炎の発現頻度と病期との関係を解析し，比較にはFisher's exact testを用いた。8点の5-FU血漿中濃度の平均値を算出し，当該患者の5-FU血漿中濃度データとした。値は平均±標準偏

差で表記した。白血球減少，口内炎，口唇炎について，重症のものが発現した患者群を重症群，軽症のものが発現した患者群を軽症群とし，両群の5-FU血漿中濃度を比較した。8点個々の値についても同様に行った。比較にはunpaired Student's *t*-test/Welch's test を用いた。なお，正規性がない場合はMann-Whitney's U testを用いた。

Ⅲ　結果

1.　副作用の発現状況

　副作用の発現状況を表1に示した。重症の白血球減少の発現頻度は39.2%，口内炎では13.7%，口唇炎では19.6%であった。いずれかの重症の副作用が発現した割合は51.0%であった。

表1　副作用の発現状況（N=51）

グレード	0	1	2	3	4	重症例（発現頻度）
白血球減少	0	5	26	19	1	20（39.2%）
口内炎	33	5	6	4	3	7（13.7%）
口唇炎	25	10	6	4	6	10（19.6%）

NCI-CTC ver.2.0を用いて評価した。
グレード3以上のものを重症，一方，グレード2以下のものを軽症とした。
特筆すべき副作用が発現したという記録がなかった症例数はグレード0に含めた。

2.　重症の副作用の発現頻度と病期との関係

　重症の副作用の発現頻度と病期との関係を表2に示した。いずれの副作用も，発現頻度は病期に依存しなかった（Fisher's exact test）。

3.　重症の副作用の発現と5-FU血漿中濃度との関係（8点平均値）

　白血球減少，口内炎，口唇炎について，重症群と軽症群の5-FU血漿中濃度（8点の平均値）を比較した（表3）。解析対象である30例全体の平均値は0.110±0.035 μg/mLであった。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った（$p=0.080$，unpaired Student's *t*-test）。一方，白血球減少および口内炎については，重症群と軽症群との間に差はなかった（unpaired Student's *t*-test）。

4.　重症の副作用の発現と個々の採血ポイントにおける5-FU血漿中濃度との関係

　白血球減少，口内炎，口唇炎について，重症群と軽症群の個々の採血ポイントにおける5-FU血漿中濃度を比較した（表4，表5，表6）。重症の白血球減少が発現した症例では，軽症群と比較して，治療開始3日目17時の5-FU血漿中濃度が高い傾向にあった（$p=0.099$，unpaired Student's *t*-test）。口内炎においては，いずれ

表2　重症の副作用の発現頻度と病期との関係

	N	いずれか		白血球減少		口内炎		口唇炎	
		N	%	N	%	N	%	N	%
全体	51	26	51.0	20	39.2	7	13.7	10	19.6
Stage I	11	8	72.7	7	63.6	3	27.3	2	18.2
Stage II	8	3	37.5	2	25.0	0	0.0	2	25.0
Stage III	17	10	58.8	8	47.1	3	17.6	3	17.6
Stage IV	10	5	50.0	3	30.0	1	10.0	3	30.0
$p^{a)}$		0.471		0.327		0.531		0.890	

a)　病期と相関解析，Fisher's exact test

表3　重症の副作用の発現と5-FU血漿中濃度（μg/mL）との関係（8点平均値）

	重症群		軽症群		$p^{a)}$
白血球減少	N=10	0.114±0.046	N=20	0.109±0.030	0.728
口内炎	N=6	0.112±0.045	N=24	0.110±0.034	0.880
口唇炎	N=4	0.139±0.027	N=26	0.106±0.035	0.080
いずれか	N=13	0.113±0.041	N=17	0.108±0.032	0.726
全体			N=30	0.110±0.035	

重症（グレード3以上）の副作用が発現した患者群を重症群，軽症（グレード2以下）の副作用が発現した患者群を軽症群とした。
a)　重症群と軽症群の比較，unpaired Student's t-test

TDM研究