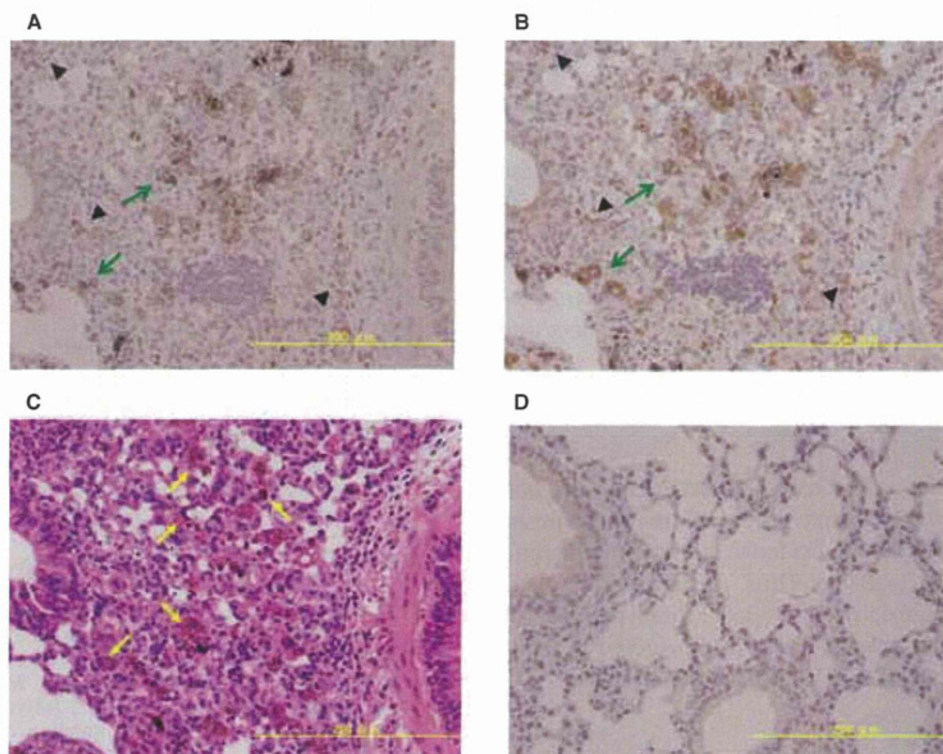

Figure 6. Immunohistochemical localisation of iNOS and NT. The panels show the alveolar region in a mouse exposed to MWCNTs, with positive staining for iNOS (A) and NT (B), and a haematoxylin and eosin stain (C). The black-coloured material is MWCNTs. Note the intense staining for iNOS and NT in both the granulomatous regions (green arrows) and epithelial cells (black arrowheads). The granulomatous regions in (C) are indicated by yellow arrows. Panel (D) shows the alveolar region in a vehicle control mouse with no significant staining for iNOS. Scale bars = 200 μm.

mutations (G:C to T:A) in DNA because it can base pair with adenine as well as cytosine (Shibutani et al. 1991; Moriya 1993). Recently, it has been reported that HεdC predominantly induces C to A or T mutations in human cells (Pollack et al. 2006; Yang et al. 2009). However, the most prominent mutation type induced by MWCNTs was G:C to C:G transversions, as was the case in our previous reports on fullerene and kaolin (Totsuka et al. 2009). In addition, Jacobsen et al. have recently reported that carbon black (Printex 90) induced base substitutions in the *cII* gene in FE1-Muta™Mouse lung epithelial cells (Jacobsen et al. 2011). The significant increases in these mutations were G:C to T:A, G:C to C:G and A:T to T:A, being similar to the results. Moreover, these mutations might be considered a hallmark of oxidative stress conditions. Oxidative products of guanine other than 8-oxodG, such as imidazolone, oxazolone (Oz), spiroiminodihydantoin, (Sp) and guanidinohydantoin (Gh), are now thought to be important causes of G to C transversions in translesion synthesis systems (Kornyushyna et al. 2002; Cadet et al. 1994; Goyal et al. 1997; Ye et al. 2003; Burrows et al. 2002; Kino & Sugiyama 2005, 2001; Kino et al. 2004). Therefore, it is suggested that Oz, Sp and Gh formation by MWCNTs might contribute to induce G:C to C:G transversions. Thus, it is important to analyse the formation of Oz, Sp and Gh in the lungs of mice treated with MWCNTs. The following hypotheses can be suggested to account for the *in vivo* genotoxic effects of MWCNTs through oxidative stress: (i) nanoparticles might trigger ROS production by iron-catalysed Fenton reactions; or (ii) nanoparticles could accumulate in cells because of phagocytosis and then enhance the production of ROS by NADPH oxidase (Aust 1994; Mossman & Gee 1993).

In the present study, inflammatory changes were introduced in the lungs of MWCNT-treated mice. Histologically, the infiltration of macrophages phagocytising tubes is thought to be a trigger, and it is clear that this inflammation originates from the host reaction towards foreign bodies. Bronchial and alveolar epithelia were influenced secondarily. It is noteworthy that the induction of lung inflammation was dependent on the total administered dose of MWCNTs. These histological findings are in line with those in previous reports indicating the induction of inflammation in the lungs by MWCNTs (Aiso et al. 2010; Ma-Hock et al. 2009; Pacurari et al. 2010; Poland et al. 2008; Takagi et al. 2008; Sakamoto et al. 2010). Intratracheally administered MWCNTs were shown to reach the visceral pleura, causing its thickening (Ryman-Rasmussen et al. 2009). Moreover, nitric oxide is known to be produced by activated macrophages in inflamed organs (Porter et al. 2006). In fact, iNOS- and NT-positive regions were frequently observed in the lungs of mice exposed to MWCNTs not only in test substance-phagocytised macrophages and granulomas but also in alveolar cells located near substance-phagocytised macrophages and granulomas (Figure 6, indicated by arrowheads). This suggests that MWCNTs induce nitric oxide production and gene mutation in the surrounding alveolar epithelial cells. Moreover, MWCNTs were found in the regional lymph nodes; such tubes would enter the lymphatic circulation and may then be distributed systemically.

Recently, many types of modified MWCNTs have been produced because of increases in their functionality and

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more widespread use (Chen et al. 2010). However, the genotoxicity of these modified nanomaterials has yet to be examined. Recent data have shown that the acute toxicity and genotoxicity of MWCNTs could be eliminated by the induction of structural defects after high-temperature treatment (Fenoglio et al. 2008). It has also been reported that MWCNT-induced toxicity, such as oxidative stress and inflammation, was increased by acid-based polymer coating but that coating MWCNTs with a polystyrene-based polymer protected against toxicity (Tabet et al. 2011). To improve their safety for occupational and general users, it is necessary to clarify the genotoxicity of modified MWCNTs.

Conclusions

It has been clearly demonstrated that MWCNTs induce both *in vitro* and *in vivo* genotoxicity. Although the mechanisms are not yet fully understood, oxidative stress and inflammation are likely to be involved. Thus, further studies of the mechanisms of genotoxicity are needed. Moreover, the levels of human exposure to MWCNTs should be studied to enable evaluation of the risk of MWCNTs to human health.

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Declaration of interest

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Supplementary material available online

Supplementary Table I, Figure 1.

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ADP-ribosylation of guanosine by SCO5461 protein secreted from *Streptomyces coelicolor*

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The *Streptomyces coelicolor* A3(2) genome encodes a possible secretion protein, SCO5461, that shares a 30% homology with the activity domains of two toxic ADP-ribosyltransferases, pierisins and mosquitocidal toxin. We found ADP-ribosylating activity for the SCO5461 protein product through its co-incubation with guanosine and NAD^+, which resulted in the formation of N^2-(ADP-ribos-1-yl)-guanosine (^{ar2}Guo), with a K_m value of 110 μM. SCO5461 was further found to ADP-ribosylate deoxyguanosine, GMP, dGMP, GTP, dGTP, and cyclic GMP with k_{cat} values of 150–370 s^{-1}. Oligo(dG), oligo(G), and yeast tRNA were also ADP-ribosylated by this protein, although with much lower k_{cat} values of 0.2 s^{-1} or less. SCO5461 showed maximum ADP-ribosylation activity towards guanosine at 30 °C, and maintained 20% of these maximum activity levels even at 0 °C. This is the first report of the ADP-ribosylation of guanosine and guanine mononucleotides among the family members of various ADP-ribosylating enzymes. We additionally observed secretion of the putative gene product, SCO5461, in liquid cultures of *S. coelicolor*. We thus designated the SCO5461 protein product as *S. coelicolor* ADP-ribosylating protein, ScARP. Our current results could offer new insights into not only the ADP-ribosylation of small molecules but also signal transduction events via enzymatic nucleoside modification by toxin-related enzymes.

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Abbreviations: MTX, mosquitocidal toxin from *Bacillus sphaericus* SSII-1; dGuo, 2′-deoxyguanosine; Guo, guanosine; ^{ar2}Guo, N^2-(ADP-ribos-1-yl)-guanosine; ^{r2}Guo, N^2-(ribos-1-yl)-guanosine; NAD^+, β-nicotinamide adenine dinucleotide; cGMP, guanosine 3′,5′-cyclic monophosphate.

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1. Introduction

ADP-ribosylation is the post-translational modification of proteins and involves the transfer of an ADP-ribose moiety from β-nicotinamide adenine dinucleotide (NAD^+) to specific residues in target proteins. Mono-ADP-ribosyltransferase activity is well-known to be present in several bacterial toxins that effectively target G proteins, elongation factors, and actins [see (Aktories and Just, 2000) for review]. Emerging studies have also revealed the existence of non-toxic mono-ADP-ribosyltransferases. Nitrogenases in *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Rhodospirillum rubrum* are regulated by dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase-activating glycohydrolase (DraG) during nitrogen fixation (Huergo et al., 2009; Masepohl and Hallenbeck, 2010), and vertebrate ecto ADP-ribosyltransferases (ARTs) target human neutrophil peptide-1 and cell surface $P2X_7$ receptors (Scheuplein et al., 2009; Stevens et al., 2009). Poly(ADP-ribose) polymerase 10 (PARP-10/ARTD10) also shows mono-ADP-ribosylation activity towards histones (Messner and Hottiger, 2011).

Some of the ADP-ribosyltransferases also target non-protein molecules. The pierisins, originally identified from *Pieris rapae* and *Pieris brassicae* as proteineous toxin against mice and cell lines (Marsh and Rothschild, 1974; Feltwell, 1982; Watanabe et al., 1999), target the N^2 amino groups of 2′-deoxyguanosine in double stranded DNA, causing mutations and an apoptotic response in cultured cells (Carpusca et al., 2006; Matsumoto et al., 2008; Orth et al., 2011; Yamamoto et al., 2009). The non-toxic CARP-1 from shellfish *Meretrix lamarckii* also target the same bases of DNA *in vitro* (Nakano et al., 2006). In contrast, tRNA 2′-phosphotransferases initially ADP-ribosylate a 2′-phosphate at the splice junction of pre-tRNA, then remove it by forming ADP-ribose 1″-2″ cyclic phosphate, resulting in the formation of a correct tRNA anticodon loop (Kato-Murayama et al., 2005; Sawaya et al., 2005; Steiger et al., 2005). Some small molecules can also be targets for ADP-ribosylation. For example, both Arr and Arr2 from opportunistic pathogens inactivate rifampicin through ADP-ribosylation (Baysarowich et al., 2008). In addition, some ADP-ribosyltransferases show low NAD^+ glycohydrolase activity that targets water molecules.

We observed from a BLAST search that the SCO5461 protein product, annotated as a secretion protein in the genome of *Streptomyces coelicolor* A3(2) (Bentley et al., 2002), shares homology with the activity domains of the pierisins and the mosquitocidal toxin from *Bacillus sphaericus* SSII-1 (MTX). MTX is an NAD^+:arginine ADP-ribosyltransferase that kills mosquito larvae (Schirmer et al., 2002a,b; Thanabalu et al., 1993), whereas pierisins are NAD^+:DNA(guanine-N^2) ADP-ribosyltransferases that induce apoptosis or gene mutation in mammalian cells in culture and *in vivo* (Shiga et al., 2006; Takamura-Enya et al., 2001; Totsuka et al., 2003; Watanabe et al., 2004). *Streptomyces* are gram-positive, soil-bacteria, and are unique organisms in terms of their metabolite profiles, most notably in relation to antibiotics, and in their properties as soil cleaners (Chater et al., 2010; Hodgson, 2000). In our present study, we demonstrated the ADP-ribosylating activity of SCO5461 and found that it has strong activity against the N^2 amino groups of guanine residues in nucleosides and mononucleotides. This is therefore the first report of an ADP-ribosyltransferase that mainly targets nucleosides, mononucleotides, and their 5′-phosphorylated forms. We also discuss the physiological roles of the ADP-ribosylation of nucleosides and mononucleotides.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and a plasmid

S. coelicolor A3(2) M145 ($SCP1^-SCP2^-$) was grown on Tryptic Soy Broth (Difco, Detroit, MI), with shaking in a Sakaguchi-flask at 28 °C. *Escherichia coli* K-12 JM109 (Toyobo, Osaka, Japan) was grown on LB for subcloning; *E. coli* K-12 ER2508 (New England Biolabs, Ipswich, MA) was grown on Terrific Broth for protein expression. A plasmid vector, pMALp2x (New England Biolabs), was used for subcloning and protein expression.

2.2. cDNA subcloning and expression of ScARP

We performed genome DNA extraction, PCR cloning and subcloning of cDNA using standard protocols (Kieser et al., 2000; Sambrook and Russell, 2001). SCO5461 and SCO5461(43–204) genes were ligated into pMALp2x. We introduced point mutations into these genes via overlap-PCR (Nakano et al., 2006). Proteins encoded in pMALp2x vectors were expressed as maltose-binding protein (MBP)-fused products in *E. coli* (Riggs, 1990). Following affinity purification, the MBP tag was cleaved from these recombinant products with factor Xa protease, followed by Mono-S column chromatography. Details of all of these procedures are included with the Supporting information.

2.3. ADP-ribosylation of nucleic acids

The standard reaction conditions employed for nucleosides and mononucleotides were as follows: nucleosides (final 1 mM) were incubated with SCO5461(43–204) protein (final 0.2 nM) and NAD^+ (final 0.01–3 mM) in 200 μl of 50 mM Hepes–NaOH pH 7.0 and 50 mM NaCl, for 10 min at 30 °C. The reaction mixture was immediately injected into an HPLC column. When oligo- or polynucleotides were used as substrates, reacted nucleotides (final 0.1 mg/ml) were injected into HPLC columns after digestion with micrococcal nuclease, phosphodiesterase II, and alkaline phosphatase (Nakano et al., 2006). The products were quantified from the A_{257} values in a standard curve generated using an equimolar mixture of ADP-ribose and Guo. Details of the digestion and HPLC conditions are included with the Supporting information.

2.4. Chemical synthesis of N^2-(D-ribofuranos-1-yl)-guanosine

The chemical synthesis of N^2-(D-ribofuranos-1-yl)-guanosine was performed in accordance with the synthesis route determined previously for N^2-(D-ribofuranos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). The

Fig. 1. Similarities between SCO5461 and its homologues. (A) Multiple alignments of the deduced amino acid sequences of SCO5461 and its homologues. Conserved motifs (LXR, STT, and reaction center glutamic acids) were boxed. Amino acid residues located two residues upstream of the reaction center are indicated in bold type. The source organisms and DDBJ/EMBL/GenBank accession numbers are indicated in Table S1. Note that SSPG_02248 from *S. lividans* TK24 (EFD66608) corresponds exactly to SCO5461 in this region and is not included in the alignment. (B) Schematic representations of the primary structure of SCO5461 and its homologues. Boxes with horizontal lines, activity domains; dotted boxes, ricin-fold like receptor-binding domains; cross-hatched ovals, secretion signals; "E", reaction center glutamic acids; arrowheads, regions sensitive to proteases. For details on binding domains, see Kanazawa et al. (2001) and Carpusca et al. (2006). (C) Dendrogram for amino acid sequences having homology to ScARP. Sequences were chosen from the DDBJ/EMBL/GenBank database using the BLASTp program (Altschul et al., 1990), and similarities were calculated within the highly-homologous regions by ClustalW (Thompson et al., 1994). A phylogenetic tree was drawn using DendroMaker for Macintosh ver. 4.1 (Tadashi Imanishi, http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html). The scale represents the number of substitutions per site. Names of genes, source organisms, and INSDC accession numbers are listed in Table S1 of the Supporting information.

details of these procedures and the ^{1}H NMR and MS data are described in the Supporting information.

3. Results

3.1. Subcloning of the SCO5461 gene, a putative ADP-ribosyltransferase from S. coelicolor

SCO5461, a putative gene from *S. coelicolor*, encodes a protein (204 amino acids, MW 22385.60) that comprises a secretion signal motif (amino acids 1–42) and a possible ADP-ribosyltransferase domain (amino acids 43–204, MW 18426.99). The latter activity domain has a 30% homology with the activity domains of the mosquitocidal toxin from *B. sphaericus* SSII-I (MTX) and pierisin-1 from *P. rapae* (Fig. 1A and B). MTX and pierisin-1 have lectin-like receptor-binding domains at their C-termini which facilitate their entry into eukaryotic cells and repress the ADP-ribosyltransferase activity of these proteins until cleaved by proteases (Carpusca et al., 2006). However, SCO5461 has no putative receptor-binding domain.

Recent genome projects have revealed homologues of SCO5461 in some actinomycete genomes (Fig. 1A and C, and Table S1 on Supporting information), the products of which contain three characteristic motifs of cholera toxin-like ADP-ribosyltransferase motifs; an LXR motif, an STT motif, and a catalytic center glutamic acid (Domenighini and Rappuoli, 1996; Otto et al., 2000). All SCO5461 homologues in actinomycete lack putative binding domains, and those in *Streptomyces lividans*, *Streptomyces scaviei*, and *Streptomyces albus* harbor secretion signals at their N-terminal ends. However, a SCO5461 homologue in *Streptosporangium roseum* lacks a putative secretion signal. Interestingly, avermectin-producing *Streptomyces avermitilis* has a pseudogene lacking a clear ORF, and streptomycin-producing *Streptomyces griseus* has no SCO5461 homologue. All known arginine ADP-ribosyltransferases, including MTX, have a glutamic acid located two residues upstream of their catalytic center glutamic acid (Laing et al., 2011). On the other hand, pierisin-1 and most SCO5461 homologues have glutamine, instead of glutamic acid, two residues upstream of their catalytic center (Fig. 1A, bold type).

3.2. ADP-ribosylation of nucleic acids by ScARP, the SCO5461 gene product

We purified the activity domain of the SCO5461 gene product as an MBP-tagged form following its expression in *E. coli* and then removed the MBP tag with factor Xa. The resulting peptide (SCO5461(43–204), MW 18903.51), which

had a preceding ISEF residues derived from the multi-cloning site sequence on its N-terminus, was confirmed to be the single predominant band on an SDS-PAGE gel (Figure S1 on Supporting information) and was therefore used in the subsequent experiments.

When SCO5461(43–204), expressed in *E. coli*, was incubated for 4 h with calf thymus DNA in the presence of NAD^+, two new peaks appeared at the same retention times seen for N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by HPLC analysis (Fig. 2A-I). Both peaks were clearly enhanced by co-injection of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine, synthesized by the incubation of deoxyguanosine and pierisin-1 in the presence of NAD^+ (Figure S2 on Supporting information). Since these peaks were much smaller than those made by pierisin-1 and calf thymus DNA, we searched for more desirable substrates. Many RNA species, including tRNA and oligo$(G)_{14}$, and oligo$(dG)_{14}$ can be more easily modified compared with calf thymus DNA, suggesting that SCO5461(43–204) recognizes single stranded oligonucleotides as substrates (Fig. 2A; details described later).

Interestingly, when SCO5461(43–204) was incubated for 10 min with guanosine (Guo) and NAD^+, two new HPLC peaks appeared (Fig. 2B-I). UV spectra of these two new peaks were found to be similar to that of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Fig. 2B-I). Similar results were obtained with deoxyguanosine (dGuo; Fig. 3 and detailed analysis described later). However, when SCO5461(43–204) was incubated with (deoxy)adenosine, (deoxy)cytidine, thymidine, or uridine, in the presence of NAD^+, no new peaks appeared (Figure S3A on Supporting information).

The replacement of glutamic acid at the putative reaction center of the SCO5461 product with aspartic acid (Figure S1 on Supporting information) resulted in a >90%-decrease in the peaks (Fig. 2B-III), and no new peaks appeared with the use of ADP-ribose instead of NAD^+ (Fig. 2B-IV), suggesting that the SCO5461 product had ADP-ribosylated guanine residues. We hereafter refer to the SCO5461 protein product as *S. coelicolor* ADP-ribosylating protein (ScARP).

We further analyzed the structure of compounds in the two newly appeared peaks in the reaction of Guo, NAD^+, and ScARP (Fig. 2B-I, indicated with an arrow and an arrowhead). As shown in Figure S3B on Supporting information, the area of both peaks on the HPLC charts increased linearly up to 1 h. The ratio of the backward peak (minor peak, indicated by an arrowhead) to the forward peak (major peak, indicated by an arrow) from the reaction of Guo, NAD^+, and ScARP then increased during a 4-h reaction. When the isolated forward peak was re-analyzed by HPLC after incubation at 30 °C, the ratio changed to about 1:1 within 4 h, suggesting that both peaks can easily anomerize to each other (Figure S3C on Supporting information). Moreover, LC-ESI-MS analysis indicated that both the compounds in these peaks have a molecular ion peak at *m*/*z* 825, corresponding to ADP-ribosylated guanosine, and an ion peak at *m*/*z* 693, arising from the loss of a ribose moiety commonly observed in nucleoside separation, and an ion peak at *m*/*z* 926 corresponding to a triethylamine addition, derived from the HPLC eluent, to the parent mass at *m*/*z* 825 (Fig. 4A). We concluded that both peaks were ADP-ribosylguanosine and

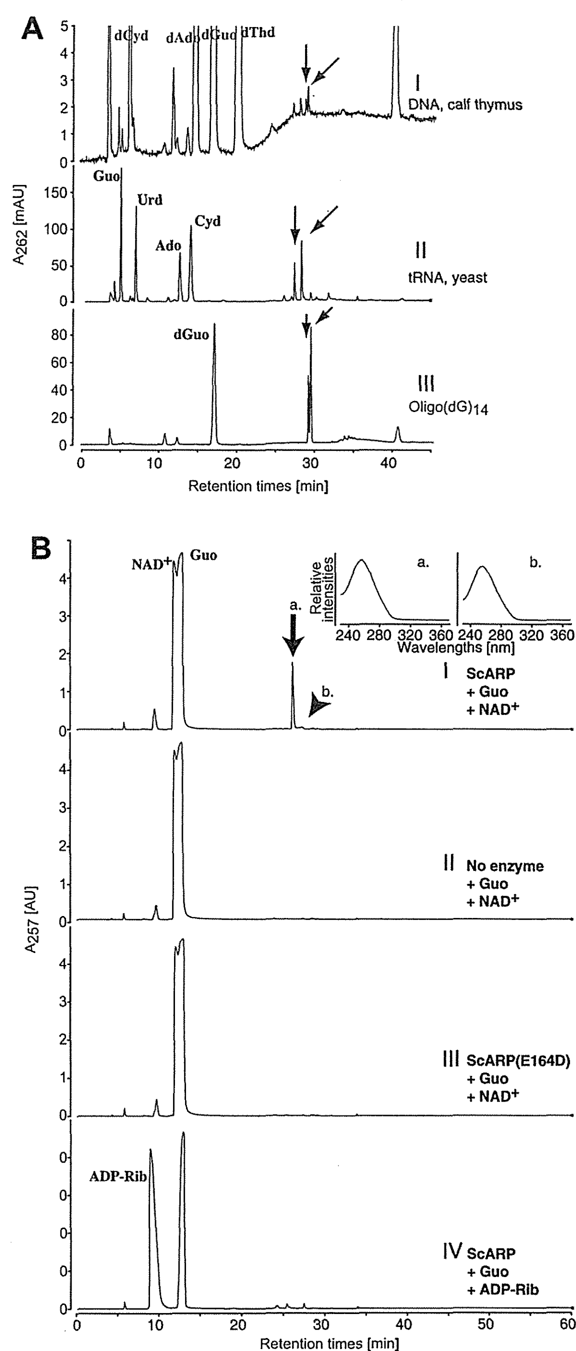

Fig. 2. Structural analysis of ADP-ribosylated nucleic acids formed by SCO5461(43–204) (ScARP). (A) HPLC elution patterns of hydrolysates of (I) calf thymus DNA, (II) tRNA, and (III) oligo$(dG)_{14}$, after incubation with ScARP (final 625 nM) and NAD^+ (final 2 mM) for 4 h. The arrows indicate newly appearing peaks. Note that unreacted NAD^+ was removed by ethanol precipitation prior to nuclease digestion. (B) HPLC elution patterns of guanosine incubated with (I) ScARP (0.2 nM) and NAD^+ (1 mM), (II) NAD^+ (1 mM), (III) ScARP(E164D) (0.2 nM) and NAD^+ (1 mM), and (IV) ScARP (0.2 nM) and ADP-ribose (1 mM). The arrow and arrowhead indicate newly appearing peaks. UV absorption spectra of compound **a** (λmax 257 nm) and **b** (λmax 256 nm), generated using a photodiode array detector, are superimposed in the elution pattern I.

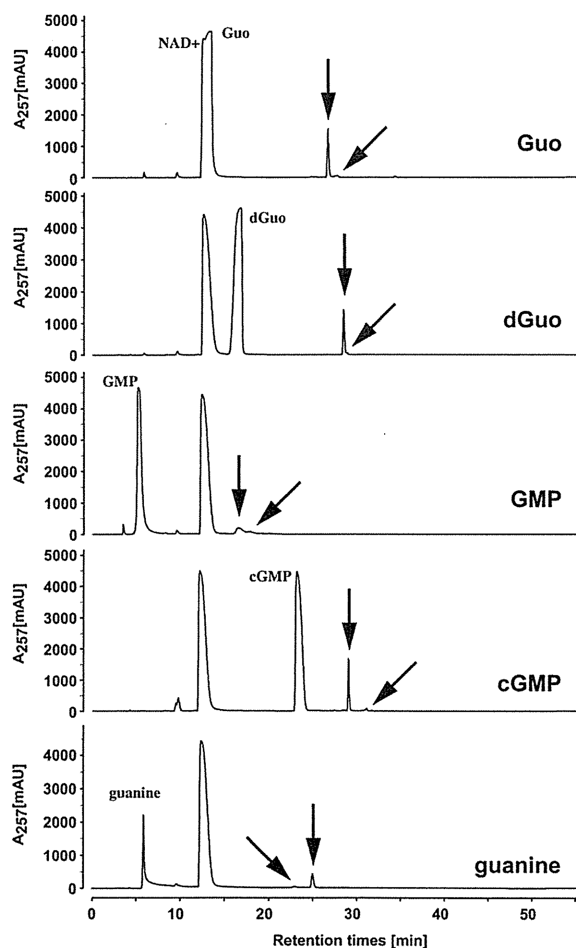

Fig. 3. ADP-ribosylation of monomers of nucleic acids by ScARP. HPLC elution patterns of Guo (top), dGuo (upper middle), GMP (middle), cGMP (lower middle), and guanine (bottom) incubated for 10 min (30 min for guanine) with ScARP (0.2 nM) and NAD$^+$ (1 mM) are shown.

the initial-reaction product was the forward peak, whereas the backward peak was its anomerized form at the C1′-position of ADP-ribose.

We further confirmed the structure of ADP-ribosylated Guo using HPLC, LC-MS, and ^{1}H NMR. We first digested ADP-ribosyl-Guo to ribosyl-Guo using phosphodiesterase I and calf intestinal alkaline phosphatase, and compared the resulting products by HPLC with independently synthesized N^2-(ribos-1-yl)-Guo (Scheme S1 on Supporting information). As shown in Fig. 4B, the retention times of the four peaks from enzymatic digest coincident with those of the synthesized (α- and β-ribofuranos-1-yl)-Guo and their further anomerized form, (α- and β-ribopyranos-1-yl)-Guo, as found for N^2-(ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). ^{1}H NMR analysis of ADP-ribosyl-Guo revealed that most of the signals could be assigned to the same as those of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001), except presence of a newly absorbance peak of around 4.5 ppm derived from a proton at the 2′ position of the ribose moiety

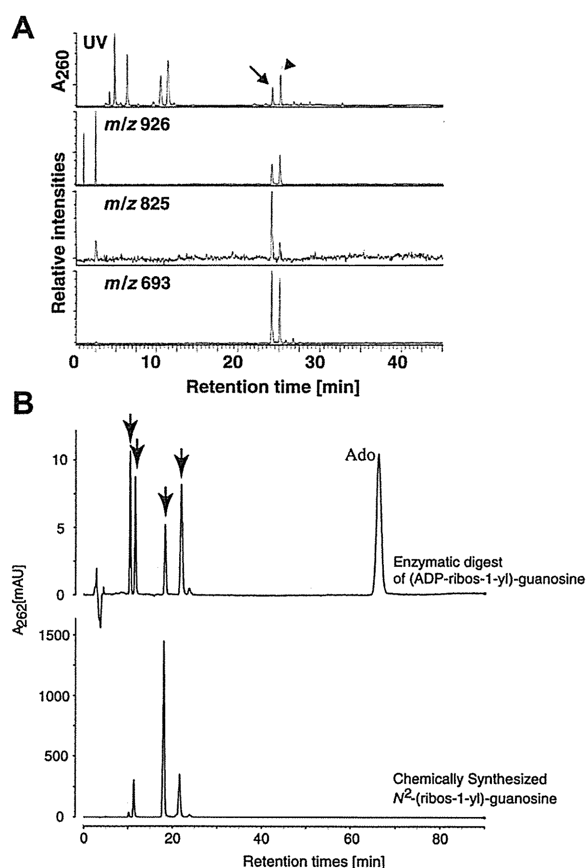

Fig. 4. Structural confirmation of N^2-(ADP-ribos-1-yl)-guanosine. (A) LC-ESI-MS elution patterns of reaction products formed from tRNA (0.1 mg/ml), NAD$^+$ (2 mM), and ScARP (625 nM). The HPLC profile (UV) and the ion chromatograms (*m/z* 926, 825, and 693) are shown. Both compounds in these peaks had a molecular ion peak at *m/z* 825 corresponding to [ADP-ribosyl-Guo + H^+]$^+$, an ion peak at *m/z* 693 arising from the loss of a ribose moiety, and an ion peak at *m/z* 926 corresponding to a triethylamine addition, derived from the HPLC eluent, to the parent mass at *m/z* 825. The forward peak is indicated by an arrow, and the backward peak is indicated by an arrowhead. (B) HPLC elution patterns of hydrolysate of (ADP-ribos-1-yl)-guanosine (top) and chemically synthesized N^2-(ribos-1-yl)-guanosine (bottom). Ado, adenosine.

with missing protons around 2.00 ppm derived from two protons of 2′ and 2″ position of the deoxyribose moiety that was presented in the ADP-ribosylated 2′-deoxyguanosine (Figure S4 on Supporting information). This result strongly indicated that the product was N^2-(ADP-ribos-1-yl)-guanosine (Fig. 5). Similar results were obtained using either HPLC or LC-MS analysis when dGuo was used instead

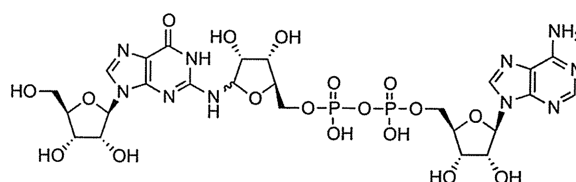

Fig. 5. Structure of N^2-(ADP-ribos-1-yl)-guanosine.

of Guo. Moreover, retention times for two peaks corresponding to ADP-ribosylated dGuo were the same as those of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (i.e. the enzymatic digest of ADP-ribosylated DNA produced by pierisin-1). We thus concluded that ScARP ADP-ribosylates the N^2 amino groups of guanine residues in Guo and dGuo in the same manner as pierisin-1 does for the N^2 amino groups of guanine residues in dsDNA.

3.3. Kinetics of NAD^+:guanosine ADP-ribosylation by ScARP

We next performed kinetic analyses to determine the structural preferences of nucleic acids for ADP-ribosylation by ScARP. Neither the 3′- and 5′-phosphate groups showed much effect on the ADP-ribosylation rate of Guo and dGuo, and cyclic GMP was also effectively modified. Guanine was also modified but at a lower rate than Guo (Fig. 3, Table 1). All of the tested DNAs and RNAs containing guanine residues were modified more slowly than their monomers; 50% of the bases in both oligo$(G)_{14}$ and oligo$(dG)_{14}$ were modified at a relatively faster rate than yeast tRNA, *E. coli* rRNA, mRNAs from HeLa cells, and synthesized dsRNA, but only a small amount of guanine residues in calf thymus dsDNA were modified (Table 1, Fig. 2A, Figure S2 on Supporting information).

We next tried to optimize the reaction conditions for the kinetic analysis of ScARP, NAD^+, and Guo. The quantity of ADP-ribosylated products increased linearly for up to 60 min (see Figure S3B on Supporting information), and the enzymatic kinetics were thus determined using 0.2 nM ScARP in a 10-min reaction. The optimum pH range was 6–7, but activities were relatively low under weak acidic conditions compared with weak basic conditions. The optimum temperature was 30 °C, and 20% of the maximum activity was retained even at 0 °C. The optimum NaCl concentration was 50 mM but the effect of the NaCl concentration was not appreciable within the 0–300 mM range (Figure S5 on Supporting information). Similar activities were observed in the presence of KCl or Na_2SO_4 (50 mM each) in the place of NaCl, and neither the addition of Ca^{2+}, Mg^{2+}, EDTA, EGTA, NH_4Cl, nor dithiothreitol (5 mM each) interfered with this activity. We used Lineweaver–Burk plot analysis to determine the K_m value for NAD^+ at 110 μM, and the k_{cat} value for Guo at 325 s^{-1} (Fig. 6; Table 1). K_m values for Guo and dGuo are 31 μM and 52 μM, respectively, supporting that both Guo and dGuo are equally targeted. Both the 3′- or 5′-phosphate groups showed little effect on the k_{cat} values. These results suggest that ScARP has relatively high activity compared with the activity domains of MTX and pierisin-1, which have k_{cat} values of 2.5 ± 1 min^{-1} for soybean trypsin inhibitor (Schirmer et al., 2002a) and 55 s^{-1} for dsDNA (Watanabe et al., 2004) respectively.

Table 1
Acceptors for ADP-ribosylation by ScARP.

Acceptors	k_{cat} [s^{-1}]	K_m [μM]
Guo	325	31
dGuo	278	52
GMP	204	
dGMP	512	
cGMP	148	
GTP	460	
dGTP	379	
guanine	63	174
oligo(G)14	0.26	
oligo(dG)14	0.20	
tRNA, yeast	0.14	
DNA, calf thymus	0.001	
Ado, Cyd, Urd	<0.0001	
dAdo, dCyd, dThd	<0.0001	

k_{cat} and K_m values were obtained using Lineweaver–Burk plot analysis.

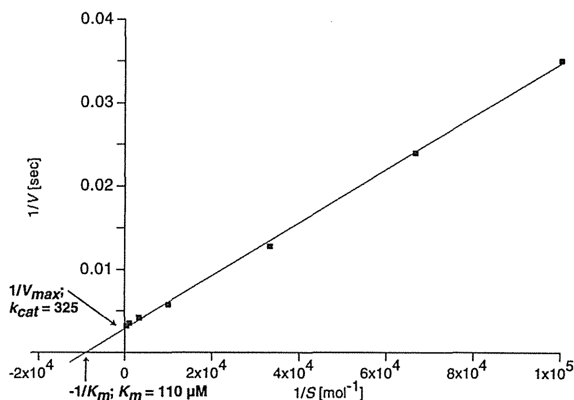

Fig. 6. Lineweaver–Burk plot analysis of ADP-ribosylation of 1 mM Guo by 0.2 nM ScARP in the presence of 0.01–3 mM NAD^+, in a 50 mM Hepes-NaOH/50 mM NaCl buffer for 10 min at 30 °C. Product amounts were determined by the A_{257} values.

3.4. Secretion of ScARP from *S.* coelicolor

This is the first report that characterizes a mono-ADP-ribosylating enzyme for guanine nucleosides and mononucleotides i.e. ScARP. Although Widdick et al. may have previously detected secreted ScARP, their reporter-gene assay could not determine twin-arginine translocation pathway-dependent secretion from a plate culture of *S. coelicolor* (Widdick et al., 2006). Since there is no UUA^{Leu} codon in the SCO5461 gene, the tRNA of which is specifically expressed upon differentiation (Lawlor et al., 1987), ScARP must not be differentiation-specific. We thus validated its ADP-ribosyltransferase activity from liquid culture medium of growing-phase *S. coelicolor* using in-gel enzymatic analysis and LC-ESI-MS (Figure S6A on Supporting information). When the active fraction was separated with an SDS-PAGE gel co-polymerized with yeast tRNA, and ADP-ribosylated by soaking the gel with $[^{32}P]NAD^+$ and radioluminographed, a band corresponding to an ADP-ribosyltransferase was detected at 18-kDa, with the same mobility as that of the activity domain of ScARP (Fig. 7). We also purified cultured medium partially, and the active fractions with or without ScARP, as the internal standard, were measured in duplicate by nano LC-MS/MS system (2DICAL, Ono et al., 2009, 2006). As shown in Figure S6B and C on Supporting information, the peak of 499.57 *m/z* (RT 35.455 min) matched the doubly-charged ITPEPVWR sequence with an iScore of 31 and an expect score of 44.0, and the peak of 482.526 *m/z* (RT 36.310 min) matched the triply-charged GPQVVFEEGFHAK sequence with an iScore of 37 and an expect score of 6.5 of SCO5461/gi21223819 in

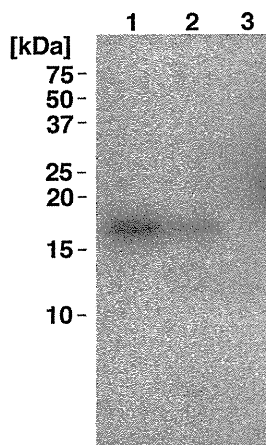

Fig. 7. In-gel analysis of NAD$^+$:tRNA ADP-ribosyltransferase activity from the culture medium of *S. coelicolor*. Lane 1, total protein from the culture medium of growing phase of *S. coelicolor*, concentrated with an Amicon Ultra MWCO 10 kDa devise (10 μg); lane 2, secretion-form, 18-kDa (10 ng) ScARP; lane 3, secretion-form, 18-kDa ScARP(E164D) (10 ng).

the MASCOT database. Both peaks were clearly enhanced in the samples supplemented with ScARP, indicating that *S. coelicolor* secreted ScARP protein in liquid culture medium.

4. Discussion

We revealed in our current study that ScARP is an enzyme lacking receptor-binding domains, secreted from *S. coelicolor*, and that ADP-ribosylates the N^2 amino groups of guanine nucleosides as well as mononucleotides. This is therefore the first report to identify an ADP-ribosyltransferase that mainly targets mononucleotides and nucleosides, since pierisin-1 shows weak ADP-ribosylation activity on dGuo (Figure S2 on Supporting information; Takamura-Enya et al., 2001), but the ADP-ribosylation of Guo by MTX was undetectable at least in the condition used in present study (Figure S3D on Supporting information). Hence, ScARP could be classified as a pentosyltransferase [EC 2.4.2.x] with a systematic designation of NAD$^+$:guanine-N^2-ADP-D-ribosyltransferase, or as an N-glycosidic cholera toxin-like-ADP-ribosyltransferase catalyzing mono-ADP-ribosylation [EC 2.4.2.30.1.X.1.2] with a systematic name NAD$^+$:mono-ADP-D-ribosyl-guanine-N^2-ADP-D-ribosyltransferase (GADPRT), according to the new extended EC numbering system proposed by Hottiger et al. (2010).

Pallen et al. (2001) have also predicted by PSI-BLAST analysis that a 219-aa secretion protein (ID 7105990), identified in the unfinished genome project of *S. coelicolor*, could be an ADP-ribosyltransferase. However, their predicted reaction center in the β-5 strand of this protein product corresponds to E189, and not E164, in the SCO5461 product. This somewhat contrasting finding may be due to the preliminary nature of the sequences in unfinished genome projects. Previous reports have described the existence of NAD$^+$:protein ADP-ribosyltransferases and ADP-ribosylated proteins not only in *S. coelicolor* (Penyige et al., 2009; Shima et al., 1996; Sugawara et al., 2002) but also in *Streptomyces grieus*, a species that has no SCO5461 homologue (Ochi et al., 1992; Penyige et al., 1996). Since ScARP also targets basic proteins, such as soybean trypsin inhibitor with a k_{cat} value of less than 0.001, ScARP might not ADP-ribosylate protein substrates that were previously identified in *S. coelicolor*.

The formation of only a single isomer and the ready anomerization of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine has been proposed as the mechanism underlying the ADP-ribosylation of deoxyguanosine residues in DNA by pierisins (Takamura-Enya et al., 2004). Our present study revealed that ScARP produces only a single isomer of N^2-(ADP-ribos-1-yl)-guanosine, and it anomerized within 4 h. Although we couldn't determine which isomer had been the initial product, an inversion at the C1′-position of ADP-ribose moiety from β to α occurs in some ADP-ribosyltransferases [see (Laing et al., 2011) for review]. Future structural analyses of ScARP co-crystallized with Guo and NAD$^+$ will likely elucidate the reaction mechanism.

ScARP is a secreted protein without a receptor-binding domain, but the accumulated data indicate that the C-terminal receptor-binding domain is indispensable for toxic activities of MTX and pierisins (Carpusca et al., 2006). Moreover, ScARP seems not to be an acute toxic enzyme, since it could be expressed in *E. coli*; even expression vectors for pierisins have not been successfully generated, possibly due to the toxic effects of their basal expression during subcloning (Yamamoto et al., 2009). Nevertheless, the wide distribution of the ScARP homologues lacking a C-terminal receptor-binding domain in streptomycetes suggests that they must have an important function. If ScARP is an enzyme which functions extracellularly, it could be possible that ADP-ribosylguanosine (^{ar2}Guo) functions in response to environmental changes. Many bacteria use guanosine derivatives in response to environmental changes, such as guanosine penta- or tetraphosphate ((p)ppGpp)-mediated stringent responses (Srivastava and Waters, 2012), or cyclic di(3′-5′)GMP (c-di-GMP)-mediated regulation of motility and biofilm formation (Krasteva et al., 2012). In this respect, it is interesting to speculate that ScARP disregulates these signals by direct ADP-ribosylation of these guanosine derivatives, or by synthesizing ^{ar2}Guo as mimicking or competitively inhibiting molecules. Since the damaged nucleotide pool induces genomic instability and gene mutation [for reviews, see Henderson et al. (2010)], it might be possible to speculate the ^{ar2}Guo-mediated increasement of DNA replication errors in bacteria living near *S. coelicolor*.

Although streptomycetes are known to secrete a large amount of extracellular enzymes to obtain soluble nutrients (Chater et al., 2010), there is a "topological paradox", similar to that found in vertebrate ARTs and CD38 (De Flora et al., 1997; Koch-Nolte et al., 2011), since both NAD$^+$ and guanosine mainly exist inside cells. Possible sources for NAD$^+$ and guanosine are dead or damaged bacteria and eukaryotes, the major nutrition source for streptomycetes. The other possible sources for guanosine are secreted extracellular nucleic acids [see (Kikuchi and Rykova, 2010) for review]. Further studies, such as screens for naturally formed ADP-ribosylated guanine nucleotides, elucidation of the responses of bacteria to ADP-ribosylated guanosine,

and phenotypic analysis of a ScARP-deletion strain will be necessary in the future to better understand the biological significance of ScARP.

The other remaining question is the origin of ADP-ribosyltransferase such as ScARP. The ScARP homologue in *S. avermitilis* (Save_pseudo in Fig. 1A and C) is overlapped by the SAV_1763 lipoprotein gene and therefore has an imperfect ORF. Save_pseudo is located on the border between the region for non-essential secondary metabolite genes and the region for essential genes (Ikeda et al., 2003). Our present hypothesis is that this gene could have once been distributed among some *Streptomyces* strains, but then disappeared by recombination with the other gene in *S. avermitilis*. There are many actinomycete genome projects currently underway, reflecting the importance of these bacteria in ecology, pharmacy, and industry. Both phylogenetic and biological studies of these microorganisms may help to elucidate the origin and physiological roles of nucleoside- and mononucleotide-specific ADP-ribosyltransferases.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary information

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxicon.2012.11.019.

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Mini-review

Metabolic syndrome: A novel high-risk state for colorectal cancer

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ABSTRACT

Metabolic syndrome (MS) and related disorders, including cancer, are steadily increasing in most countries of the world. However, mechanisms underlying the link between MS and colon carcinogenesis have yet to be fully elucidated. In this review article we focus on the relationships between various individual associated conditions (obesity, dyslipidemia, diabetes mellitus type 2 and hypertension) and colon cancer development, and demonstrate probable related factors revealed by *in vivo* and *in vitro* studies. Furthermore, molecules suggested to be involved in cancer promotion are addressed, and the potential for cancer prevention by targeting these molecules is discussed.

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1. Introduction

Many disorders can be induced by excessive accumulation of visceral adipose tissue, and the combination of related symptoms, so-called metabolic syndrome (MS), is attracting increasing attention as a major health problem since it can lead to conditions such as cardiovascular disease. Recently, MS has also attracted much interest as a risk factor for several cancers, including colon cancer. The World Cancer Research Fund and American Institute for Cancer Research have evaluated causal relationships between accumulation of visceral adipose tissue and cancer, and concluded 'confident evidence' for colorectum and pancreas cancers [1]. In Japan, overweight and obesity, defined as a body mass index (BMI) of 25 or more, are similarly reported to be associated with several cancers, such as colorectum cancer in males, breast cancer in postmenopausal females and liver cancer in those with a history of hepatitis C virus infection [2–4].

In this review article, relationships between the symptoms of MS and colorectal carcinogenesis are focused on in animal models. Commonly used animals for MS models are rodents because of their size. The models are classified into three groups: diet-induced obesity models (C57BL/6J mice and F344 rats), monogenic models (*ob/ob* mice, *db/db* mice, ZDF rats and KK-A^y mice), and polygenic models (TSOD mice and OLETEF rats). A high-fat/-fructose diet, or mice with genetic alterations such as mutation of leptin, leptin receptor and agouti genes are commonly used. Suitable animal models of MS-associated carcinogenesis might be mice with intact leptin and leptin receptors because leptin signaling stimulates cell growth, and may affect carcinogenesis.

2. Metabolic syndrome

MS is common in Western countries, and is currently increasing almost ubiquitously across the globe. In addition to developed countries, MS is increasing in developing countries in adults and particularly in children [5]. Moreover, obesity and overweight are rapidly increasing in both urban and rural areas in the under developed countries of sub-Saharan Africa and South Asia [6].

Various diagnostic criteria for MS have been proposed by many national/international organizations [7–10]. Consensus statements for diagnosis of MS are almost the same, and these are the presence of any three abnormal findings out of five. i.e. (1) waist circumference (males: ⩾90 cm; females: ⩾80 cm), (2) blood triglyceride (TG) levels ⩾150 mg/dL (1.7 mmol/L), (3) blood high-density lipoprotein (HDL) cholesterol levels (males < 40 mg/dL (1 mmol/L); females <50 mg/dL (1.3 mmol/L), (4) blood pressure (systolic blood pressure ⩾130 mmHg and/or diastolic blood pressure ⩾85 mmHg or drug treatment for hypertension), and (5) blood sugar (fasting blood sugar ⩾100 mg/dL (5.6 mmol/L) or drug treatment for diabetes mellitus type 2 (T2DM)) [11]. However, further work is required for the components regarding waist circumference, which rely on population and country-specific definitions [12].

A major pathogenesis of this syndrome could be accumulation of visceral adipose tissue, characterized by increased numbers of macrophage infiltration along with low-grade inflammation [13]. In addition to low-grade inflammation, other factors that may contribute to colorectal cancer development would be dyslipidemia, insulin resistance, subsequent adipocytokine imbalance and activation of the renin-angiotensin system, which are further documented in detail in this paper Fig. 1.

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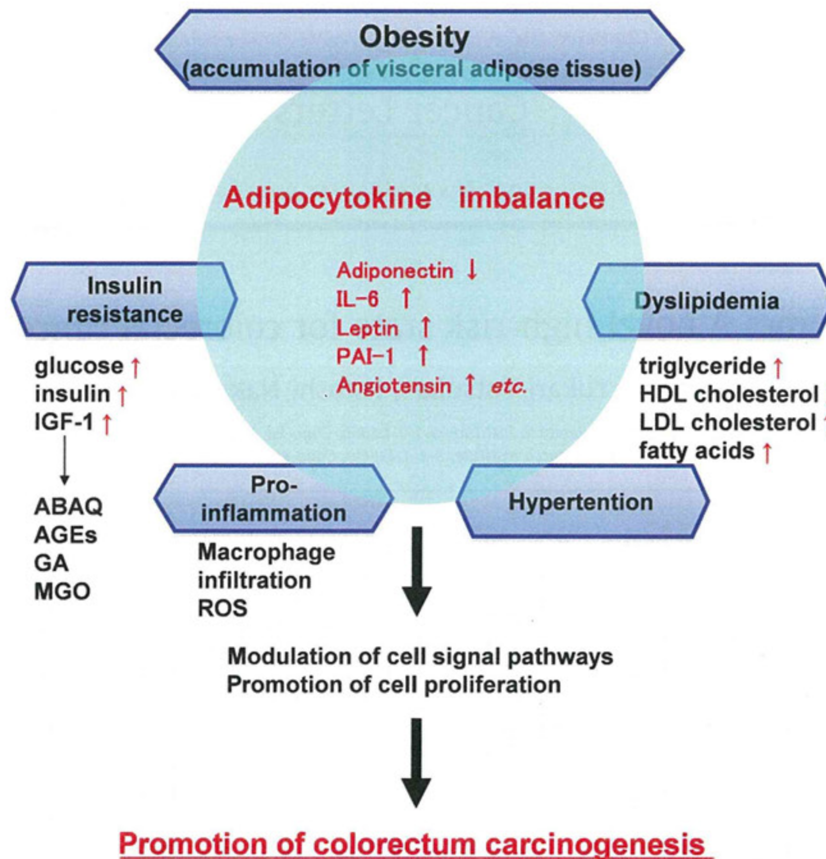

Fig. 1. Assumed relationship between metabolic syndrome and imbalance of adipocytokine production linked to colorectal cancer development. AGEs, advanced glycation end products; GA, glyceraldehydes; HDL, high-density lipoprotein; IGF-1, insulin like growth factor-1; IL-6, interleukine-6; LDL, low-dencity lipoprotein; MGO, methylglyoxal; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species.

3. Dyslipidemia

Hypertriglyceridemia is associated with an elevated risk (HR = 1.71) of colon cancer in Japanese men [14]. In the case of a precursor lesion of colorectal cancer, most epidemiological studies have consistently showed that serum TG levels are associated with their increase [15–18]. Thus, it is considered that serum TG, lipoprotein lipase (LPL), a key enzyme that catalyzes the hydrolysis of TG, could play important roles in carcinogenesis.

In animal models of human familial adenomatous polyposis (FAP), *Apc*[1309] (C57BL/6J[Apc/ApcD1309]) [19] and *Min* mice [20,21], elevated serum TG has been observed with suppression of mRNA levels for LPL in the liver and small intestine. Although no significant differences were observed between *Apc*[1309] mice and wild-type mice at 6 weeks of age, the average serum TG value in *Apc*[1309] mice at 12 weeks was found to be markedly increased almost 10-fold (~600 mg/dL) as compared to that at 6 weeks. A similar increase of TG levels (almost 400 mg/dL) was observed in *Min* mice at 15 weeks compared to the 8 weeks time point.

The anti-T2DM agent, pioglitazone, is a potent peroxisome proliferater-acitivated receptor γ (PPAR γ) ligand with a weak binding affinity for PPARα. PPAR responsive elements exist in the promoter region of the *LPL* gene, and pioglitazone has been confirmed to increase LPL mRNA levels in the liver and intestinal epithelial cells in *Apc*-deficient mice. Serum levels of TG at 12 weeks of the *Apc*[1309] mice were reduced to 44% and 50% by 100 and 200 ppm of pioglitazone treatment, respectively, with a 33% decrease in the total numbers of polyps (Table 1) [19]. *Min* mice treated with 100–1600 ppm pioglitazone for 14 weeks also showed a decrease of intestinal polyps to 63–9% of the control number [20]. Administration of 100 and 200 ppm bezafibrate, a PPAR α ligand, which also elevates LPL mRNA, to *Apc*[1309] mice reduced serum levels of TG dose dependently up to 55% ($P < 0.05$), with a reduction in the total numbers of polyps by 13% and 25% ($P < 0.05$), respectively [20]. We further treated *Min* mice with the LPL selective inducer NO-1886, demonstrated to possess no PPAR agonistic activity, unlike bezafibrate or pioglitazone [22,23], and showed 400 and 800 ppm doses to significantly decrease the total number of intestinal polyps to 48% and 42%, respectively, of the untreated control value, in mice (Table 1) [24]. Of note, NO-1886 caused a marked increase in *LPL* mRNA levels in the liver and the small intestine [24]. Based on these results, suppression of serum TG levels by increasing LPL activity is suggested to contribute to a reduction of intestinal polyp formation under *Apc*-deficient conditions, and both TG and LPL could be good molecular targets for colon cancer prevention.

Table 1
Summary of tumor suppressive effects of chemopreventive agents.

Agent	Dose (ppm)	Mouse model	Suppression to the untreated control group (%)	Refs.
Pioglitazone	200	*Apc*[1309]	67	[48]
Pioglitazone	1600	Min	9	[49]
Bezafibrate	200	*Apc*[1309]	75	[49]
NO-1886	800	Min	42	[53]
SK-216	100	Min	56	[42]

4. Diabetes

Insulin resistance is characteristic of metabolic syndrome, associated with high levels of fasting glucose, insulin and insulin-like

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Table 2
Representative dicarbonyl compounds: occurrence and consequent mutations.

Compound[a]	Mutation spectrum	Target base	Increase in diabetic patients[b]
MGO	G:C → T:A [41] G:C → C:G	G, A [34]	3.5-fold [32]
GO	G:C → T:A [42] G:C → C:G	G, A, C [35]	2.2-fold [32]
GA	Unknown	G [36]	2-fold [38][c]

[a] Abbreviations: MGO, methylglyoxal; GO, glyoxal; GA, glyceraldehyde.
[b] Compared with healthy control.
[c] Detected as amino acid adducts.

growth factor (IGF-1) in the blood. It is considered that these conditions are linked to T2DM, and a higher risk of colon cancer [25,26]. For example, hyperglycemia, hyperinsulinemia and high level of IGF-1 have been demonstrated to increase cell viability and proliferation observed in an *in vitro* setting [27].

Multiple genetic alterations in tumor-related genes have been identified in various types of cancers [28]. With obesity or under T2DM conditions, an increased level of reactive oxygen species (ROS) has been reported in multiple sites, such as blood, liver and adipose tissue. In animal experiments, Furukawa et al. demonstrated that production of ROS in adipose tissue increased body weight-dependently, and ROS production was stimulated by fatty acids *via* NADPH oxidase activation [29]. Moreover, there have been several reports of significantly elevated oxidative DNA damage in blood of T2DM patients [30]. ROS attack of nucleotide bases in DNA yields a variety of alterations and damaged nucleosides that escape repair have the capacity to introduce mutations during DNA replication [31]. Based on these findings, T2DM may contribute to induction of mutations and colon carcinogenesis *via* increased oxidative stress.

In diabetes (both type 1 and type 2) patients, glucose concentrations in blood are at high levels compared with healthy subjects all day long. It has been reported that reduced sugars, including glucose, are non-enzymatically converted into dicarbonyl compounds, such as methylglyoxal (MGO), glyceraldehyde (GA), under physiological conditions [32]. Such dicarbonyl compounds react irreversibly with amino groups of physiological components, such as protein, DNA and lipid by the Maillard reaction, to form glycation adducts or so-called Advanced Glycation End products (AGEs) [33–36]. These have been detected as amino acid- [37,38], deoxyribonucleoside (nucleobase)- [39] and phospholipid-adducts [40], in both types of diabetic patients. Glycation products of DNA are known to induce mutations in mammalian [41,42] and bacterial cells [43], such as, for example, G:C to C:G and G:C to T:A transversions in the *supF* gene in simian kidney cells associated with N^2-(1-carboxyethyl)-2′-deoxyguanosine produced by the reaction of 2′-deoxyguanosine with MGO (Table 2) [41].

Furthermore, we discovered a novel Maillard reaction product formed from L-tryptophan and glucose, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-de]quinolin-7-one, ABAQ, showing mutagenicity toward various Salmonella strains in the presence of S9 mix [44]. Because of a consistent increase in blood glucose levels under T2DM conditions its production might be enhanced in T2DM individuals. We are now investigating the presence of ABAQ *in vivo* using urine samples collected from DM rat models and DM patients.

5. ROS and inflammation

As mentioned in the previous section, DNA damage induced by ROS is likely to play an important role in carcinogenesis, and obesity increases ROS levels in adipose tissue and blood. In MS patients, abdominal fat tissue attracts macrophages by induction of several chemokines, such as monocyte chemoattractant protein-1 (MCP-1), and forms crown-like structures [13]. Activated macrophages are known to produce ROS and inflammatory cytokines, and thus obesity is now considered to be a pro-inflammatory condition.

ROS directly effects cell proliferation and apoptosis through modification of gene expression followed by activation of transcription factors, such as members of the AP-1 and NF-κB pathways [45]. Activation of AP-1 results in induction of cyclin D1, which in turn promotes entry into mitosis, while NF-κB induces inflammatory cytokines and growth factors, which enhance the inflammation status. A recent report demonstrated that ROS and prostaglandin E_2, which play important roles in inflammation in colon cancer tissue, modulate DNA methylation patterns [46], control gene expression and may thereby contribute to the multistage carcinogenesis process.

Lipid peroxidation mediated by ROS has also been recognized to play a key role in carcinogenesis, for example by activation of transcriptional factors [47]. Free and ester forms of unsaturated fatty acids and cholesterol are easily attacked by ROS, and are oxidized by a chain mechanism. Colorectal cancer risk in a case-control study showed positive relationships with erythrocyte membrane compositions of palmitic and oleic acids, but negative links with linoleic ($18{:}2n-6$) and arachidonic acids [48]. *Min* mice with a hyperlipidemic state demonstrate elevated values for palmitic and oleic acids in plasma and erythrocyte membranes, and higher plasma levels of linoleic acid, indicating these to be important in intestinal polyp formation [49]. In addition, detailed analysis of serum lipids in *Min* mice using reverse-phase liquid chromatography/electrospray ionization mass spectrometry revealed that hydroperoxidizable TG precursors containing linoleic acid were deposited at the tips of villi with aging, and these hydroperoxidized TG were also increased in serum [50]. Such increases of oxidizable TG precursors in serum and small intestinal mucosa could be reduced by treatment with pitavastatin, a novel lipophilic statin [50], with concomitant reduction of intestinal polyp development [51]. These results indicated that quantitative and qualitative lipid changes affect the course of intestinal polyp formation in *Min* mice, and support the idea that oxidative stress might lead to the development of colon cancer.

6. Adipocytokine imbalance

Obese mice, such as the KK-A^y strain, are highly susceptible to induction of colon premalignant lesions, aberrant crypt foci (ACF), and development of colorectal carcinomas on exposure to azoxymethane (AOM) [52]. KK-A^y mice were established by cross-mating KK, T2DM model mice, with C57BL/6J-A^y mice [53,54], which carry the *Agouti* gene (*Ay*), and feature severe hyperphagia, hyperinsulinemia and dyslipidemia. C57BL/6J mice are generally used as non-obese controls [55,56]. The numbers of AOM-induced ACF per mouse and tumor per mouse developing in KK-A^y mice (almost 70 and 8, respectively) also appeared higher than in other obese mice, *ob*/*ob* or *db*/*db* mice, not possessing intact leptin or leptin receptors [52]. In addition to severe hyperinsulinemia and hypertriglyceridemia, the KK-A^y mouse exhibits abdominal obesity, and resultant elevation of serum adipocytokines, such as interleukin-6 (IL-6), leptin and plasminogen activator inhibitor-1 (Pai-1) compared with values for lean C57BL/6J mice. In the visceral fat tissue, significant over-expression of pro-inflammatory adipocytokine mRNAs such as for IL-6, leptin, MCP-1, Pai-1 and tumor necrosis factor (TNF)-α were confirmed; in contrast, that for adiponectin was decreased. The consequent adipocytokine imbalance is suggested to be involved in the promotion of colon carcinogenesis.

Our recent findings for two adipocytokines, adiponectin and PAI-1, and their relevance to intestinal tumorigenesis provide

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further support for this idea. Adiponectin is a 30 kDa protein, present at high levels in plasma (range, 3–30 μg/mL), inversely correlated with the BMI [57,58]. Moreover, low plasma adiponectin levels are associated with insulin resistance, high serum glucose levels, and coronary artery disease [59–61] as well as with increased risk of various cancers, including colorectal cancer [62,63].

Thus, we investigated how low levels of adiponectin might be involved in colon carcinogenesis using *Min* mice. Adiponectin-deficient *Min* mice of both sexes exhibited a 2- or 3-fold increase in the total number of intestinal polyps compared to those of adiponectin-wild *Min* mice at the ages of 9 and 12 weeks [64]. In addition, adiponectin-deficient C57BL/6 J mice treated with AOM showed increased incidences and multiplicities of colorectal adenomas and adenocarcinomas. AMPKα activation through the adiponectin receptor, AdipoR1, inhibits Akt activation followed by mammalian target of rapamycin (mTOR) inactivation [63,65], presumably through abolished signaling from AdipoR1, enhancing cell growth and tumor development.

In primary cell culture, fibroblasts from adiponectin-deficient C57BL/6J mice over-express Bcl-2 compared to those of adiponectin-wild C57BL/6J mice [64,66]. Adiponectin deficiency also affects production of other adipocytokines. Adiponectin-deficient *Min* mice exhibit an increase in serum Pai-1 levels with adiponectin gene dosage [64], in agreement with the tendency for elevation observed with adiponectin-deficiency at the age of 55 weeks in C57BL/6J mice [64]. Treatment with an AMPK activator, metformin, was also found to lower amounts of hepatic Pai-1 mRNA in *Min* mice, in line with earlier reports [67,68]. Thus, it is conceivable that Pai-1 levels are generally depressed by adiponectin.

PAI-1, a serine protease inhibitor (serpin) protein, which inhibits the function of tissue plasminogen activator and urokinase-type plasminogen activator by direct binding, demonstrates increased levels with obesity and the metabolic syndrome. PAI-1 can be induced by TG, very low-density lipoprotein, transforming growth factor β (TGFβ) and various growth factors [69–72]. There is also evidence that the serum PAI-1 concentration may be a reliable indicator of a poor prognosis in colorectal cancer [73–79].

In our experiments, serum Pai-1 levels in the 15-week-old male *Min* mice could be shown to be 8 times higher than in wild-type mice, while hepatic Pai-1 mRNA levels were 11-fold increased. Administration of a PAI-1 inhibitor, SK-216, at 25, 50 and 100 ppm doses in the diet for 9 weeks reduced serum Pai-1 levels and hepatic Pai-1 mRNA levels of *Min* mice compared to the wild-type levels. Moreover, *Min* mice receiving SK-216 at 50 and 100 ppm exhibited significantly reduced total numbers of intestinal polyps, to 64% and 56% of the untreated group value, respectively (Table 1). Serum TG levels were also decreased by 43% at the dose of 100 ppm [80]. These results indicate that Pai-1 induction associated with hypertriglyceridemia may contribute to intestinal polyp formation with *Apc* deficiency. Thus, adiponectin and PAI-1 are considered to be key molecules involved in obesity-associated cancers.

7. Angiotensin-renin system

Activation of the renin–angiotensin system (RAS) has been implicated in the etiology of hypertension, obesity and metabolic syndrome [81]. Angiotensin II (Ang II) elicits its biological activities through two well-defined receptors, type 1 (AT1R) and type 2 (AT2R), to elevate blood pressure, and agents that block AT1R, angiotensin-converting enzyme (ACE) activity and calcium influx block such elevation. It is not clear whether hypertension affects neoplasia, but accumulating evidence suggests that activation of RAS is involved in development of various cancers, such as in the breasts, colorectum, kidneys and lungs [82].

AT1R expressed in a wide variety of tissues activates downstream MAPK and STAT signal pathways [83]. Thus, Ang II- AT1R -mediated signals induce expression of protooncogenes such as *c-fos*, *c-myc* and *c-jun*, and thereby promote cell proliferation [84,85]. In animal models, the AT1R blockers (ARBs) captopril and telmisartan have been shown to suppress the development of ACF and more advanced preneoplastic lesions, β-catenin accumulated crypts, in male *db/db* obese mice [86]. Moreover, captopril or telmisartan decreased the mRNA levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 in the white adipose tissue of AOM-treated *db/db* mice.

ACE inhibitors block the formation of Ang II and have been demonstrated to attenuate tumor growth in experimental animals [87–90] and to reduce the risk of several human cancers [91]. AT2R expression is low in adult tissues, although detectable in heart, kidneys, pancreas, adrenal glands, uterus, ovaries and brain [92], and AT2R-mediated signals counteract AT1R-mediated actions [82,93]. It is interesting that down-regulation of cytochrome P450 2E1 expression in the liver of AT2R-null mice resulted in an increase in the number of AOM-induced colon tumors [94]. Calcium blockers are also primarily utilized to control peripheral blood pressure. Some of them, such as verapamil, are known to inhibit p-glycoprotein (encoded by *Mdr1a* gene), and the number of polyps in *Min* mice undergoing verapamil administration was significantly decreased [95].

The available findings with anti-hypertensive agents appear clinically significant because these drugs are widely used for patients with hypertension who frequently are obese. Inhibition of RAS might be an effective strategy for prevention of colon cancer.

8. Future aspects

Understanding the molecules involved in obesity-associated cancer may provide clues to cancer preventive strategies in obese individuals. There appears to be a convergence of effects of dyslipidemia, insulin resistance, inflammation and adipocytokines. Targeting related molecules and signaling pathways may therefore be a good preventive and/or therapeutic approach. Some studies suggest that weight loss after gastric bypass surgery is associated with a reduced incidence of cancer [96]. Its ability to reduce the risk of obesity-associated cancers needs to be confirmed in future investigations. In addition, factors reducing the risk of obesity-associated cancers with physical activity require clarification as a high priority.

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Induction of glandular stomach cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-nitrosoindole-3-acetonitrile

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Helicobacter pylori (H. pylori) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/10^8 nucleotides in total), detected in DNA samples from the glandular stomach by ^{32}P-postlabeling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for 3 weeks, induced well—and moderately—differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under *H. pylori* infection at 54–104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection.

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia, and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3–5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6,7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8–10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12–14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19,20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde as mutagen precursors. 1-Nitrosoindole-3-acetonitrile (NIAN), an *N*-nitroso-substituted compound formed by treatment of indole-3-

Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile

Abbreviations: DMSO: dimethyl sulfoxide; H&E: hematoxylin and eosin; *H. pylori*: *Helicobacter pylori;* MG: Mongolian gerbil; MNNG: *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; MNU: *N*-methyl-*N*-nitrosourea; NIAN: 1-nitrosoindole-3-acetonitrile.

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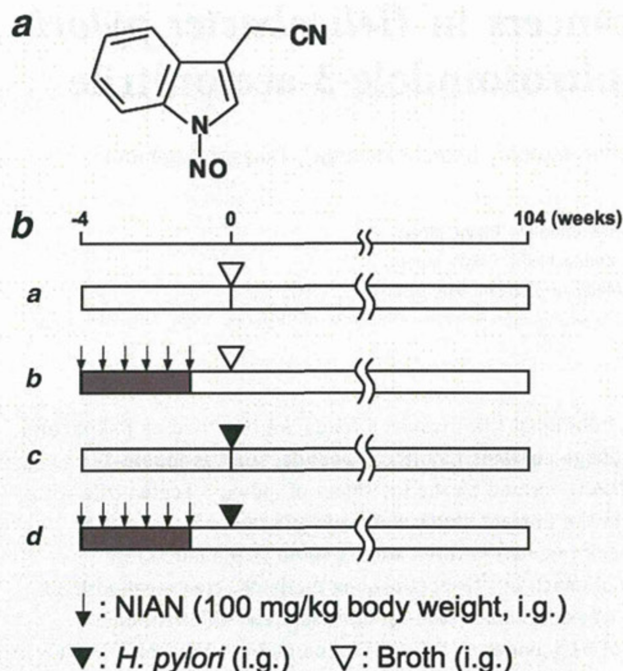

Figure 1. Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after the final administration, the animals were inoculated with *H. pylori* (ATCC 43504) (groups C and D) or sterilized broth (groups A and B).

acetonitrile with nitrite under acidic conditions, is a direct-acting mutagen in *S. typhimurium* and Chinese hamster lung cells,[20–22] and it is confirmed to form DNA adducts and to induce DNA single-strand scission in the rat glandular stomach.[23,24] Therefore, NIAN could play some role in gastric cancer development, as in the case of the well-known direct-acting mutagens, MNNG and MNU, in animal experiments.[16,17,25]

The Mongolian gerbil (MG) is reported to be susceptible to colonization by *H. pylori*, and *H. pylori* infection greatly enhances MNNG or MNU-induced gastric carcinogenesis in MGs.[26,27] Therefore, the MG is considered to be a useful animal model for evaluating the gastric cancer risk of direct-acting *N*-nitroso compounds, with or without *H. pylori* infection.

Chinese cabbage, containing nitrate and indole compounds, is commonly consumed in East Asian countries, including Japan, Korea and China, in which gastric cancer mortality is very high. In the present study, DNA adducts were detected with NIAN treatment in the glandular stomach of MGs, and the carcinogenicity of NIAN for gastric cancer *in vivo* was examined. The results clearly demonstrated that gastric cancer developed with a combination of NIAN administration and *H. pylori* infection in MGs. Possible involvement of indole compounds and nitrate derived from various foodstuffs, including Chinese cabbage, in gastric cancer development in humans is discussed.

Material and Methods

Materials

Indole-3-acetonitrile was purchased from Tokyo Food Techno (Tokyo, Japan), sodium nitrite from Wako Pure Chemical Industries (Osaka, Japan) and ammonium sulfamate from Kanto Chemical (Tokyo, Japan). Brucella broth was obtained from Becton Dickinson (Cockeysville, MD) and horse serum from Nippon Bio-Supply (Tokyo, Japan).

Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1*a*. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hr at room temperature in the dark, as previously reported.[21] Nitrosation was stopped by addition of ammonium sulfamate at a final concentration of 50 mM. The reaction solution was filtered and the residue was washed with deionized water, then with *n*-hexane. The residual paste was dried and stored at −80°C until use. The preparation was >93% pure as judged by its UV absorbance on HPLC.

Bacterial culture

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured in brucella broth supplemented with 10% heat-inactivated horse serum for 24 hr at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), as previously described.[28]

Animal treatment

Specific pathogen-free male, 6-week-old MGs (MGS/Sea, Kyudo, Fukuoka, Japan) were housed in a biohazard room, air-conditioned at 24°C ± 2°C and 55% humidity, on a 12 hr light–dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and water.

To analyze the formation of DNA adducts in the glandular stomach of MGs by NIAN treatment, NIAN was dissolved in 50% dimethyl sulfoxide (DMSO), and administered to three MGs by gavage of 0.5 ml solution, two times a week at a level of 100 mg/kg body weight. Two further MGs served as a control group receiving the solvent alone (0.5 ml). At 8 hr after administration of NIAN, both groups of animals were sacrificed under ether anesthesia, and their stomachs were resected and stored at −80°C until use. DNA was extracted by a standard procedure with enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

The protocol for long-term gastric carcinogenicity in MGs treated with NIAN + *H. pylori* infection is illustrated in Figure 1*b*. The animals were randomly divided into four groups (groups A–D). Groups A and C were given 50% DMSO without NIAN (0.5 ml) whereas groups B and D were orally administrated NIAN (0.5 ml, 100 mg/kg body weight) dissolved in 50% DMSO by gavage, two times a week for 3 weeks. At one week after the last administration, the