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Anti-tumor properties of orally administered glucosamine and *N*-acetyl-D-glucosamine oligomers in a mouse model

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ABSTRACT

The current study evaluated the anti-tumor activities of *N*-acetyl-D-glucosamine oligomer (NACOS) and glucosamine oligomer (COS) after their oral administration in a tumor (colon 26)-bearing mouse model. Compared to the control group, NACOS and COS groups showed significantly suppressed tumor growth, and apparent, marked apoptosis in tumor tissues. Furthermore, serum interleukin-12p70 and interferon-γ levels significantly increased in the NACOS and COS groups compared to the corresponding levels in the control group. Collectively, the results indicate the oral administration of NACOS and COS could enhance innate immunity. Results of experiments in Myd-88 knockout mice revealed that the apparent effects were related to both Myd-88-dependent and Myd-88-independent pathways. The data indicated that oral administration of NACOS and COS produced anti-tumor effects through the induction of apoptosis and stimulation of the immune system, which suggests that NACOS and COS are candidate anti-tumor functional foods.

1. Introduction

Recently, naturally occurring compounds regarded as chemo preventive agents have garnered significant interest as treatments for the prevention and/or intervention of neoplastic progression before the occurrence of invasive malignant disease (Muzzarelli, 1997, 2010; Chen et al., 2005, 2006a,b). Previous reports have indicated that *N*-acetyl-D-glucosamine oligomer (NACOS) and glucosamine oligomer (COS) possess anti-tumor properties (Harish-Prashanth & Tharanathan, 2005; Huang, Mendis, Rajapakse, & Kim, 2005; Shen, Chen, Chan Jeng, & Wang, 2009; Suzuki et al., 1986; Tokoro, Tatewaki, Mikami, Suzuki, & Suzuki, 1989; Wang et al., 2008). Furthermore, it was revealed that the tumor inhibitory effects of NACOS and COS are potentially related to their ability to induce lymphocyte cytokines thorough increased T-cell proliferation. Essentially, the anti-tumor mechanisms of NACOS and COS are enhanced by acquired immunity via acceleration of T-cell differentiation, which in turn increases cytotoxicity and maintains T-cell activity (Suzuki et al., 1986). However, most reports that describe the *in vivo* anti-tumor effects of NACOS and COS have evaluated the intraperitoneal or intravenous administration of NACOS and COS, while few reports have investigated the beneficial effects of oral administration of NACOS and COS in mouse or rat tumor models. To our knowledge, no reports have described differences in the effects of oral administration of NACOS and COS on tumor growth in a tumor-bearing mouse model. In the present study, we examine the effects of the oral administration of GlcNAc oligomers and GlcN oligomers on tumor growth and survival time using a colon-26 tumor-bearing mouse model.

2. Materials and methods

2.1. Animals

BALB/c mice (4-week-old, females) were purchased from CLEA Japan (Osaka, Japan). Myeloid differentiation primary response gene-88 (Myd-88) knockout mice (BALB/c background, 7–9-week-old, females) were purchased from Oriental BioService, Inc., (Kyoto, Japan). All mice were maintained under conventional conditions.

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The Animal Research Committee of Tottori University approved the care and use of the mice in this study.

2.2. Reagents

NACOS and COS, derived from crab shells, were provided by Koyo Chemical Co. Ltd., (Tokyo, Japan). The NACOS was comprised of monomer (24.3%), dimer (16.4%), trimer (16.2%), tetramer (14.9%), pentamer (13.0%), hexomer (7.6%), and heptamer (1.4%). The COS was comprised of dimer (8.9%), trimer (19.6%), tetramer (23.7%), pentamer (25.4%), hexomer (8.4%), heptamer (3.1%), and octomer (1.7%).

2.3. Preparation of the tumor-bearing mouse model

Experimental tumor models were prepared according to our previously described methods (Azuma et al., 2012). In brief, colon-26 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin in a 37 °C incubator under 5% CO_2 with naturally evaporating distilled water. For the injection of colon-26 cells, mice were anesthetized by inhalation of 3% isoflurane (Intervet, Inc., Tokyo, Japan). A total of 1×10^5 colon-26 cells (1×10^6 cells/mL) were subcutaneously injected into the dorsal regions of the mice.

2.4. Tumor growth study

Fifty-six BALB/c mice were randomized into seven groups: the control group ($n = 8$), the NACOS 1% group ($n = 8$), the COS 1% group ($n = 8$), the NACOS 2% group ($n = 8$), the COS 2% group ($n = 8$), the NACOS 4% group ($n = 8$) and the COS 4% group ($n = 8$). Excepting the control group, each group received a normal powdered diet (CE-2; CREA Japan, Osaka, Japan) with 1, 2, or 4% (w/w) NACOS or COS. The control group was fed a normal powdered diet. Before transplantation of the tumor cells, the mice were bred for 28 days, and were fed a powdered diet with or without each percentage (w/w) of NACOS or COS. After breeding for 28 days, tumor cells (0.1 mL/head; 1×10^6 cells/mL) were injected into the dorsal region of each mouse (day 0). Each mouse was also fed the powdered diet with or without NACOS or COS for 14 days after the injections of tumor cells. All mice were subsequently euthanized by inhalation of 5% isoflurane followed by cervical dislocation, and serum samples were obtained on day 14. Tumor samples were taken, and the tumor volumes (mm^3) calculated by measuring the mediastinum, transverse line, and depth of each tumor. Following collection, tumor tissues were fixed in 10% buffered formalin.

2.5. Histopathological evaluation

In the control, NACOS 4%, and COS 4% groups, each tumor sample was sectioned for histopathological evaluation (5 μm), stained with hematoxylin-eosin (HE), and examined microscopically. The apoptotic rate of the tumor tissues was also examined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphatate biotin nick end labeling (TUNEL) staining. TUNEL staining was performed according to our previous methods (Takagi et al., 2014), tissue sections (3 μm) on glass slides were deparaffinized, washed with ethanol and water, and soaked in diluted water. TUNEL staining was performed using the In situ Apoptosis Detection kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Each section was examined microscopically, and the number of TUNEL positive cell in each tumor sample was calculated. The calculation was performed in 10 fields at 400× magnification by using tumor sections from four mice in each group. The mean score of 40 fields was considered to be the number of cell divisions in each group.

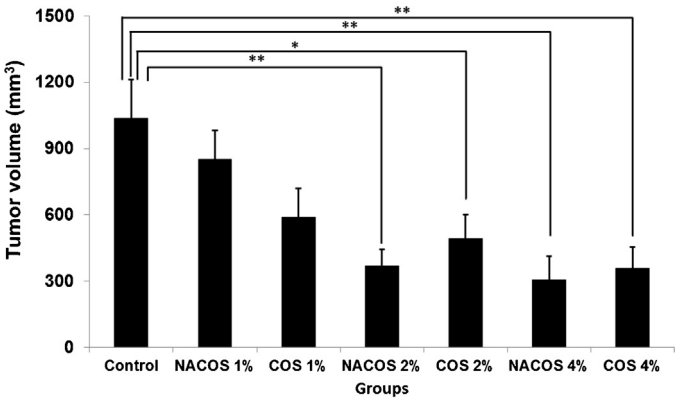

Fig. 1. Effects of oral administration of NACOS and COS on tumor growth. Data represent the mean ± standard error. For the NACOS 1%, COS 1%, NACOS 2%, COS 2%, NACOS 4%, and COS 4% groups, $n = 8$. For the control group, $n = 10$. ** indicates $p < 0.01$ and * indicates $p < 0.05$ compared to the control group (Tukey–Kramer test). NACOS: *N*-acetyl-D-glucosamine oligomer; COS: glucosamine oligomer.

2.6. Measurements of serum cytokine levels

The serum interleukin (IL)-12p70, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) levels were quantified using a murine sandwich enzyme-linked immunosorbent assay (ELISA; Quantikine®, R&D Systems Inc., Minneapolis, USA) according to the manufacturer's protocols.

2.7. MyD-88 knockout mouse study

Nine Myd-88 knockout mice were divided into three groups ($n = 3$), the control/Myd-88 ($n = 3$), the NACOS 4%/Myd-88 ($n = 3$), and the COS 4%/Myd-88 groups ($n = 3$). The NACOS 4%/Myd-88 and COS 4%/Myd-88 groups received the normal powdered diet (CE-2; CREA Japan, Osaka, Japan) with a supplement of 4% (w/w) NACOS or COS, while the control/Myd-88 group was fed a normal powdered diet. Before transplantation of the tumor tissues, the mice were bred for 28 days and were fed a powdered diet with or without NACOS or COS. After breeding for 28 days, each mouse underwent the injection of tumor cells (0.1 mL/head; 1×10^6 cells/mL) into the dorsal region (day 0). Each mouse was fed the powdered diet with or without NACOS or COS for 14 days after the injections of tumor cells. All mice were subsequently euthanized by inhalation of 5% isoflurane followed by cervical dislocation on day 14. Following euthanasia, tumor tissues were collected, and the tumor volumes (mm^3) were calculated by measuring the mediastinum, transverse line, and depth of each tumor.

2.8. Statistical analysis

The statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test, and data were expressed as the mean ± standard error. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of oral administration of NACOS and COS on tumor growth

The results of tumor growth are shown in Fig. 1. In the NACOS 1% group, the tumor volume ($852.5 \pm 129.3\ mm^3$)

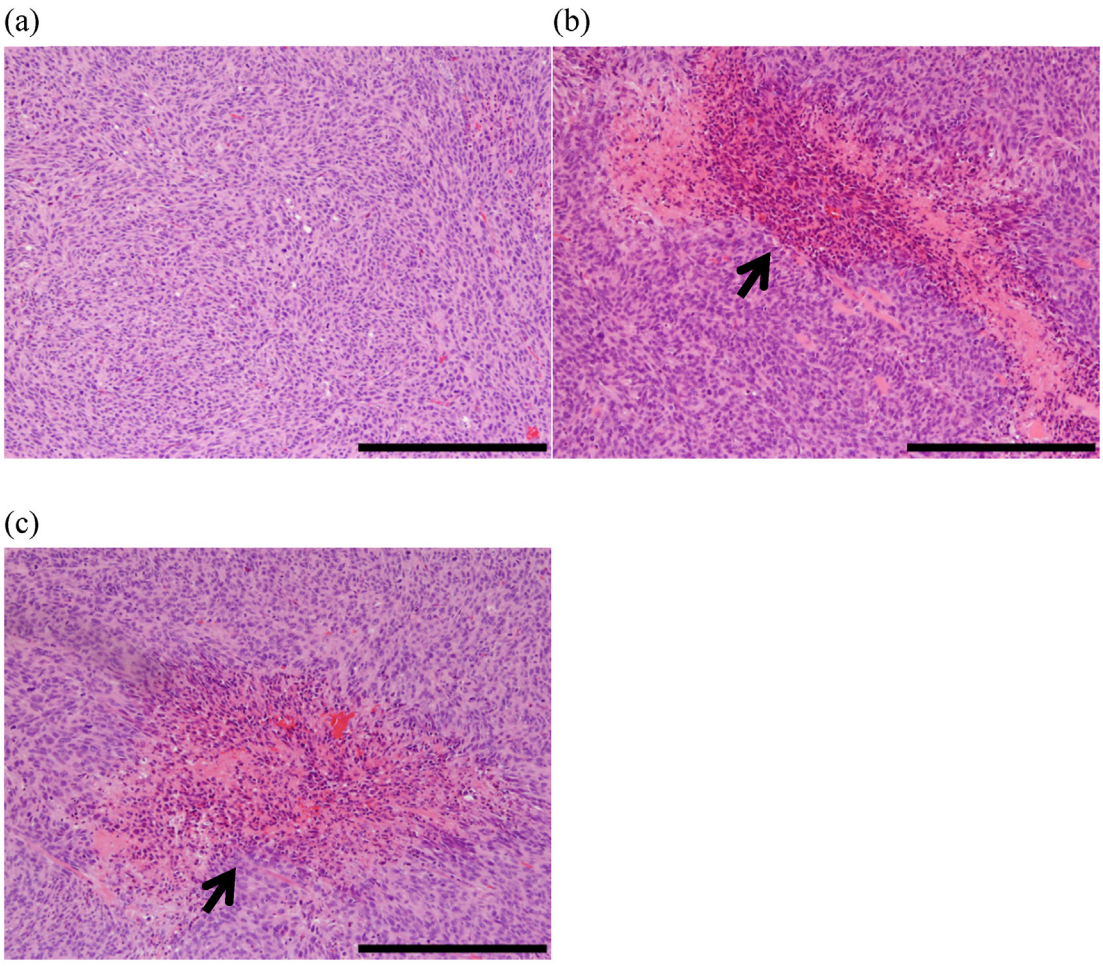

Fig. 2. Effects of NACOS and COS on histopathological changes of tumor tissue. All images are from one of eight to ten mice in the control (a), NACOS (b), and COS (c) groups. The necrosis regions are shown by arrows. Bar = 200 μm. NACOS: *N*-acetyl-D-glucosamine oligomer; COS: glucosamine oligomer.

was slightly lower compared with that of the control group ($1037.0 \pm 175.2\ mm^3$). Similarly, the tumor volume in the COS 1% group, ($589.4 \pm 129.6\ mm^3$) was less than that of the control group, but the differences between the tumor volumes in the experimental and control groups were not statistically significant. In the COS 2% group, the tumor volume ($492.8 \pm 109.8\ mm^3$) was significantly lower than the control group tumor volume ($p < 0.05$). Likewise, the tumor volumes calculated in the NACOS 2% ($369.8 \pm 74.0\ mm^3$), NACOS 4% ($307.5 \pm 107.0\ mm^3$), and COS 4% ($358.3 \pm 98.5\ mm^3$) groups were significantly lower than tumor volumes observed in the control group ($p < 0.01$).

The results of the effects of oral administrations of NACOS or COS determined by histopathological changes in tumor tissue using hematoxylin-eosin (HE) staining are shown in Fig. 2. In the control group, active cell proliferation was frequently observed (Fig. 2a), while cell proliferation was markedly suppressed in the NACOS and COS groups (Fig. 2b and c). Additionally, necrotic cells were widely observed in the NACOS and COS groups (shown by arrows in Fig. 2b and c).

3.2. Effects of oral administrations of NACOS and COS on apoptosis in tumor tissues

We also examined the effects of oral administrations of NACOS or COS on apoptosis using TUNEL staining. The numbers of TUNEL-positive cells in the control, NACOS 4%, and COS 4% groups are shown in Fig. 3. The number of TUNEL-positive cells in the NACOS 4% group (70.1 ± 13.0 cells/field) were significantly increased ($p < 0.05$) compared to the number of TUNEL-positive cells observed in the control group (28.8 ± 6.3 cells/field). In the COS 4% group, the number of TUNEL-positive cells (61.25 ± 13.7 cells/field) was increased compared with that of the control group, but the difference was not significant.

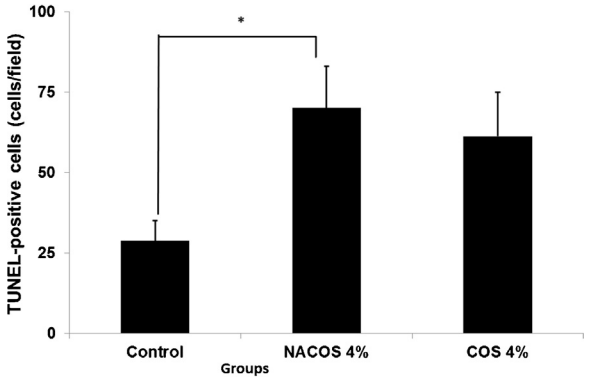

Fig. 3. Effects of oral administration of NACOS and COS on the number of the TUNEL-positive cells in tumor tissues. Data represent the mean ± standard error. * $p < 0.05$ compared to the control group (Tukey–Kramer test). NACOS: *N*-acetyl-D-glucosamine oligomer; COS: glucosamine oligomer.

Table 1
Effects of oral administration of NACOS and COS on serum cytokines levels.

	Control	NACOS 4%	COS 4%
IFN-γ (pg/mL)	0.6 ± 0.2	8.3 ± 0.5**	8.4 ± 0.3**
IL-12 (pg/mL)	11.2 ± 1.3	25.3 ± 3.5**	23.5 ± 3.0**
TNF-α (pg/mL)	13.9 ± 1.6	13.4 ± 1.3	12.7 ± 0.8

Mean ± standard error; $n = 6$ in each group.
** $p < 0.01$ compared to the control group (Tukey–Kramer test).

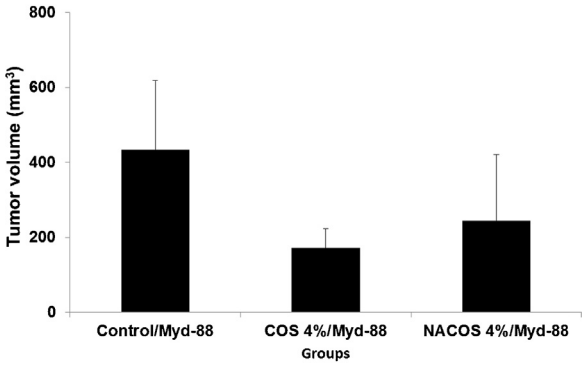

Fig. 4. Effects of oral administration of NACOS and COS on the tumor growth in Myd-88 knockout mice. Data represent the mean ±standard error (Tukey–Kramer test). NACOS: *N*-acetyl-D-glucosamine oligomer; COS: glucosamine oligomer.

3.3. Effects of oral administrations of NACOS and COS on serum cytokines levels

Serum cytokine levels are shown in Table 1. In the NACOS 4% and COS 4% groups, serum IFN-γ and IL-12p70 levels were significantly increased compared with levels in the control group ($p < 0.01$). However, there were no differences in serum TNF-α levels among the control, NACOS 4% and COS 4% groups.

3.4. Effects of oral administration of NACOS or COS on tumor growth in Myd-88 knockout mice

The results are shown in Fig. 4. The tumor volumes in the NACOS ($245 \pm 176\,mm^3$) and COS ($172 \pm 51\,mm^3$) groups were lower when compared to the tumor volumes of the control group ($434 \pm 184\,mm^3$), but the differences were not statistically significant.

4. Discussion

The results of the current study indicated that oral administrations of NACOS and COS equally suppressed tumor growth in the colon-26 bearing mouse model. In the NACOS 4% and COS 4% groups, the numbers of TUNEL positive cells in the tumor tissues were significantly increased compared to the control group. Previously, Kim et al. (2012) reported that COS possessed elevated anti-cancer activities, and induced apoptosis in human myeloid leukemia HL-60 cells. Combined with our results, the existing evidence indicates that one of the anti-tumor mechanisms of NACOS and COS is the ability of the compounds to induce apoptosis.

Previous reports indicated that the tumor inhibitory effect of COS was most likely related to the ability of COS to induce lymphocyte cytokines by increasing T-cell proliferation. Mainly, adaptive immunity enhanced the antitumor mechanism of COS by accelerating T-cell differentiation, which in turn increased cytotoxicity and maintained T-cell activity (Suzuki et al., 1986). Previous reports have demonstrated that the antitumor effects of various low-molecular weight chitosan, such as water-soluble 21- or 46-kDa molecules with low viscosity, decreased tumor growth and final tumor weight in sarcoma 180-bearing mice, due to an increase in natural killer (NK) cell activity (Kobayashi, Watanabe, Suzuki, & Suzuki, 1990; Maeda & Kimura, 2004). A separate report indicated the low-molecular-weight water-soluble chitosan and COS might be useful in preventing tumor growth, by serving as immunomodulator to enhance cytotoxic activity against tumors (Kimura & Okuda, 1999). In addition, it was reported that in cases of skin disease, the low-molecular-weight water-soluble chitosan and COS activated macrophages via the production of cytokines such as IFN-γ and IL-12 from the intraepithelial lymphocytes (Zhou et al., 2010). To the best of our knowledge, no prior reports have described the relationship between oral administration of NACOS or COS and cytokine production or activity. However, our data strongly suggest that oral administration of NACOS and COS stimulated the production of IFN-γ and IL-12. However, further studies focusing on NK cell activities induced by oral administration of NACOS or COS are required.

The stimulation of innate immunity is essential for the activation of adaptive immunity (Arancibia et al., 2007). In particular, Toll-like receptors (TLR) on the surface of intracellular organelles recognize specific structures on bacteria, viruses, and fungi (Akira, Takeda, & Kaisho, 2001). In fact, chitin activates TLR-2 and Myd-88 in a novel IL-17A/IL-17AR-based innate immunity pathway (Da Silva, Hartl Liu, Lee, & Elias, 2009). Adapter molecules such as Myd-88 and the TIR-domain-containing adapter-inducing interferon-β (TRIF) play important roles in inducing the production of cytokines via TLRs (Akira, 2000; Akira, Uematsu, & Takeuchi, 2006). TLR-4 stimulates cytokine production via both Myd-88 and TRIF signaling pathways (Akira et al., 2006). In the present study, we used Myd-88 knockout mice to demonstrate that tumor growth was suppressed by oral administration of NACOS and COS; however, the differences were not statistically significant. Conversely, in our studies involving normal mice, tumor growth suppression following oral administration of NACOS or COS was significant. The results suggest that the *in vivo* anti-tumor effects of NACOS and COS stem not only from Myd-88 dependent pathways, but also from Myd-88 independent pathways.

In vitro studies have indicated that p21 was up regulated, while proliferating cell nuclear antigen, cyclin A, and cdk-2 were down-regulated following the addition of COS to the tumor cell line. However, there has been no report that described the effects of oral NACOS and COS administration on cell cycles. To gain a further understanding of the detailed mechanisms involved, additional studies are required.

5. Conclusion

In conclusion, tumor growth was equally suppressed by oral administration of NACOS or COS. Collectively, the data indicate that oral administration of NACOS and COS have anti-tumor effects through the induction of apoptosis and the stimulation of the immune system, which suggests that NACOS and COS are potentially beneficial when administered as a functional anti-tumor food.

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