

Histochemical study of intestinal mucins after administration of silver nanoparticles in Sprague–Dawley rats

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Abstract To investigate the effects of silver nanoparticles on the histological structure and properties of the mucosubstances in the intestinal mucosa, Sprague–Dawley rats were divided into four groups (10 rats in each group): vehicle control, low-dose group (30 mg/kg), middle-dose group (300 mg/kg), and high-dose group (1,000 mg/kg), and administered silver nanoparticles (60 nm) for 28 days, following OECD test guideline 407 and using GLP. The control sections contained no silver nanoparticles; however, the treated samples showed luminal and surface particles and the tissue also contained silver nanoparticles. A dose-dependent increased accumulation of silver nanoparticles was observed in the lamina propria in both the small and large intestine, and also in the tip of the upper villi in the ileum and protruding surface of the fold in the colon. The silver nanoparticle-treated rats exhibited higher numbers of goblet cells that had released their mucus granules than the controls, resulting in more mucus materials in the crypt lumen and ileal lumen. Moreover, cell shedding at the tip of the villi was frequent. Lower amounts

of neutral and acidic mucins were found in the goblet cells in the silver nanoparticle-treated rats, plus the amount of sialomucins was increased, while the amount of sulfomucins was decreased. In particular, in the colon of the silver nanoparticle-treated rats, sialylated mucins were detected in the lamina propria, the connective tissue under the epithelia. Therefore, the present results suggest that silver nanoparticles induce the discharge of mucus granules and an abnormal mucus composition in the goblet cells in the intestines.

Keywords Silver nanoparticles · Mucins · Intestines · Histochemistry · Intestinal goblet cells

Introduction

Various studies have already established the strong antibacterial property of silver nanoparticles (Feng et al. 1999; Sondi and Salopek-Sondi 2004; Morones et al. 2005; Li et al. 2005; Percival et al. 2005; Li et al. 2006; Kim et al. 2007). The extremely small size of nanoparticles means their surface area is large relative to their volume and their properties are enhanced when compared to the bulk material. In the case of silver nanoparticles, this allows them to interact easily with other particles and increases their antibacterial efficiency. Silver nanoparticles kill all types of fungal infections, bacteria, and viruses, including antibiotic-resistant strains. Meanwhile, the strong toxicity exhibited by various chemical forms of silver toward a wide range of microorganisms is also widely known (Liau et al. 1997; Gupta and Silver 1998; Nomiya et al. 2004).

The U.S. Agency for Toxic Substances and Disease Registry (ATSDR) previously published a review of the toxicology of silver nitrate, silver chloride, silver sulfide,

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and silver oxide, in which they stated that the health effects resulting from short- and long-term exposure to air containing specific levels of silver are not known (ATSDR 2005). Nonetheless, a previous study by the current authors on the 28-day oral toxicity of silver nanoparticles (60 nm) at doses of 30 mg/kg, 300 mg/kg, and 1,000 mg/kg, following OECD test guideline 407 and good laboratory practices (GLP), indicated that exposure to over 300 mg of silver nanoparticles could result in slight liver damage, plus a gender-related difference was noted in the accumulation of silver in the kidneys, with a twofold higher accumulation in the female kidneys when compared with the male kidneys (Kim et al. 2008).

It has already been shown that silver nanoparticles can enter the human body through several ports, and there have been several excellent reviews on the intestinal uptake of particles (Florence and Hussain 2001; Hussain et al. 2001). In this context, mucins are large glycoproteins secreted by mucous cells in the intestinal epithelium that act as a medium for lubrication, transport between the luminal contents and the epithelial cells, and protection against mechanical damage and penetration by microorganisms and bacterial toxins (Allen et al. 1976; Neutra and Forstner 1987; Forstner et al. 1995; Belly et al. 1999). Mucins also serve as a protective barrier against acid and pepsin digestion (Kemper and Specian 1991).

Accordingly, this study investigated the changes in the histological structures and properties of mucins to assess the effects of oral exposure to silver nanoparticles on the intestinal mucosa in rats.

Materials and methods

Silver nanoparticles

The silver nanoparticles (52.7–70.9 nm, average; 60 nm) were purchased from Namatech Co., Ltd. (Korea) and at least 99.98% pure (Kim et al. 2008). The vehicle, 0.5% aqueous Carboxy methylcellulose (CMC), was obtained from Sigma (USA).

Animals and conditions

Four-week-old male and female, specific pathogen-free (SPF) Sprague–Dawley rats were purchased from Orient-Bio (Korea) and acclimated for 2 weeks before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (maximum of 3 rats per cage) in a room with controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 7\%$), and a 12-h light/dark cycle. The rats were fed rodent chow (Harlan Teklab, Plaster International Co., Korea) and

filtered water ad libitum. At 5 weeks, the rats were divided into four groups (10 rats in each group): vehicle control (0.5% carboxy methyl cellulose), low-dose group (30 mg/kg per day), middle-dose group (300 mg/kg per day), and high-dose group (1,000 mg/kg per day). At 6 weeks, the rats were exposed to silver nanoparticles following OECD test guideline 407 (OECD 1995), based on 28 days of repeated oral administration (dosing volumes were 10 ml/kg) using GLP.

Histological analysis

At the conclusion of the 4-week experiment, the rats were 10 weeks old. Before the necropsy, food was withheld for 24 h and the rats anesthetized with ether. After collecting their blood, the rats were sacrificed by cervical dislocation. The ileum, colon, and rectum were all carefully removed, then weighed and fixed in a 10%-formalin solution containing neutral phosphate buffered saline. Thereafter, the organs were embedded in paraffin, stained with hematoxylin and eosin (H–E) and a Periodic acid Schiff (PAS) reaction, and examined under a light microscope.

Histochemistry of mucins

To determine the general histochemistry of the mucins, the following staining procedures were used: a PAS reaction to detect neutral mucins, alcian blue (AB) staining at pH 2.5 to detect acidic mucins, AB staining at pH 2.5 and a PAS reaction to characterize the neutral mucins and acidic mucins, and high iron diamine (HID) and AB staining at pH 2.5 to distinguish between sulfated and nonsulfated (sialyated) mucins.

Results

Histological findings

The experimental samples at the high and middle dose revealed luminal and surface particles, plus silver nanoparticles were found within the tissue. Higher numbers of silver nanoparticles were also found in the lamina propria (connective tissue under the epithelia) in both the small and large intestine, and in the tip of the upper villi in the ileum and protruding surface of the fold in the colon (figures not shown).

In the control rat ileum, the goblet cells in the villi and crypt epithelium were revealed as densely stained mucus granules that filled the apical cytoplasm (Fig. 1a). Meanwhile, in the silver nanoparticle-administered rats, the ileal mucosa exhibited two different regions, where the first showed no change in the architecture of the epithelia

Fig. 1 AB pH 2.5-PAS staining of ileum mucosa from control and silver nanoparticle-administered rats. In the silver nanoparticle-administered rats (**b**), higher numbers of goblet cells were found to have released their mucus granules, resulting in more mucus materials in the crypt lumen and ileal lumen, plus the staining intensity of the goblet cells was lower when compared with that for the control rats (**a**). Magnification $\times 400$

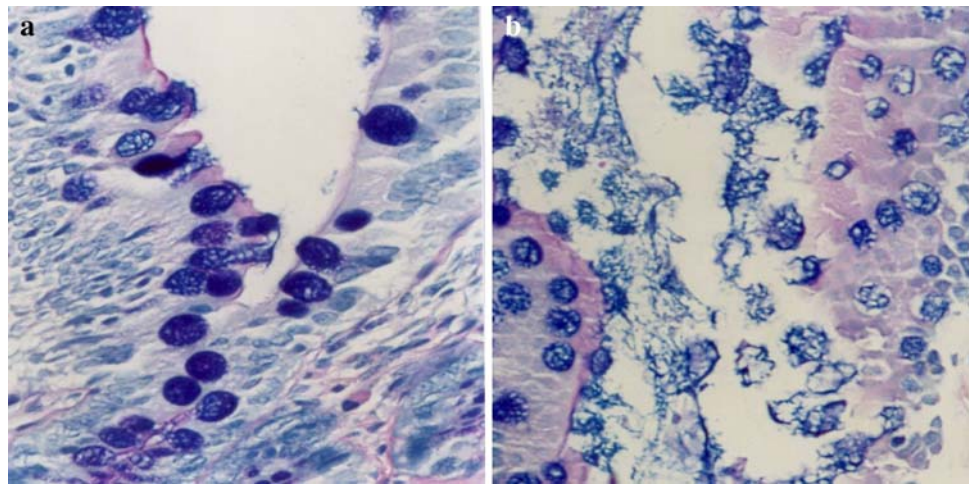
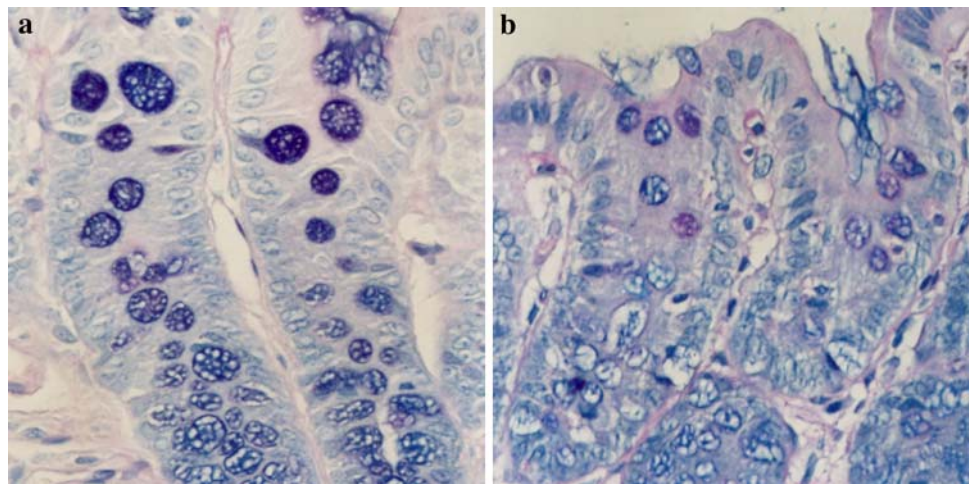


Fig. 2 AB pH 2.5-PAS staining of rectum mucosa from control and silver nanoparticle-administered rats. In the control rectum mucosa (**a**), goblet cells with densely stained granules were observed along the length of the crypt. Meanwhile, in the silver nanoparticle-treated rectum mucosa (**b**), the number of stained mucus cells was decreased, the crypt lumen was expanded, and mucus was released from the goblet cells. Magnification $\times 400$



compared to the control rats, while the other showed disordered goblet cells. In the silver nanoparticle-treated rats, the numbers of goblet cells that had released their mucus granules were higher than those in the controls, there were also large amounts of mucus material in the crypt lumen and ileal lumen (Fig. 1b), and cell shedding at the tip of the villi was frequent.

In rats, the proximal colon differs from the distal colon with respect to the surface structure, the form of the crypt, and the depth of the crypt. However, this study was limited to the proximal region of the colon, and no significant changes in the goblet cells from the proximal colon were observed for the silver nanoparticles-treated rats. In contrast, the goblet cells in the rectum of the silver nanoparticle-treated rats were found to have released their mucus granules and were occasionally destroyed (Fig. 2b).

Histochemistry of mucins

The histochemical results for the mucins in the ileum, colon, and rectum mucosa using PAS, AB pH 2.5, AB pH 2.5-PAS,

and HID-AB pH 2.5 staining are given in Tables 1, 2, and 3. The numerical values from 1 to 4 correspond to an increasing intensity of staining. However, the results represent a composite of subjective evaluations covering a range of values, thus the findings may have differed for any one individual. A numerical evaluation of the staining of the brush border of the epithelial cells was not attempted.

Neutral and acidic mucosubstances were both demonstrated in goblet cells from the villi and crypts of the ileum mucosa in the control rats, and sulfated mucins were more prominent than nonsulfated (sialyated) mucins. Furthermore, while neutral mucosubstances were found in epithelial goblet cells throughout the large intestine, acid mucosubstances were generally predominant, with sulfated mucins being particularly prominent in the distal colon. Meanwhile, sialyated mucins, with variations in the individual amounts present, were most prominent in the lower regions of the crypts, yet mixed with sulfomucins in the upper regions. Finally, in the distal colon, sulfated mucins were predominant throughout, with occasional cells showing some sialyated mucins.

Table 1 PAS, AB pH 2.5, AB pH 2.5–PAS, and HID-AB pH 2.5 staining properties of mucosubstances in goblet cells from rat ileum mucosa after administration of silver nanoparticles

Cells	Stains			
	PAS	AB pH 2.5	AB pH 2.5-PAS	HID-AB pH 2.5
CG				
VGC	3–4R	3B	4BP	3N
UCGC	2R	3B	3BP	2N > 2NB, 1B
LCGC	2R	2B	3BP	2N > 2NB, 1B
AG				
I				
VGC	0–1R	0–1B	0–1BP	0–1 N
UCGC	0–1R	1B > 0	1BP	1N/1NB > 1B, 0
LCGC	1R	1B > 0	1BP	1N/1NB > 1B, 0
II				
VGC	3R	2–3B	4BP	3N
UCGC	1–2R	2B	3BP	2N/2NB > 1B
LCGC	1–2R	1–2B	3BP	2N/2NB > 1B

Numbers indicate the degree of staining (4, very intense; 3, intense; 2, moderate; 1, weak; ±, faint; 0, absent; >, most marked)

Key to symbols: *R* Red, *PAS* positive; *B* blue, AB-positive; *N* Black, HID-positive; *BP* bluish purple, mixture of AB- and PAS-positive mucins, with AB-positive mucins predominating; *NB* black to blue, HID-positive mucins predominating over AB-positive mucins; *BN* Blue to black, AB-positive mucins predominating over HID-positive mucins; *VGC* villous goblet cells; *UCGC* upper crypt-goblet cells; *LCGC* lower crypt-goblet cells; *CG* control group; *AG* administered group

Symbols separated by/indicate a mixture of cells with different mucin content

Table 2 PAS, AB pH 2.5, AB pH 2.5–PAS, and HID-AB pH 2.5 staining properties of mucosubstances in goblet cells from rat colon mucosa after administration of silver nanoparticles

Cells	Stains			
	PAS	AB pH 2.5	AB pH 2.5-PAS	HID-AB pH 2.5
CG				
SGC	3–4R	3B	4BP/4P	3 N
UCGC	3–4R	3B	4BP/4P	3 N > 3NB
LCGC	2R	2B	3B>3BP	2 N > 2B/2NB
AG				
SGC	3R	3B	4BP/4P	3 N
UCGC	3R	2B	3BP–3P	3 NB > 3N
LCGC	1–2R	1–2B	2B > 2BP	2 NB > 2 N/2B

Numbers indicate the degree of staining (4, very intense; 3, intense; 2, moderate; 1, weak; ±, faint; 0, absent; >, most marked)

Key to symbols: *SGC* surface-goblet cells

Other symbols are the same as in Table 1

As shown in Tables 1, 2, and 3, goblet cells from the ileum, colon, and rectum mucosa of the silver nanoparticle-administered rats showed decreased amounts of neutral and

Table 3 PAS, AB pH 2.5, AB pH 2.5–PAS, and HID-AB pH 2.5 staining properties of mucosubstances in goblet cells from rat rectum mucosa after administration of silver nanoparticles

Cells	Stains			
	PAS	AB pH 2.5	AB pH 2.5-PAS	HID-AB pH 2.5
CG				
SGC	2R	2–3B	3BP/3P	2N
UCGC	2R	2–3B	3BP/3P	2N
LCGC	1–2R	1–2B	2B > 2BP	1N > 1NB
AG				
SGC	2R	2–3B	3BP/3P	2N
UCGC	0–2R	0–2B	0–2BP > 2P	2N > 2NB/1BN/1B
LCGC	0–1R	0–1B	1B > 1BP	1NB > 1N/1BN/1B

Numbers indicate the degree of staining (4, very intense; 3, intense; 2, moderate; 1, weak; ±, faint; 0, absent; >, most marked)

Key to symbols: *SGC* surface-goblet cells

Other symbols are the same as in Table 1

acidic mucins (Figs. 1b, 2b), and this was even more marked in the regions shedding goblet cells. In particular, in the ileum of the silver nanoparticle-treated rats, the mucin distribution differed greatly between the normal and abnormal regions. A significant decrease of both neutral and acidic mucins was observed in the ileum mucosa (Fig. 1b). Plus, in the silver nanoparticle-treated rats, the proportion of sulfated mucins decreased, while the proportion of nonsulfated (sialylated) mucins increased, however, the total amount of acidic mucins decreased (Figs. 3b, 4b). In particular, an HID-positive reaction was detected in the lamina propria (connective tissue under the epithelia) of the colon, indicating the presence of sialylated mucins, which was not found in the control rats (Fig. 5).

Discussion

The antibacterial activity of silver nanoparticles has resulted in their widespread use in bedding, washers, water purification, toothpaste, shampoo and rinse, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, and humidifiers (Maynard 2006; KISTI 2006). Silver nanoparticles have also recently been shown to be a promising antimicrobial material (Sondi and Salopek-Sondi 2004). However, the toxicity of silver nanoparticles in biological systems remains a controversial research area, due to the limited availability of information.

In a previous inhalation study, the present authors found no significant toxicity in Sprague–Dawley rats that had been repeatedly exposed to silver nanoparticles via inhalation, based on 6 h each day, 5 days a week, for a total of 4 weeks. Plus, no significant changes were found in the male and female body weights or hematology and blood

Fig. 3 HID-AB pH 2.5 staining of colon mucosa from control and silver nanoparticle-administered rats. The silver nanoparticle-administered rats (**b**) exhibited a higher amount of sialomucins and lower amount of sulfomucins when compared with the controls (**a**). Magnification $\times 400$

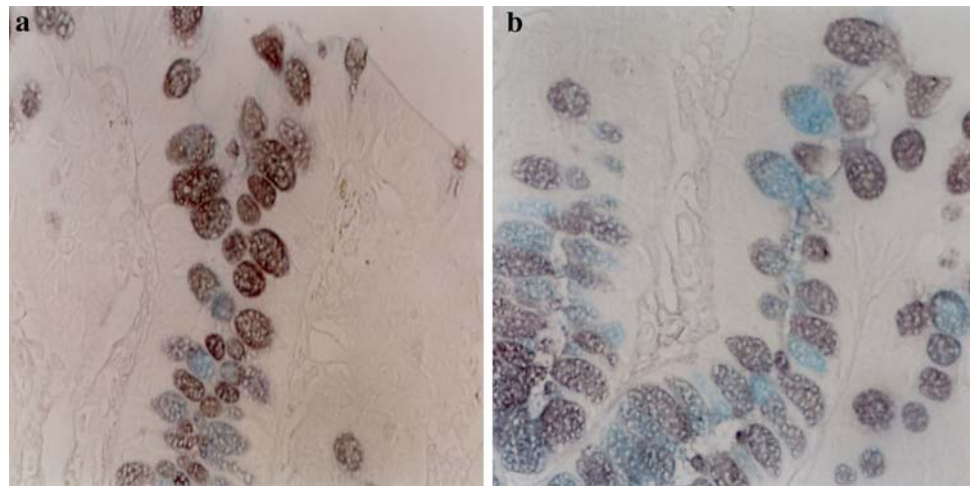


Fig. 4 HID-AB pH 2.5 staining of rectum mucosa from control and silver nanoparticle-administered rats. The silver nanoparticle-administered rats (**b**) exhibited increased amounts of sialomucins and decreased amounts of sulfomucins when compared with the controls (**a**). Magnification $\times 400$

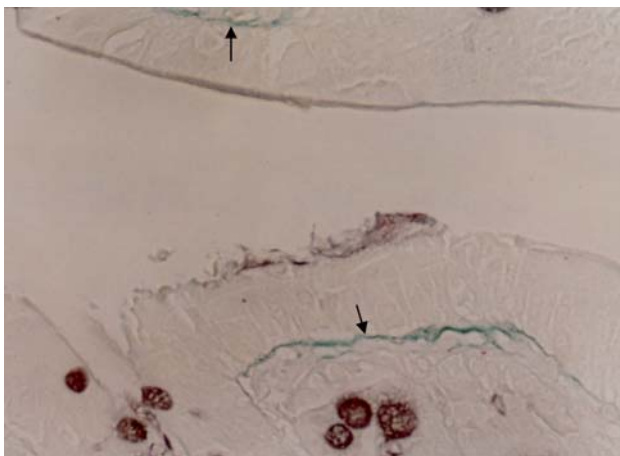
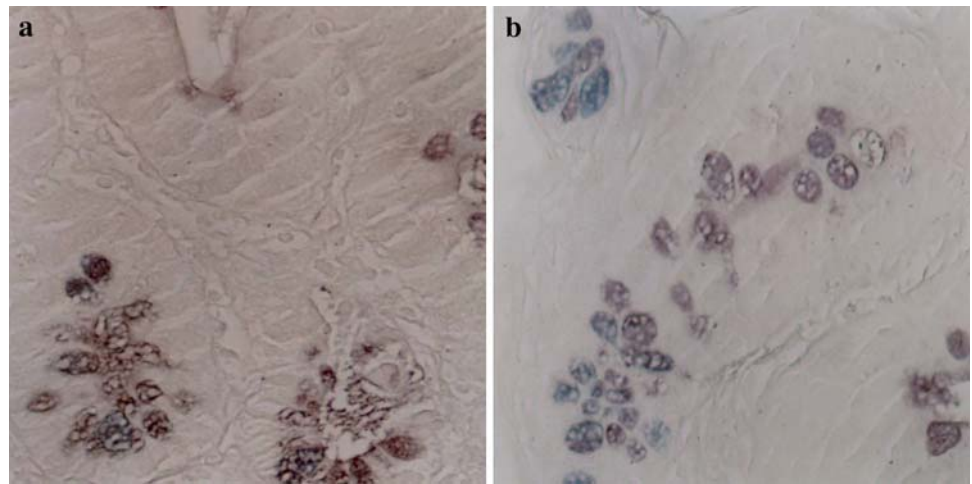


Fig. 5 HID-AB pH 2.5 staining of rectum mucosa from silver nanoparticle-administered rats. An HID-positive reaction (*arrows*) was detected in the lamina propria of the colon, indicating the presence of sialomucins, which was not found in the control rats. Magnification $\times 400$

biochemical values relative to various concentrations of silver nanoparticles during the 28-day experiment (Ji et al. 2007). Meanwhile, in another oral toxicity study using male and female Sprague–Dawley rats, the present authors found that oral exposure to silver nanoparticles (60 nm) at concentrations of 30, 300, and 1,000 mg/kg for 28 days had no effect on micronucleated polychromatic erythrocytes or bone marrow cells. However, the repeated oral doses of silver nanoparticles did induce liver toxicity and had a coagulation effect on peripheral blood. The histopathologically evaluated liver toxicity included dilatation of the central vein, bile duct, and hyperplasia (Kim et al. 2008).

In this study, the experimental samples revealed luminal and surface particles and the tissue also contained silver nanoparticles. An increased accumulation of silver nanoparticles was observed in the lamina propria (connective tissue under the epithelia) in both the small and large intestine.

The mucus secreted into the lumen from intestinal goblet cells forms a gel layer that covers the mucosal surface of the intestinal tract, acting as a semipermeable barrier between the lumen and epithelium. The stability of this mucus layer is then essential in preserving the integrity of the intestinal epithelium, and any breakdown of this protection can lead to mucosal injury. Therefore, this study investigated whether silver nanoparticles would modulate the secretion of intestinal mucus, a major component of physiological defense mechanisms. The oral administration of silver nanoparticles was found to induce the discharge of mucus from goblet cells, mostly in the ileum and rectum, yet not in the proximal colon. Thus, since goblet cells releasing mucus were frequently found in the silver nanoparticle-administered rats, this indicates that the silver nanoparticles did not allow the cells to be replenished by mucin synthesis. As the gel layer formed by the mucus on the surface of the intestinal epithelium has a barrier function, the present data may be relevant physiologically to the defense mechanisms of the gastrointestinal tract. Goblet cells secrete mucins via an unregulated constitutive pathway that is dependent on the continuous movement of mucin granules from the Golgi apparatus to the apex of the cell and by a regulated process dependent on the sudden release of mucins from the granules. This regulatory process is controlled by a wide variety of stimuli, including nerve activation and inflammatory mediators (Neutra et al. 1982; Branka et al. 1997; Plaisancie et al. 1998; Verburg et al. 2002; Smirnova et al. 2003; Blanchard et al. 2004). In this study, silver nanoparticles were demonstrated to be one of these stimuli.

This study also found that the intestinal goblet cells from the silver nanoparticle-administered rats showed a decreased amount of neutral and acidic mucins. Meanwhile, the proportion of sulfated mucins was decreased, while the proportion of sialylated mucins was increased, although the total amount of acidic mucins was decreased. The increase in sialylated mucins observed in this study was similar to the intestinal mucin change in the case of active ulcerative colitis (Filipe and Dawson 1970), small intestine carcinoma (Filipe and Fenger 1979), and the administration of organophosphorus pesticides to rats (Lee 1979; Jo and Kim 1987), although the toxicants were different. Filipe and Branfoot (1974) reported that the transitional mucosa, the normal mucosa adjacent to carcinomas, in the large intestine is characterized by an increase of sialylated mucins, accompanied usually by a decrease or absence of sulfated mucins, which are predominant in the normal mucosa of the large intestine. In addition, higher quantities of neuraminidase-sensitive sialic acids in the mucosa around a tumor have also been reported (Filipe 1971; Filipe and Cooke 1974; Dawson et al. 1978). Thus, evidence of sialylated mucins in the mucosa around a tumor may be an index of the tumor's aggressiveness. As such, the presence

of sialylated mucins in the lamina propria (connective tissue under the epithelia) of the colon in this study, which was not found in the control rats, would seem to indicate that repeated silver nanoparticle-exposure may produce pathologic regions.

In conclusion, this study suggests that silver nanoparticles are a powerful intestinal secretagogue and induce an abnormal mucin composition in the intestinal mucosa. Thus, further studies are needed to determine the relevance of these findings to the pathophysiology of the gastrointestinal tract.

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