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Localization of membrane-associated sialomucin on the free surface of mesothelial cells of the pleura, pericardium, and peritoneum

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Abstract Strong anionic sites, as recognized by deposition of cationic colloidal iron even at pH 1.5, were distributed on the free surfaces of the mesothelia of the mouse pleura, pericardium, and peritoneum. Methylation inhibited colloidal iron staining on the surface, and successive saponification restored it. Digestion with neuraminidase or hydrolysis of sialic acid with H_2SO_4 erased the colloidal iron staining. Lectin *Limax flavus* agglutinin (LFA), which is specific for sialic acid, labeled the free surface of the mesothelium. All these findings strongly suggested that the surface substance contained sialic acid. Moreover, prior treatment with LFA inhibited the mesothelial surface stain with colloidal iron. In transmission electron microscopy, the colloidal iron (pH 7.3)-stained substance took the shape of fine strands of 50–300 nm in length. These characteristics of the substance on the mesothelial surface correspond well with biochemical properties of membrane-associated sialomucin, whose strong and abundant negative charges produce repulsive forces between facing serosal surfaces. This may contribute to prevent serosal adhesion and to reduce friction during movements of organs.

Introduction

The free surface of the peritoneal mesothelium is coated with glycoconjugates containing anionic or negatively charged sites, which have been demonstrated using various cationic probes, such as colloidal iron (Curran et al. 1965; Ohtsuka and Murakami 1994), cationized ferritin (Leak 1986), thorium dioxide, and ruthenium red (Andrews and Porter 1973). The strong anionic sites, ionized even at pH 1.5, spread all over the free surfaces of the parietal, mesenteric, and visceral peritoneum; the surface coats with negative charges are considered to act repulsively to maintain the peritoneal cavity (Ohtsuka and Murakami 1994). Some histochemists suggested that both sialomucin and sulfomucin exist on the mesothelium to provide such anionic sites (Katsuyama et al. 1977). On the contrary, our former enzyme digestion and transmission electron microscopy studies demonstrated that the free surface of the peritoneal mesothelium possessed a membrane-associated mucin-like substance which contained abundant sialic acid (Ohtsuka and Murakami 1994).

Previously, it has been reported that *Triticum vulgaris* lectin (wheat germ agglutinin, WGA) specifically labeled the free surface of the peritoneal mesothelium (Hjelle et al. 1991). However, this lectin labels both *N*-acetylglucosamine and *N*-acetylneuraminic acid (Chevalier et al. 1987). *Limax flavus* agglutinin (LFA), which is used in our present study to determine specific sugar elements on the mesothelium, has higher specificity for sialic acid (Miller et al. 1982; Roth et al. 1984).

The present light and electron microscopic study shows that the pleura, pericardium, and peritoneum commonly have strong anionic sites associated with the plasma membrane of the free surface of the mesothelium, and confirms again, using the cationic colloidal iron method combined with chemical modifications, enzyme digestions, and some lectin labeling, that the strong anionic sites, ionized even at pH 1.5, derive from sialic acid.

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Materials and methods

Light microscopy

Animals and tissue preparation

Ten- to 15-week-old mice [Crj:CD-1 (ICR); Charles River Japan] were used under deep anesthesia with diethyl ether. Perfusion-fix-

ation with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2–7.4) was performed through the left ventricle or the inferior vena cava. Tissue pieces of the lung, heart, diaphragm, liver, and jejunum were excised together with their serosa, and immersed in the same fixative for 6–8 h. The fixed tissues were processed by the usual paraffin sectioning technique to obtain sections of 5- to 7-μm-thick.

Cationic colloidal iron staining

The sections were incubated in our cationic colloidal iron (Murakami et al. 1986), whose pH value was variably changed between 1.0 and 7.5. After rinsing with distilled water, sections were treated with 1% $K_4Fe(CN)_6$ in 0.1 M HCl for the Prussian blue reaction, counterstained with nuclear fast red, and observed with a photo-microscope (BX-10, Olympus).

Methylation and saponification

The sections were methylated by incubation for 4 h in a mixture of 0.8 ml of 31% HCl and 100 ml of absolute methanol at 37°C (mild methylation) (Spicer 1960). Control sections were incubated for 4 h in methanol at 37°C. Some methylated sections were saponified by incubation in 1% KOH in 70% ethanol for 5–20 min at 25°C. Methylated, methylated-saponified, and control sections were processed for cationic colloidal iron staining (pH 1.5).

Enzyme digestion

Some sections were pretreated with neuraminidase (from *Streptococcus* 6646 K; Seikagaku), hyaluronidase (from *Streptomyces hyalurolyticus*; Seikagaku), chondroitinase ABC (from *Proteus vulgaris*; Seikagaku), heparitinase (from *Flavobacterium heparinum*; Seikagaku), or keratanase (from *Pseudomonas* sp.; Seikagaku) as previously described (Murakami et al. 1994; Ohtsuka and Murakami 1994), and then they were stained with cationic colloidal iron at pH 1.5. As negative controls, sections were incubated in buffer solutions without any enzymes. In order to check the digestion abilities of these enzymes, sections containing the renal glomerulus, umbilical cord, articular synovia (Nishida et al. 1995) or brain nerve cells with sulfated proteoglycan surface coats (Murakami et al. 1994) were used.

Chemical desialylation

For the purpose of hydrolysis of sialic acids, some sections were incubated for 1 h in 0.1 N H_2SO_4 at 80°C (Spiro and Bhoyroo 1974). Control sections were incubated in distilled water at 80°C. Then, the sections were stained with colloidal iron.

Lectin binding study

The lectins used and their specific sugar affinities are as follows: *L. flavus* agglutinin (LFA; EY Laboratories) having an affinity for *N*-acetylneuraminic acid > *N*-glycolylneuraminic acid (Miller et al. 1982); *Vicia villosa* agglutinin (VVA; Vector) with affinity for *O*-glycosidically linked *N*-acetylgalactosamine (Tollefsen and Kornfeld 1983).

The tissue sections were labeled with the lectins as previously described (Murakami et al. 1995). Briefly, the sections were blocked with 1% bovine serum albumin in 0.01 M phosphate buffered-saline, pH 7.4 (PBS), briefly rinsed with PBS, and completely covered for 1 h at room temperature with 5–40 μg/ml of biotinylated lectin, VVA or LFA, in PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1 mM $MnCl_2$. After rinsing 3 times, specimens were treated for 30 min with 5 μg/ml of peroxidase-conjugated streptavidin (Vector), and rinsed again. Then, labeled sites were localized with diaminobenzidine (DAB) solution (0.024% DAB and 0.006% H_2O_2 in TRIS-HCl buffer, pH 7.6), and the sections were counterstained with Mayer's hematoxylin.

To confirm lectin binding specificity, the sections were pre-incubated with 0.2 M *N*-acetylneuraminic acid for LFA and 0.2 M *N*-acetylgalactosamine for VVA, and then incubated with biotinylated lectins in the presence of the sugars. DAB reaction with peroxidase-streptavidin alone did not show non-specific staining on the serosal surface.

After treatment with the lectin, LFA or VVA, some sections were stained with the cationic colloidal iron method instead of processing for streptavidin-peroxidase and DAB reaction.

Electron microscopy

Small pieces of the mouse lung, heart, and jejunum were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, rinsed in 5% sucrose in 0.1 M cacodylate buffer, dehydrated with ethanol, and embedded in LR white resin (hard grade; London Resin). Ultrathin sections of the specimens were stained with cationic colloidal iron at pH values of 1.0–7.5 (Ohtsuka et al. 1993). After exposure to OsO_4 vapor, specimens were observed with a transmission electron microscope (H-7100; Hitachi).

Results

Light microscopy

Our cationic colloidal iron was confirmed to keep stable positive charges at a wide pH range of 1.0–7.5 and to be useful in detecting negatively charged sites which ionized at the pH value of the staining mixture. When mouse pulmonary tissue was stained with this colloid at a pH value of 7.3 (Fig. 1A), at which most anionic sites ionized, almost all tissue elements, including the alveolar epithelium, septal connective tissues, endothelium, and pleura showed the Prussian blue reaction. In the specimens stained with colloidal iron at pH 1.5, a distinct Prussian blue reaction was observed on the free surface of the mesothelium of the pulmonary pleura (Fig. 1B). At this pH, the parietal pleura (Fig. 1C), pericardium (Fig. 1D), and peritoneum (Fig. 1E) showed an intense Prussian blue reaction on the free mesothelial surface. The staining manner of the mesothelial surface showed no differences between the parietal and visceral serosa, and between the pleura, peritoneum, and peritoneum.

Mild methylation with HCl-methanol at 37°C completely eliminated the Prussian blue reaction for colloidal iron on the serosal surface (Fig. 2A). When specimens were saponified after methylation, the blue reaction was restored on the surface (Fig. 2B). Neuraminidase treatment before colloidal iron staining erased the Prussian blue reaction on the free surface of the mesothelium (Fig. 2C). Any other enzyme digestions with hyaluronidase, chondroitinase ABC, heparitinase, and keratanase did not affect colloidal iron staining at pH 1.5. Hydrolysis of sialic acid with H_2SO_4 made specimens lose stainability against cationic colloidal iron on the free mesothelial surface (Fig. 2D).

The free surface of the mesothelium was distinctly labeled with the lectins VVA (Fig. 3A) and LFA (Fig. 3B).

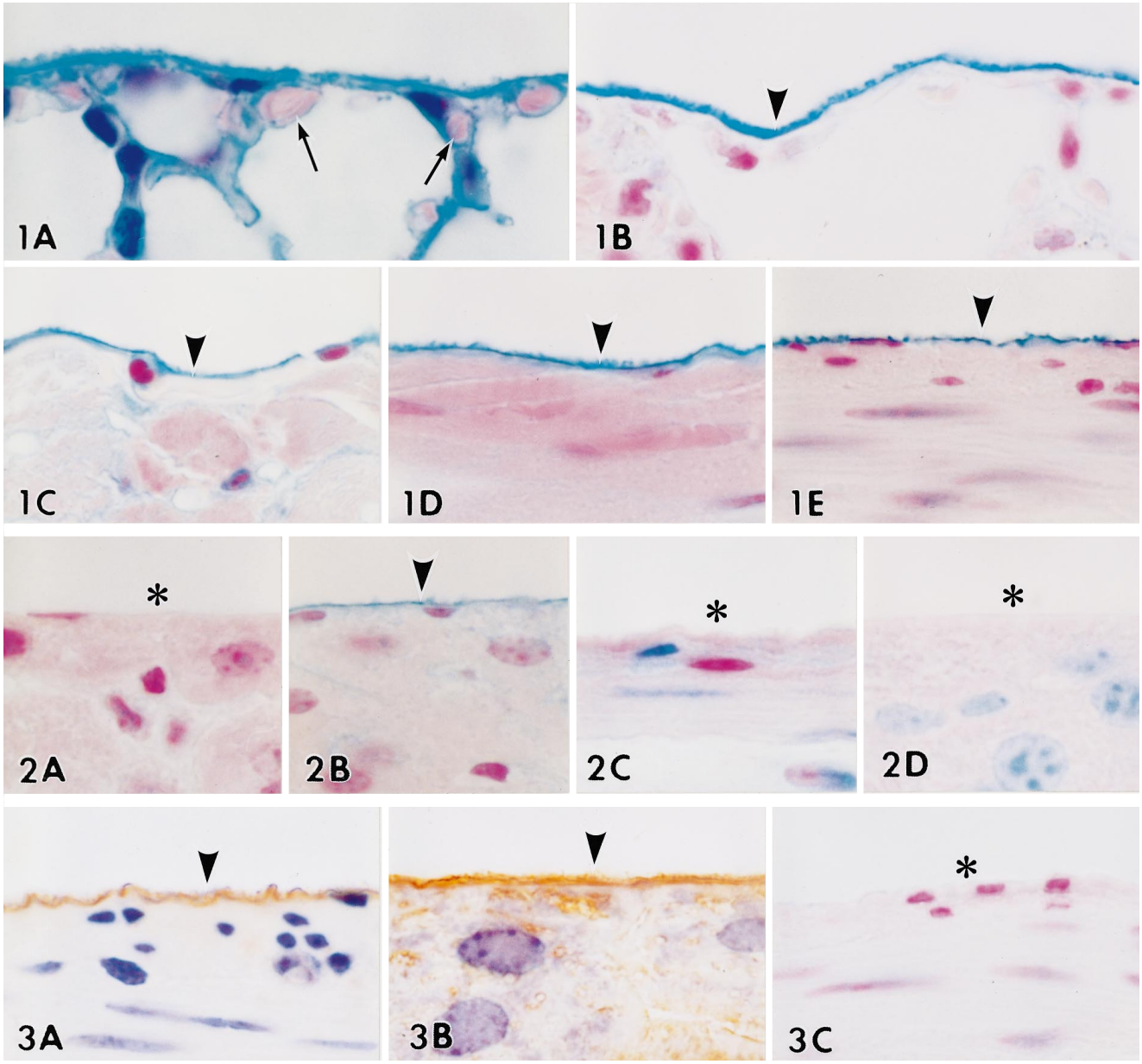

Fig. 1 Light micrographs of the mouse lung (**A, B**), diaphragm (**C**), heart (**D**), and jejunum (**E**) which have been stained with cationic colloidal iron at pH values of 1.5–7.3, treated for the Prussian blue reaction, and counterstained with nuclear fast red. **A** At pH 7.3, almost all pulmonary tissue elements except erythrocytes (*arrows*) show the blue reaction, because even weak anionic groups can ionize at this pH level. **B–E** At pH 1.5, only strong anionic groups are stained. Note the restricted blue reaction on the mesothelial surfaces of the pleura (*arrowheads* **B, C**), pericardium (*arrowhead* **D**) and peritoneum (*arrowhead* **E**). **A–E** ×1300

Fig. 2 Mouse hepatic (**A, B, D**) and jejunal (**C**) sections which were stained with the cationic colloidal iron method (pH 1.5) plus nuclear fast red after chemical modification or enzyme digestion. **A** Prior methylation eliminates the Prussian blue reaction on the free mesothelial surface (*asterisk*). **B** Methylation and successive saponification restores the blue reaction on the surface (*arrowhead*). **C** Neuraminidase digested sections show no blue reaction on the mesothelium (*asterisk*). **D** Desialylation with 0.1 N H_2SO_4 also erases the blue reaction on the mesothelial surface (*asterisk*). **A–D** ×1300

Fig. 3 Mouse jejunal (**A**) and hepatic (**B**) sections labeled with biotinylated lectins, *Vicia villosa* agglutinin (**A**) and *Limax flavus* agglutinin (LFA; **B**), and processed for the peroxidase-diaminobenzidine reaction. Note the distinct labeling with these lectins on the free surface of the serosa (*arrowheads*, **A, B**). **C** A section of the small intestine treated with LFA and stained with cationic colloidal iron shows no Prussian blue reaction on the free mesothelial surface (*asterisk*). **A–C** ×1300

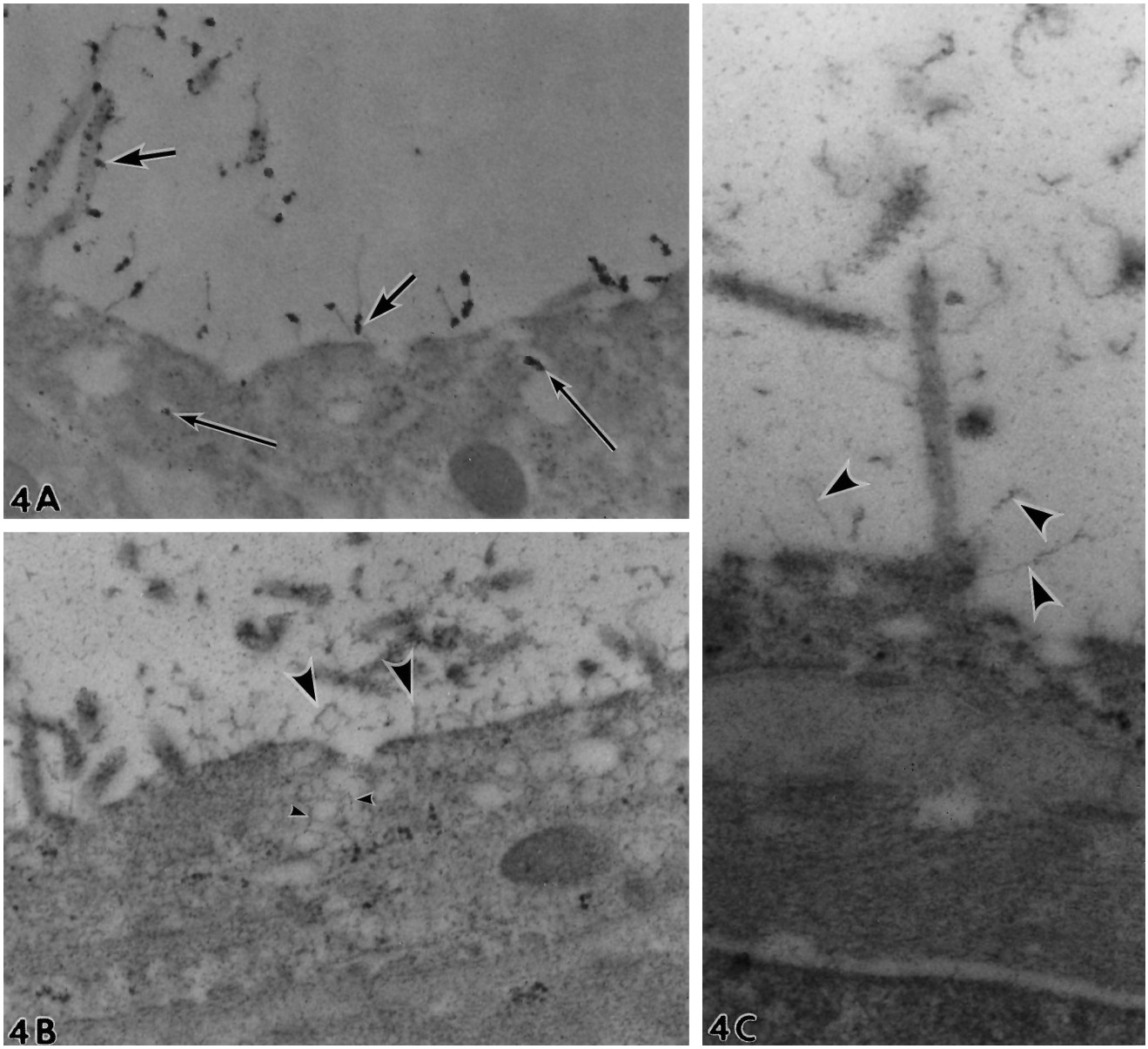

Fig. 4 Transmission electron micrographs of the mouse jejunal serosa stained with cationic colloidal iron at pH 1.5 (**A**) or at pH 7.3 (**B, C**). When the serosal sections are stained at pH 1.5, deposited colloidal iron particles aggregate on the free surface of the mesothelial cells, forming dots or globules (*thick arrows* **A**). The sections stained at pH 7.3 show colloidal iron deposition in a strand-like manner on the mesothelial surface (*large arrowheads* **B**). Note the cytoplasmic vesicles contain colloidal iron depositions, which are also observed as globules at pH 1.5 (*thin arrows* **A**) and as strands at pH 7.3 (*small arrowheads* **B**). In a high power view, fine strands deposited with colloidal iron spread into the peritoneal cavity (*arrowheads* **C**). **A, B** ×45 000, **C** ×80 000

Treatment with LFA prior to cationic colloidal iron staining inhibited the Prussian blue reaction on the free mesothelial surface (Fig. 3C). Treatment with VVA did not affect the Prussian blue reaction on the serosal surface.

Electron microscopy

In the specimens stained with cationic colloidal iron at pH 1.5, the fine electron-dense particles of colloidal iron deposited on the free mesothelial surface of the peritoneum (Fig. 4A), pleura (Fig. 5A), and pericardium (Fig. 6A). At this pH, particles of colloidal iron (1–1.5 nm) on the mesothelial surface frequently accumulated to form globules or dots (10–20 nm in diameter). Within the mesothelial cells, dot-like depositions of colloidal iron were observed in the cytoplasmic vesicles (Fig. 4A).

In the specimens stained with colloidal iron at pH 7.3, deposition of the colloidal particles showed a fine strand structure on the free mesothelial surface of the peritoneum (Fig. 4B, C), pleura (Fig. 5B), and pericardium (Fig. 6B). The length of the strand was 100–300 nm in the peritoneum, 50–150 nm in the pleura, and

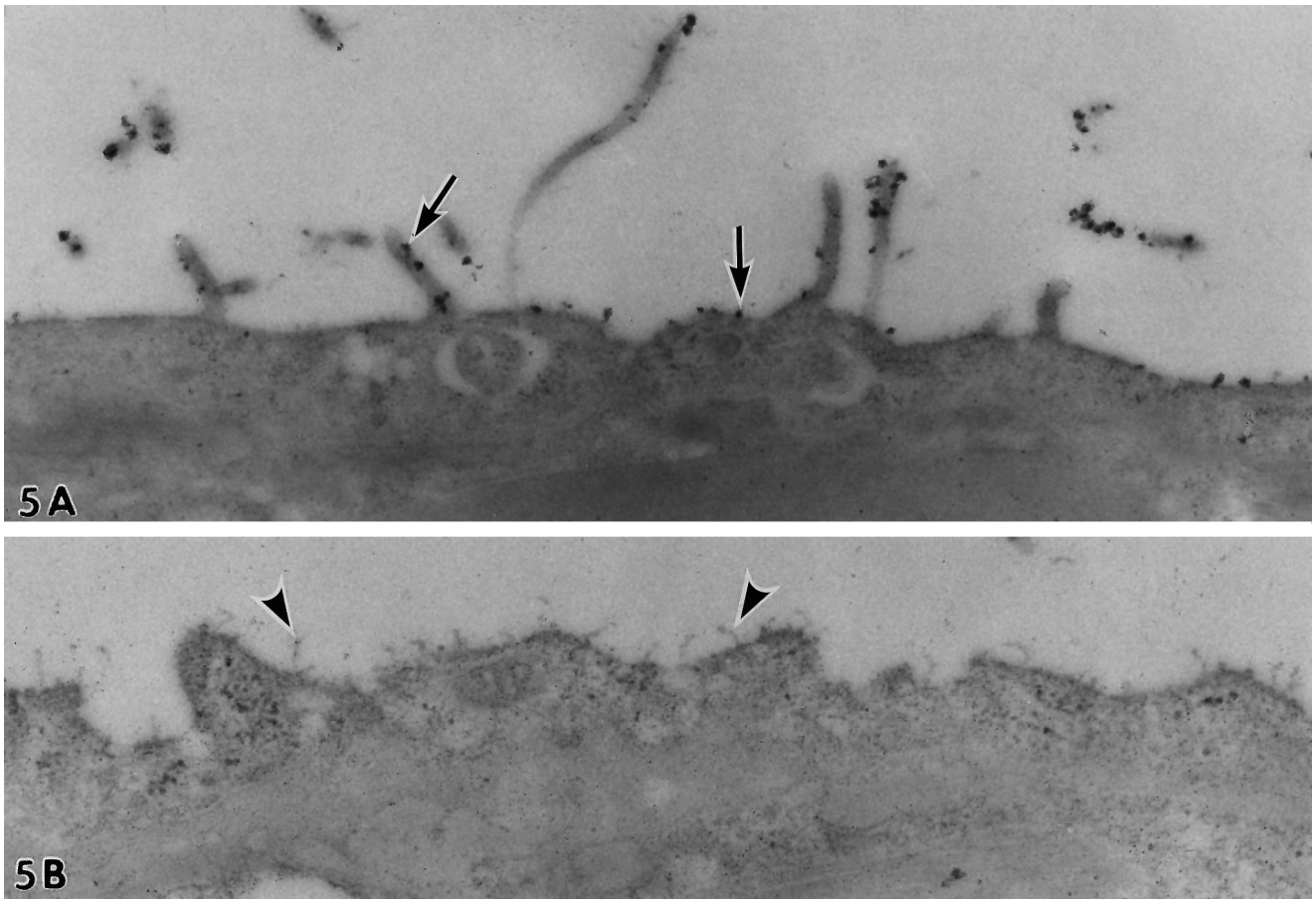

Fig. 5 Free surface of the lung pleural mesothelium stained with cationic colloidal iron at pH 1.5 (**A**) or 7.3 (**B**). When the sections were stained at pH 1.5, the colloidal iron particles accumulated to form dot-like depositions on the free surface of the mesothelium (*arrows* **A**). Note, at pH 7.3, the strand-like deposited colloidal iron on the free surface of the mesothelial cells (*arrowheads* **B**). **A** ×30 000, **B** ×45 000

25–100 nm in the pericardium; it was longest in the peritoneum, and shortest in the pericardium. One end of the strand attached to the surface of the cytoplasmic membrane of the mesothelial cells. In some sections, similar fine strands stained with colloidal iron were observed in cytoplasmic vesicles within the mesothelial cell (Fig. 4B).

Discussion

The present light and electron microscopic study using a cationic colloidal iron method has confirmed that all the free mesothelial surfaces of the peritoneum, pleura, and pericardium have abundant anionic sites, which ionize even at a pH value of 1.5. Anionic sites on the mesothelial surface have been shown using ruthenium red (Andrews and Porter 1973; Gotloib et al. 1988), thorium dioxide (Andrews and Porter 1973), dialyzed iron (Curran et al. 1965; Katsuyama et al. 1977), high iron diamine (Katsuyama et al. 1977), and cationized ferritin (Leak 1986). Our previous cationic colloidal iron studies have shown that the anionic sites commonly exist on the free surfaces of the parietal, mesenteric, and visceral serosa in the rat and human peritoneum (Ohtsuka and Murakami 1994). Furthermore, "anionic" colloidal iron studies have shown that the free surface of the peritoneal mesothelium has few cationic sites even at the neutral pH of 7.2 (Ohtsuka and Murakami 1994). Thus, all the free surface of the coelomic mesothelium is covered with substances which contain abundant anionic but few cationic sites.

Some histochemists have reported that the surface coat on the mesothelium consists of sialomucin as well as sulfomucin (Katsuyama et al. 1977). However, in our present study, prior mild methylation, neuraminidase digestion, H_2SO_4 hydrolysis or LFA treatment completely eliminated cationic colloidal iron staining at pH 1.5; saponification after methylation restored it; hyaluronidase, chondroitinase ABC, heparitinase, and keratanase had no digestive effect. All these findings strongly support the notion that mesothelial surface anionic sites ionized at pH 1.5 are derived from carboxyl groups of sialic acid, but do not suggest the existence of sulfate groups or sulfated proteoglycans.

The lectin LFA is highly specific for *N*-acetyl- and *N*-glycolylneuraminic acids (Miller et al. 1982), and has been used to detect sialic acids (Roth et al. 1984). The

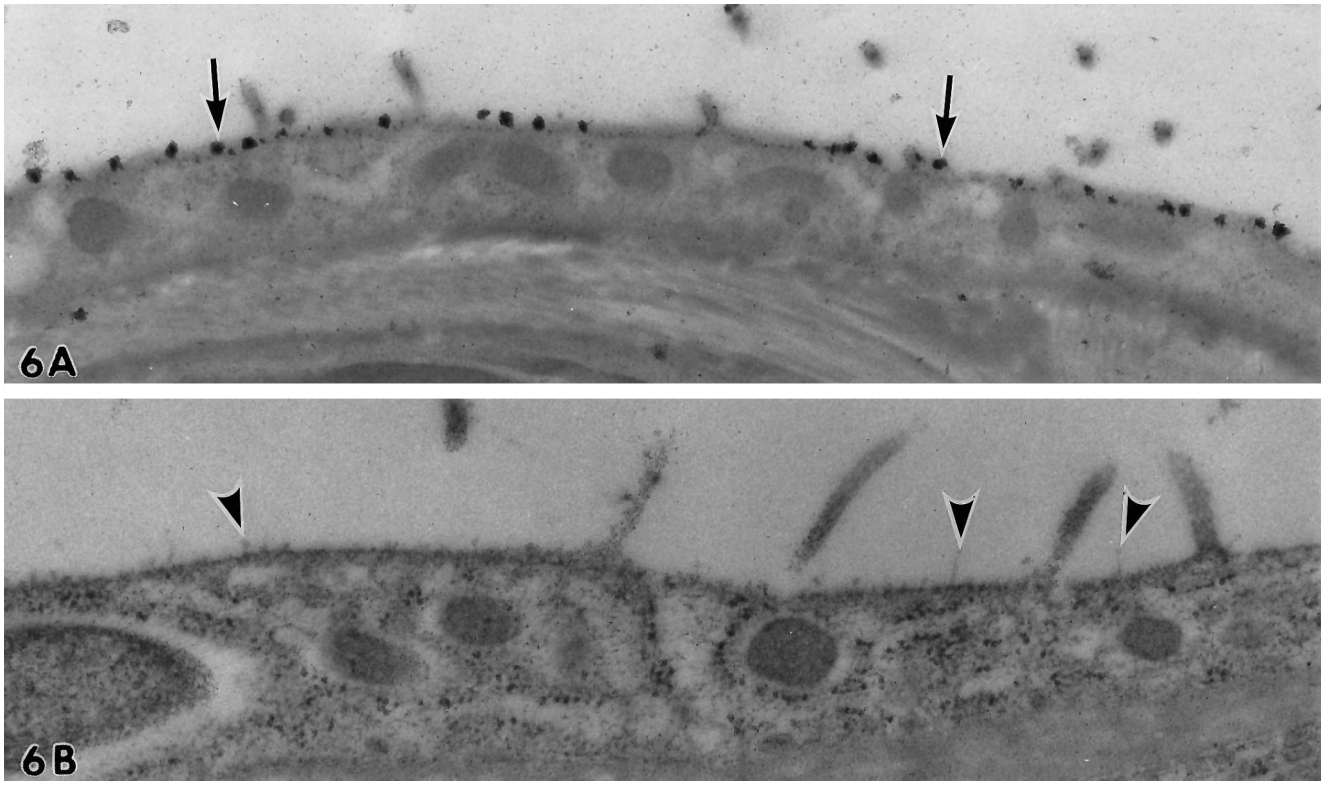

Fig. 6 Visceral pericardium stained with cationic colloidal iron at pH 1.5 (**A**) or at pH 7.3 (**B**). When the sections were stained at pH 1.5, the colloidal iron particles accumulated to form globular depositions on the free mesothelial surface (*arrows* **A**). Note, at pH 7.3, the strand-like deposited colloidal iron on the free surface of the mesothelial cells (*arrowheads* **B**), though the strands are shorter than those of the pleura and peritoneum. **A** ×30 000, **B** ×45 000

present binding to LFA on the free surface of the mesothelium indicates the abundant presence of sialic acids. It has been reported that cultured human, rabbit, and rat mesothelial cells have specific sugars which bind to *T. vulgaris* lectin (Hjelle et al. 1991). This lectin is known to bind both *N*-acetylneuraminic acid and *N*-acetylglucosamine (Holthöer et al. 1981). The results of our present study are explained by the existence of *N*-acetylneuraminic acid on the mesothelial cell surface and that our cationic colloidal iron deposits to ionized carboxyl groups of this acid. The lectin VVA binds to *O*-glycosidically linked *N*-acetylgalactosamine (Tollefsen and Kornfeld 1983). This kind of sugar is an essential element of mucopolysaccharides or glycosaminoglycan.

It is noteworthy that LFA treatment interfered with the cationic colloidal iron staining at pH 1.5. This strongly suggests that LFA and colloidal iron share common binding sites on the mesothelial surface mucin, or that binding of LFA to sialic acid covers or hides the carboxyl group of this acid. Thus, deposition of cationic colloidal iron at pH 1.5 on the mesothelial surface is equivalent to sialic acid detection by LFA.

In the electron microscope, it is interesting that, when the serosa is stained with cationic colloidal iron at pH 1.5, colloidal particles tend to accumulate to form globules, while at pH 7.3, the colloidal particles are arranged as fine strands. This difference of arrangement may be explained by the effects of the pH on the three-dimensional structure of the stained substance, that is, this substance takes a strand-like shape at pH 7.3 while it is globular at pH 1.5. Transmission electron microscopic studies of ruthenium red-stained samples showed that many fine strands (about 15-nm-thick) interconnected with each other by adjacent microvilli (Andrews and Porter 1973). In our present and previous electron microscopic studies (Ohtsuka and Murakami 1994), very fine strands are present in the specimens stained with cationic colloidal iron at pH 7.3, though the thickness of the strands is about 5 nm. This difference in thickness may occur due to the size of the cationic probes used. The length of the strands is longer in the peritoneum than in the pericardium. This may represent a difference in friction between facing serosa, since the abdominal organs move slowly but the heart beats more frequently and quickly.

The substance stained with cationic colloidal iron on the free mesothelial surface has the following characteristics: (1) at the physiological pH of 7.3, it takes the shape of fine strands whose length is approximately 50–300 nm; (2) it contains abundant sialic acid molecules; and (3) one end of the strand connects with the plasma membrane. These histochemical and morphological characteristics correspond well to the biochemical properties of membrane-associated sialomucin, which can reduce cellular adhesion or aggregation capacity (Hilkens et al. 1992). The present electron microscopic

finding that colloidal iron deposits in the cytoplasmic vesicles may suggest that sialomucin is produced in the mesothelial cell.

It has been reported that a sialomucin, episialin, is present at the luminal surface of mesothelial cells (Hilkens et al. 1992). The present cell-surface sialomucin detected by the colloidal iron method may be episialin, or at least may include it. Though the free mesothelial surfaces of the serosa contact and rub against each other, all of the surfaces possess the negatively charged substance at the front, which may act by electrostatic repulsion to avoid serosal adhesion and to reduce friction during organ movement.

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