

Interleukin-6 expression on the biliary epithelia during inflammation-associated biliary carcinogenesis in bilioenterostomized hamsters

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Running title: Interleukin-6 expression and biliary carcinogenesis

Key words: biliary carcinoma, interleukin-6, cholangitis, hamster,

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Abstract

Background: Chronic inflammatory conditions of the biliary tree strongly predispose patients to biliary carcinoma. The aim of this study was to evaluate the role of interleukin-6 (IL-6) expression during biliary carcinogenesis in bilioenterostomized hamsters.

Materials and Methods: Syrian hamsters were subjected to either a choledochoduodenostomy (CD, n=11) or a simple laparotomy (SL, n=10) and then received *N*-nitrosobis(2-oxopropyl)amine (BOP) treatment. The animals were sacrificed 20 weeks after surgery and the development of biliary carcinoma, the presence and degree of cholangitis, and IL-6 expression on the biliary epithelia were examined histologically.

Results: In the CD group, 8 hamsters (73%) demonstrated persistent cholangitis and 6 (55%) of them developed intrahepatic biliary carcinoma, while no hamster without cholangitis showed any biliary carcinoma. In the SL group, cholangitis was recognized in 4 hamsters (40%) and no development of biliary carcinoma was identified. A significantly high incidence of tumor development ($p=0.024$) and a close correlation between the presence of cholangitis and the occurrence of biliary carcinoma ($p=0.013$) were thus evident in the CD group. Moreover, the degree of cholangitis was significantly higher in the CD hamsters ($p=0.041$) and an IL-6 over-expression was identified in 5 hamsters that had undergone a CD, with a scattered expression on the intra- and extrahepatic biliary epithelia. Despite

the fact that the induced biliary carcinomas showed a multicentric occurrence in the liver, these tumors originated from within the restricted area where IL-6 was expressed.

Conclusions: A deregulated IL-6 over-expression on the biliary epithelia may therefore be involved in inflammation-associated biliary carcinogenesis in hamsters that have undergone a bilioenterostomy.

Introduction

Chronic inflammatory conditions involving the biliary tree such as mechanical irritation by means of cholelithiasis (1, 2), chronic cholangitis with hepatolithiasis (3), bile stasis and bacterial infection (4) are considered to be high-risk conditions for developing biliary carcinoma. Biliary carcinoma also occurs long after a biliary-enteric anastomosis (5) or sphincteroplasty (6) for benign diseases. Persistent cholangitis enhances biliary carcinogenesis through an acceleration of cell kinetic activity of the biliary epithelia in hamsters (7, 8).

The role of cytokines and growth factors has recently been the focus of a lot of attention in the development and progression of tumors. The inflammatory cytokine interleukin-6 (IL-6), originally identified as a T cell-derived factor which triggers antibody secretion and the final maturation of B cells (9), is a multifunctional cytokine produced by a wide variety of cells, including macrophages (10), lymphocytes (11), fibroblasts (12) and several carcinoma cells (13-17) while also mediating inflammatory reactions and immune-mediated processes (18, 19). On the other hand, IL-6 acts as a growth-regulating factor that interacts with specific membrane receptors on the tumor cell surface to induce proliferation or prolongation of survival of the tumor cells (20). However, whether the over-expression of IL-6 per se can promote cancer formation remains unknown.

This study evaluated the potential role by which over-expression of IL-6 on the biliary epithelia may contribute to the development of biliary carcinoma *in vivo*, in association with persistent cholangitis in hamsters that had undergone a bilioenterostomy.

Materials and Methods

Animals

Seven-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. The animals were housed one per cage with sawdust bedding under standard laboratory conditions in the Laboratory Animal Center for Biochemical Research at Nagasaki University Graduate School of Biomedical Sciences. All experiments were performed following the Guidelines for Animal Experimentation of Nagasaki University.

Carcinogenic studies

After the removal of the gallbladder, a choledochoduodenostomy (CD) was performed on 11 hamsters following intraperitoneal administration of sodium pentobarbital (50 mg/kg of body weight) (21). The control hamsters underwent a simple laparotomy (SL) alone (n=10). All animals were given weekly subcutaneous injections of *N*-nitrosobis(2-oxopropyl)amine (BOP) (Nakarai Tesque, Kyoto, Japan) at a dose of 5mg/kg body weight in 0.9% saline. BOP

injections were started 4 weeks after surgery and continued for 9 weeks. At postoperative week 20, the hamsters were sacrificed and the liver and biliary systems were removed en bloc. After fixation in 10% neutral formalin, the specimens were cut into 6 blocks and embedded in paraffin. Histological sections were stained with hematoxylin and eosin and then examined by a pathologist with no knowledge of the study. The number of histologically verified biliary carcinomas was counted. Carcinomas were defined as lesions that showed signs of disruption of epithelial cell polarity and evidence of an invasive event in accordance with the WHO classification of tumors in hamsters (22).

Inflammatory changes

To evaluate the relationship between cholangitis and biliary carcinogenesis, the grade of cholangitis was scored in accordance with the infiltration of inflammatory cells as follows: grade 0, no cholangitis; grade 1, mild invasion of inflammatory cells around the bile duct; grade 2, severe invasion of inflammatory cells around the bile duct; grade 3, abscess formation in the liver (7,8).

Immunohistochemical staining for IL-6

Immunohistochemical staining for IL-6 was performed using the indirect immunoperoxidase method. Tissue sections embedded in paraffin were dewaxed in xylene and treated with microwave and incubated with anti-mouse IL-6 monoclonal antibodies (DAKO, Tokyo, Japan) after the blocking of endogenous

peroxidase. The slides were stained with a biotin-labeled secondary antibody (DAKO, Tokyo, Japan) and a peroxidase-labeled avidin-biotin complex with DAB was used as a substrate. The grade of the expression of IL-6 was determined according to the percentage of positively stained biliary epithelial cells as follows; (-): 0%, (+): up to 5%, (++): between 5% and 50%, and (+++): more than 50%. Positive staining for IL-6 with a grade of either (++) or (+++) was considered indicate an over-expression of IL-6.

Statistical analyses

The incidence of tumor development and the presence of cholangitis were statistically analyzed using the chi-square test and Fisher's exact test. The Mann-Whitney's *U*-test was used to compare the cholangitis score and the number of tumors per animal between the groups. The logistic regression analysis was used to clarify the correlation between the presence of cholangitis and the occurrence of biliary carcinoma.

Results

Cholangitis and biliary carcinogenesis

Cholangitis of any extent was identified in 8 (73%) and 4 (40%) hamsters that underwent a CD and SL, respectively (**Table 1**). In the CD hamsters, a high-scored cholangitis of grades 2 or 3 was evident and the average cholangitis score

was 1.3, which was significantly higher than that of 0.4 in the SL hamsters ($p=0.041$). Biliary carcinoma developed in 6 (55%) hamsters that had undergone a CD, with a multicentric occurrence in the liver. On the other hand, no hamster that underwent an SL showed any biliary carcinoma. There was a significant difference in the incidence of biliary carcinoma between the 2 groups ($p=0.024$). In the CD group, 6 (75%) of the 8 hamsters with cholangitis developed biliary carcinoma, while no hamster without cholangitis showed biliary carcinogenesis. A significant correlation between the presence of cholangitis and the development of biliary carcinoma was thus evident in the CD hamsters ($p=0.013$).

IL-6 expression and biliary carcinogenesis

The over-expression of IL-6 on the biliary tree was recognized only in hamsters that underwent a CD and was identified as stained granules seen in the cytoplasm of the biliary epithelia, where the biliary epithelia showed various degrees of hyperplastic changes (**Figure 1**). The IL-6 expression was seen in both the intra- and extrahepatic biliary tree (**Table 2**). The IL-6 over-expression on the intrahepatic bile ducts was recognized in 5 CD hamsters and it was demonstrated with a scattered expression in the liver. All of these hamsters were associated with persistent cholangitis and developing biliary carcinoma. In addition, the average cholangitis score in the CD hamsters with biliary carcinoma was 2.0, which was significantly higher than that of 0.4 in the CD hamsters without carcinoma ($p=0.019$). On the other hand, only one CD hamster developed biliary

carcinoma without showing any evidence of IL-6 expression on the biliary tree. Interestingly, all biliary carcinomas originated from within the restricted area where IL-6 was expressed despite their multicentric occurrence (**Figure 2**) and IL-6 expression was also identified in the carcinoma cells (**Figure 3**).

Discussion

Biliary tract malignancies represent diagnostic and therapeutic challenges, in part because of their aggressive behavior and a lack of understanding of their underlying pathogenetic mechanisms. Chronic inflammation of the biliary tree has been implicated in biliary tumorigenesis where chronically inflamed biliary epithelia render them increasingly susceptible to biliary carcinoma (1-6). However, the carcinogenic mechanism still remains unclear.

BOP is a potent carcinogen in the biliary tract and pancreas of hamsters (23). BOP-induced biliary carcinoma shows a histological resemblance to carcinoma in humans (22) and has thus made it possible to observe various early stage lesions which are often difficult to observe in humans (21, 24). Moreover, BOP induces biliary carcinomas within a relatively short period of time although biliary carcinomas develop spontaneously in hamsters long after they undergo a bilioenterostomy (8). In this study, 6 (55%) of the 11 hamsters that underwent a choledochoduodenostomy followed by BOP-treatment developed intrahepatic biliary carcinoma and almost all these hamsters were proven to have moderate to

severe cholangitis. Meanwhile, no hamster without cholangitis, including the hamsters that had undergone a simple laparotomy, showed any biliary carcinogenesis. A significant correlation between the presence of cholangitis and the development of biliary carcinoma was thus evident in the CD hamsters. Based on our scoring system, cholangitis was noted in 40% and 73% of the SL and the CD hamsters with BOP treatment, respectively. BOP induces DNA damage of the biliary epithelial cells, which may result in BOP-induced chemical cholangitis. The baseline incidence of cholangitis in our model is thus 40%, although the grade of cholangitis is mild in the SL hamsters. These results indicated that complicated inflammatory reactions are induced in the biliary tree following a choledochoduodenostomy and enhance biliary carcinogenesis.

Cytokines are low-molecular weight soluble proteins that transmit signals between cells and are involved in several disorders. Several cytokines have important role in inflammatory reactions. Cancer is also a complex process in which cytokines play an important role. An inflammation-related cytokine, IL-6 shows pleiotrophic effects with both mitogenic and cytoprotective actions on the biliary epithelia (25-28) while increasing in the blood and bile of patients with cholangiocarcinoma or in cholangiocarcinoma cells *in vitro* (29, 30). Yokomuro et al., (26) demonstrated that exogenous IL-6 and hepatocyte growth factor (HGF) are mitogens that predispose normal human biliary epithelial cells to cholangiocarcinoma *in vitro* and that neoplastic transformation is associated with constitutive production of these growth factors with acquisition of IL-6/gp130

and HGF/met-based autocrine growth control circuits. In addition, *in vitro* studies have demonstrated that inflammatory cytokines induce DNA damage and inhibit DNA repair by a nitric oxide dependent mechanism in cholangiocarcinoma cells (31) and even in normal hamster gallbladder epithelial cells (32). In the present study, IL-6 was demonstrated to show a scattered expression on the biliary tree and was identified only in the CD hamsters with moderate to severe cholangitis. Moreover, BOP-induced biliary carcinomas showed a multicentric occurrence and yet all these tumors, except for a carcinoma in one CD hamster without IL-6 expression, originated from within the restricted area where IL-6 was expressed. Therefore, the results of this *in vivo* study suggested that persistent cholangitis due to a choledochoduodenostomy induced over-expression of IL-6 on the biliary epithelia and, as a consequence, the enhanced IL-6 predisposed non-neoplastic biliary epithelial cells to acquire a potential of differentiating into biliary carcinoma. BOP-induced biliary carcinomas also expressed IL-6 in this study, however, the significance of IL-6 expression on the carcinoma cells is unclear. The exogenous administration of IL-6 onto cultured biliary epithelial cells or the use of IL-6 null mice will contribute to a better understanding of the mechanisms of inflammation-associated biliary carcinogenesis.

In conclusion, continual irritation of the biliary tree by inflammatory stimuli could result in a deregulated IL-6 expression on the biliary epithelia that may be involved in the biliary carcinogenesis in hamsters following a choledochoduodenostomy. Based on this *in vivo* tumorigenesis model, by which

the understanding of the mechanisms of inflammation-related biliary carcinogenesis was enhanced, control of the inflammatory reaction by the selective inhibition of the IL-6 pathway may offer an opportunity for the prevention of biliary carcinoma formation in several inflammatory cholangiopathies, such as primary sclerosing cholangitis, hepatolithiasis, or recurrent cholangitis complicating biliary-enteric anastomosis.

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Figure Legends

Figure 1 Immunohistochemical staining for IL-6 in a hamster that had undergone a choledochoduodenostomy. IL-6 is expressed clearly in the cytoplasm of epithelial cells of the intrahepatic bile duct (400×). A marked dilation of the intrahepatic bile duct lined by stratified hyperplastic epithelium is seen.

Figure 2 An illustration demonstrating the prevalence of IL-6 expression and the development of biliary carcinoma on the liver specimens of a hamster that had undergone a choledochoduodenostomy. The open circles (□) indicate the area where the IL-6 expression is identified on immunohistochemical examination. The closed boxes (■) indicate the area where the development of biliary carcinoma is recognized. All biliary carcinomas originate from within the restricted area where IL-6 is expressed.

Figure 3 IL-6 expression with immunohistochemical staining on the cytoplasm of carcinoma cells in a hamster that had undergone a choledochoduodenostomy (×400).

Table 1. Relation between cholangitis and biliary carcinogenesis in hamsters treated with BOP

Group	No. of hamsters examined	No. (%) of hamsters with/without cholangitis		Average of cholangitis score	No. (%) of hamsters with carcinoma		Total no. of carcinomas induced	Total no. of carcinomas /CBA	Incidence of CBA with/without cholangitis	
		with	without		IHBD	EHBD			with	without
SL	10	4 (40)	6 (60)	0.4	0	0	0	0	0/4 (0%)	0/6 (0%)
CD	11	8 (73)	3 (27)	1.3 ^a	6 (55) ^b	0	30	5.0	6/8 (75%) ^c	0/3 (0%)

BOP: *N*-nitrosobis (2-oxopropyl) amine

SL: simple laparotomy, CD: choledochoduodenostomy

IHBD : intrahepatic bile duct, EHBD : extrahepatic bile duct

CBA : carcinoma-bearing animal

a: Significantly different from SL (p=0.041)

b: Significantly different from SL (p = 0.024)

c: Significant correlation between the presence of cholangitis and biliary carcinogenesis (p=0.013)

Table 2. IL-6 expression and biliary carcinogenesis in hamsters treated with BOP following a choledochoduodenostomy

Hamsters	Occurrence of intrahepatic biliary carcinoma	No. of carcinomas developed	Cholangitis score	IL-6 expression	
				IHBD	EHBD
1	no	0	1	-	+
2	no	0	0	-	-
3	no	0	1	+	+
4	no	0	0	-	-
5	no	0	0	+	++
6	yes	1	3	++	++
7	yes	1	3	-	-
8	yes	3	1	+++	+++
9	yes	21	2	+++	+++
10	yes	2	1	++	++
11	yes	2	2	++	+++

BOP: *N*-nitrosobis(2-oxopropyl)amine

IL-6 expression : (-); no expression, (+); up to 5%, (++); between 5% and 50%, (+++); more than 50%

IHBD : intrahepatic bile duct

EHBD : extrahepatic bile duct

Fig. 1

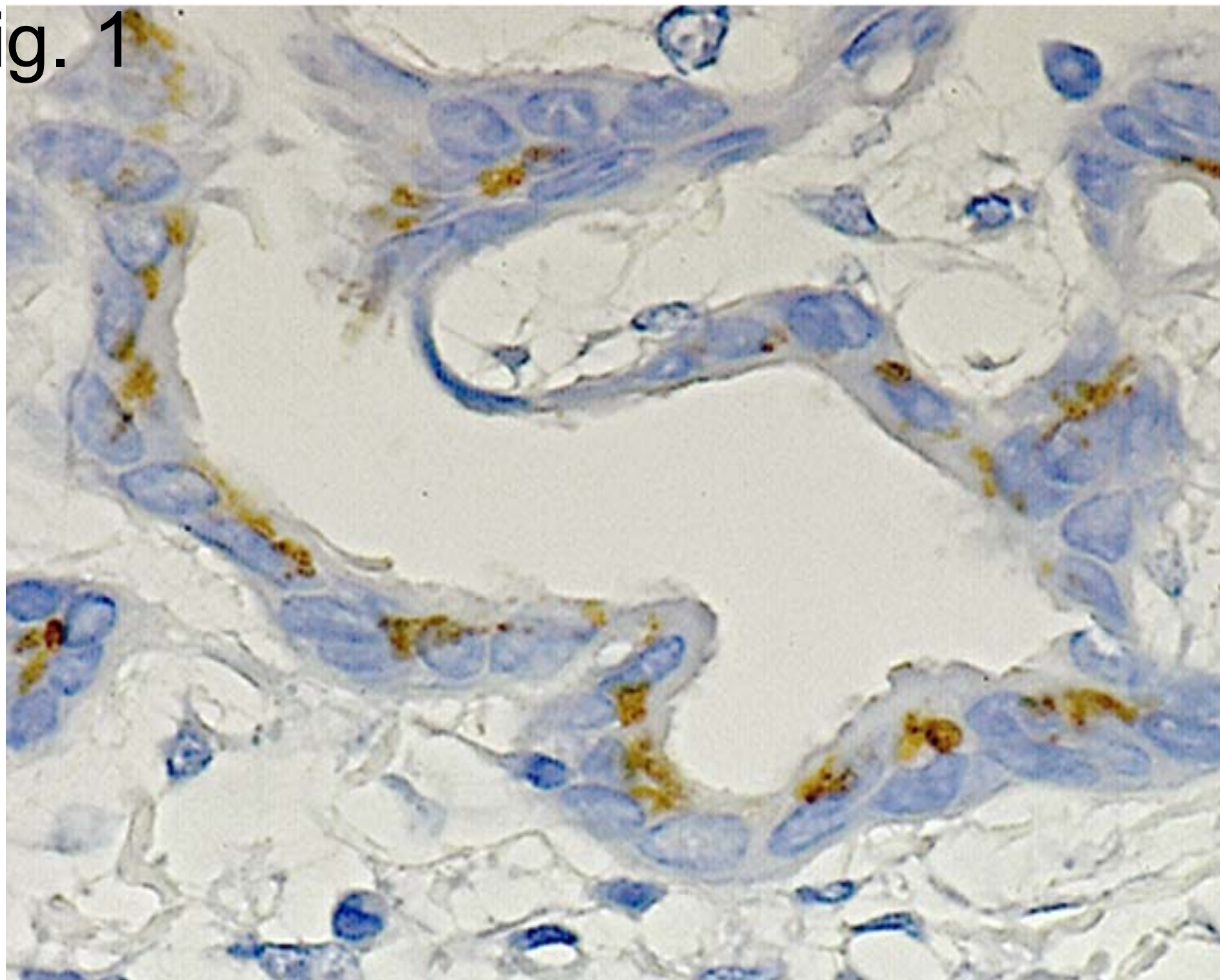


Fig. 2

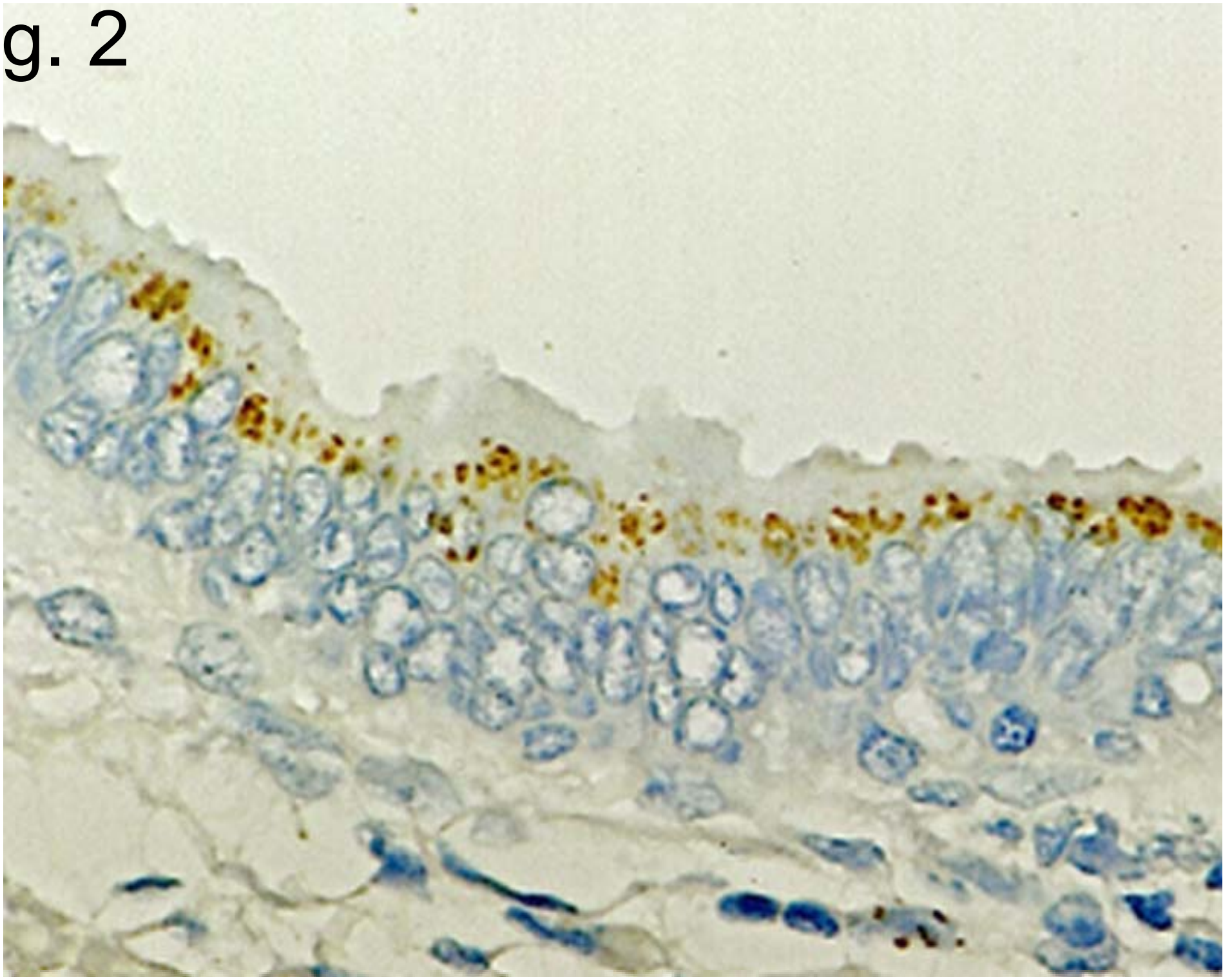


Fig. 3

